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THE ASSOCIATION BETWEEN SPERM ANEUPLOIDY AND MALE INFERTILITY: SCREENING, AETIOLOGY AND POSSIBLE ROUTES TO ALTERNATIVE THERAPY.

A thesis submitted for the degree of Doctor of Philosophy

By

Helen Ghislaine Tempest

Department of Biological Sciences

Brunel University

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// Abstract

One in six couples wishing to start a family are infertile. The many causes of infertility include genetic defects that can be single gene, multifactorial or chromosomal (including Y deletions, karyotype abnormalities and gamete aneuploidy). This thesis is concerned with the association between infertility and increased sperm aneuploidy. Specific questions are: should males be screened for sperm aneuploidy before intracytoplasmic sperm injection (ICSI)? Is there a relationship between individual semen parameters and sperm aneuploidy for specific chromosome pairs? What is the role of genome organisation in male gametes and its association with infertility? Whether use of alternative therapy (in this case, traditional Chinese Medicine (TCM)) can be used to improve sperm disomy levels. Statistical analysis of questionnaire data revealed that infertility specialists believed there to be merit in screening sperm aneuploidy levels before ICSI. Evidence is presented for possible chromosome-specific and semen parameter specific mechanisms for sperm aneuploidy as is evidence of genome organisation that may be perturbed in infertile males. Finally, in six males studied, sperm aneuploidy levels improved significantly coincident with TCM. Closer investigation of the biological activity of individual therapeutic herbs and treatment cocktails revealed strong anti-oestrogenic and anti-oxidant properties. This suggests a possible mechanism of action of the herbs and provides the basis from which future placebo controlled clinical trials might continue. Possible criticisms of the work presented here include the unavailability of blood samples from many of the patients (thus preventing karyotype analysis) and the absence of a second control group in our studies on semen parameters. Nevertheless significant steps have been made towards establishing the need for, and the implementation of, a pre-ICSI screening test. Moreover progress has been made towards further understanding the aetiology of sperm aneuploidy and towards the implementation of a new treatment that may, ultimately, augment, or even replace ICSI.

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Abbreviations list

A	Asthenozoospermia
ABP	Androgen binding protein
ADLP	Adrenoleukodystrophy protein
AIS	Androgen insensitivity syndrome
AR	Androgen receptor
AS	Angelman syndrome
ART	Assisted reproductive techniques
AT	Asthenoteratozoospermia
AZF	Azoospermia Factor
BPY	Basic proteins Y
BWS	Beckwith-Wiedemann syndrome
CAIS	Complete androgen insensitivity syndrome
CAM	Complementary and alternative medicine
CBAVD	Congenital bilateral absence of the vas deferens
CDY	Chromodomain Y
CFTR	Cystic fibrosis transmembrane conductance regulator
DAZ	Deleted in azoospermia
DBY	Dead box Y
DFFRY	Drosophila fat facets related Y
DHT	Dihydrotestosterone
FISH	Fluorescent *in-situ* hybridisation
FSH	Follicle stimulating hormone
FRAP	Ferric reducing anti-oxidant potential
GnRH	Gonadotrophin-releasing hormone
hCG	Human chorionic gonadotrophin
HGMP	Human genome mapping project
HMG	Human menopausal gonadotrophin
HPG	Hypothalamic-pituitary-gonadal axis
ICSI	Intracytoplasmic sperm injection
IMS	Infertile male syndrome
ISH	*In-situ* hybridisation
IVF	In-vitro fertilisation
LH	Luteinizing hormone
MI	Meiosis I
MII	Meiosis II
MAIS	Mild androgen insensitivity syndrome

MIF	Mullerian inhibitory factor
NOR	Nucleolar organising region
O	Oligozoospermia
OA	Oligoasthenozoospermia
OAT	Oligoasthenoteratozoospermia
OHT	Hydroxy-tamoxifen
OT	Oligoteratozoospermia
PAIS	Partial androgen insensitivity syndrome
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PEs	Phthalate esters
PGD	Preimplantation diagnosis
PMDS	Persistant Mullerian duct syndrome
PRY	Protein tyrosine BAS like (PTB-PL) related Y
PWS	Prader –Willi syndrome
RBM	RNA binding motif
RPL	Recurrent pregnancy loss
RT	Room temperature
SBMA	Spinal and bulbar muscular atrophy
SEM	Standard error of the mean
STS	Sequence-tagged-site
T	Teratozoospermia
UPD	Uniparental disomy
UTY	Ubiquitous transcribed tetratricopeptide repeat gene Y

Chapter 1

General introduction

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Chapter 1: Genetic basis of infertility

1. Introduction

Infertility is defined as a failure of a couple to conceive after 1 year of trying, and is estimated to affect 1 in 6 couples wishing to start a family. The term 'infertility' thus includes that of not only complete sterility but also that of sub-fertility, (where the capacity for producing offspring is diminished). It is estimated that the rate of infertility affects males and females equally (Shah et al., 2003).

Infertility can be caused by a number of factors; including endocrine abnormalities, psychological trauma, infection, immunological infertility, lack of ovulation, obesity, varicocele, exposure to toxic chemicals and environmental factors including: smoking, alcohol, chemotherapy (Martin et al., 1998a; Wyrobek et al., 1995), mechanical stoppage, obstruction and age. However more recently a wide range of genetic defects have been associated with infertility. Genetic defects are estimated to account for 50% of male infertility (Bhasin et al., 1994). Although this figure is spurious, it is probable that the majority of these factors affecting reduced fertility may have some genetic basis, however small. Factors including obesity, endocrine abnormalities, susceptibility to infections and psychological problems are likely (Shah et al., 2003).

1.1 Identification and classification of infertility

When a couple are having difficulties conceiving GP's will refer them to specialists including: andrologists, urologists, endocrinologists and gynaecologists, amongst others. In this situation the female partner is sent for a number of tests in order to determine levels of FSH (follicle stimulating hormone) and LH (luteinizing hormone) important for the development and quality of the eggs and to check for ovulation. A

transvaginal ultrasound is used to check uterus lining and monitoring of follicle development, also a hysterosalpinogram is undertaken to check for tumours, blockages, pelvic adhesions, endometriosis and a post coital test to ensure that the sperm can survive and penetrate the cervical mucus (www.nhsdirect.nhs.uk).

The male partner undergoes a semen assessment, of which the main factors analysed are volume, count, concentration, motility and morphology amongst other tests (See table 1.1 for normal reference values).

Table 1.1- reference guide for normal semen parameters

Category	*Reference guide*
Volume	> 2 mls
Ph	7.2 – 8.0
Sperm concentration	> 20 million/ml
Total sperm count	> 40 million
Motility	> 50% forward progression (category A and B) or > 25% rapid progression (category A)
Morphology	> 14% normal morphology (Strict Kruger criteria)

According to the results of this semen assessment, males are classified by the following: normozoospermia (all parameters are within the normal range), oligozoospermia, (sperm concentration of less than 20 million/ml or severe oligozoospermia, less than 5 million/ml). The classification of asthenozoospermia is used in cases of less than 50% forward progression (or less than 25% rapid forward progression), and teratozoospermia in males with less than 14% normal morphology (according to the strict Kruger

guidelines). Males can have any combination of these semen parameter abnormalities for example males with oligoasthenoteratozoospermia (OAT) have a disturbance in all three major categories of count, motility and morphology. Individuals for which no sperm is found within the ejaculate are referred to as azoospermic.

Over the last decade or so research into genetic causes of male and female infertility has rapidly expanded and genetic factors have been associated with infertility and assigned to specific chromosome locations however, the majority still remain undeciphered.

1.2 Genetic reasons for infertility

The genetic basis of infertility can be divided into the following:

1) Infertility involving a single gene defect.
2) Infertility involving multifactorial (polygenic) defects.
3) Infertility involving a chromosomal defect.

1.2.1 Infertility syndromes due to a single gene defect

To date a number of infertility syndromes/phenotypes have been attributed to a single gene defect and these defects can be inherited in several ways including: sex linked, autosomal dominant or autosomal recessive. The most common single gene defects are considered in the following section and listed in table 1.2.

1.2.1.1 Autosomal recessive syndromes

1.2.1.1.1 Cystic fibrosis

Cystic fibrosis is one of the most common genetic disorders affecting Caucasians of European descent, estimated to affect 1 in 2,500 children with a carrier frequency in the Caucasian population of 1 in 25. Among the clinical manifestations around 95% of males have been found to be infertile presenting with obstructive azoospermia, (Johnson, 1998). Congenital bilateral absence of the vas deferens (CBAVD) has been demonstrated in nearly all affected males regardless of the severity of the cystic fibrosis, (Lissens et al., 1996).

Mutations have been found within the cystic fibrosis transmembrane conductance regulator gene (CFTR), located on the long arm of chromosome 7 (Hargreave et al., 1998). Screening for mutations within the CRTR gene is undertaken within infertility clinics, to date over 900 mutations within the CFTR gene have been identified (www.genet.sickids.on.ca/cftr/). In many cases however, CFTR mutations are not identified as only approximately 30 of these mutations are screened regularly (Hargreave, 2000; Lamb and Lipshultz, 2000). The relationship between the absence of the vas deferens and CFTR mutations has been well established, however, there are also reports of CFTR mutations in men with severe oligozoospermia, who do not present with absence of the vas deferens. The CFTR complex has thus been suggested relate to spermatogenesis (van der Ven et al., 1996), although at present its role of the CFTR complex remains unclear (Hargreave, 2000). Mutations within the CFTR gene have not been directly associated with female infertility, but females with cystic fibrosis are subfertile with an increased risk of complications during pregnancy (Cohen et al., 1980).

A correlation exists between those studies reporting a higher incidence of males with mutations within CFTR and those that have analysed more mutations. Therefore individuals presenting with CBVAD would benefit from increased screening of mutations within the CFTR gene. (Hargreave, 2000).

1.2.1.1.2 Other autosomal recessive syndromes

Immotile cilia syndrome and Kartagener syndrome (also associated with situs inversus, bronchiectasis and chronic sinusitis) both fall into a group of related disorders collectively known as primary ciliary dyskinesia (PCD) characterised by immotility or dysmotility of ciliary structures, including airway and sperm axonemes. (Carson and Collier, 1988). In many cases affected males exhibit infertility due to abnormalities in sperm motility.

Bardet-Biedle syndrome features typically include obesity, retinitis, mental retardation, variable cardiac and renal defects and hypogonadism resulting in spermatogenic impairment (Greene et al., 1989).

Persistent Mullerian duct syndrome (PMDS) is a rare form of male pseudohermaphroditisum, whereby patients have the genotype 46, XY and in the majority of cases both external genitalia and secondary sexual characteristics appear normal. Patients also possess fallopian tubes and a uterus as a result of the failure of the regression of the Mullerian duct, due to defects in either the Mullerian inhibitory factor (MIF) produced by the Sertoli cells, or the MIF receptor (Imbeaud et al., 1996; 1995; 1994).

Usher syndrome is the most frequent cause of hereditary dual loss of sight and hearing, however, some patients have also been identified as having degeneration in the sperm axoneme (Hunter et al., 1986). Abnormal microtubular organisation of the axoneme in sperm tails and other cell types has also been found, resulting in the production of sperm with poor motility.

Cerebellar ataxia with hypogonadism is rare among the population and is often the result of parental consanguinity (as reviewed in Hargreave, 2000; Rushton and Genel, 1981). Phenotype includes disturbances in speech and gait, lack of secondary sexual characteristics, absence of libido, small and firm testes and hypothalamic pituitary dysfunction with impaired response and increases in LH and FSH.

5α-reductase deficiency, results in a failure to convert testosterone (secreted by the foetal testis) to dihydrotestosterone (DHT) due to defects in 5α-reductase, (responsible for the intracellular conversion of testosterone to DHT) (reviewed in Mak and Jarvi, 1996), and is characterised by male pseudohermaphroditism. The disease phenotype includes; perineoscrotal hypospadias, blind vaginal pouch, female habitus without breast development and the absence of female internal genitalia (reviewed in Mak and Jarvi, 1996). The main cause of infertility however is structural abnormalities of the external genitalia, and in some cases decreased spermatogenesis. Less severe forms may present with hypospadias and reduced penile size (Imperato et al., 1974).

There are several disorders, in which androgen function is altered and which are associated with adrenal hyperplasia as some of the enzymes involved in these disorders

are also involved in adrenal synthesis of androgens (due to a deficiency of androgen synthesis). The three following disorders are included in this category.

3-β-hydroxysteroid dehydrogenase deficiency syndrome, is responsible for the conversion of pregnenolone to progesterone. The disease phenotype results in incomplete masculinization as a result of point mutations in the gene encoding this enzyme (Simard et al., 1996).

Deficiency of the enzyme 17-α-hydroxylase perturbs the conversion of progesterone to 17-hydroxyprogesterone, clinical manifestations include defective virilization due to impaired testosterone synthesis in the foetal testis (Geller et al., 1997).
17-β-hydroxy dehydrogenase is responsible for converting androstenedione into testosterone, mutations in this enzyme result in pseudohermaphrodites with complete or incomplete female genitalia, with inguinal or intra-abdominal testes (Andersson et al., 1996).

1.2.1.2 Autosomal dominant syndromes

1.2.1.2.1 Myotonic dystrophy

Myotonic Dystrophy is transmitted as an autosomal dominant trait with variable penetrance affecting males and females. Patients exhibit muscle wasting, frontal balding, hypogonadism, testicular atrophy and loss of libido. Male infertility is seen in approximately 30% of cases, however some degree of testicular atrophy is seen in at least 80% of cases, mainly affecting the seminiferous tubules, with normal Leydig cells present and severe tubular sclerosis and spermatogenesis being affected. The genetic

defect involves a gene encoding a serine/threonine protein kinase, which contains an unstable CTG triplicate repeat pattern.

1.2.1.2.2 Polycystic kidney disease

This disorder is characterised by numerous cysts in the kidney, liver, spleen, lung, testis, epididymis and seminal vesicle. Two loci have been identified as being involved including PKD1 (accounting for the majority of cases) and PKD2 (located on chromosome 16p13.3 and 4q respectively) (reviewed in Mak and Jarvi, 1996). Male patients often present with infertility due to the multiple cysts found throughout the epididymis and seminal vesicles, which can result in obstructive azoospermia.

1.2.1.2.3 Noonan syndrome

Noonan syndrome patients exhibit a similar phenotype to that of Turner syndrome (refer to section 1.4.2.1), including short stature, webbed neck, cardiovascular abnormalities and cryptorchidism and testicular atrophy. Approximately 50% of affected males display azoo- or oligospermia and delayed puberty (Elsawi et al., 1994). However unlike Turner syndrome (45, XO), patients display a 46, XY karyotype, the locus involved being mapped to chromosome 12q22 (Jamieson et al., 1994).

1.2.1.3 X-linked syndromes

Kallmann syndrome individuals exhibit hypogonadotrophic hypogonadism, as a result of an endocrinological defect whereby there is no hypothalamic secretion of gonadotrophin – releasing hormone (GnRH). In these cases follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone levels are low or undetectable.

Patients' fertility can be restored when treated with a combination of FSH or human menopausal gonadotrophin (HMG) and human chorionic gonadotrophin (hCG).

Aarskog-Scott syndrome and is associated with facial, skeletal and genital abnormalities (Porteous and Goudie, 1991). Affected males often have reduced fertility frequently displaying cryptorchoidism, there may also be specific defects involving the sperm acrosome related to this syndrome (Meschede et al., 1996).

Adrenomyelopathy arises as a result of small deletions and point mutations within the adrenoleukodystrophy protein (ADLP) gene (Braun et al., 1995). Clinical onset of this disease is around 30 years of age, phenotype includes: progressive paralysis, peripheral neuropathy, adrenocortical failure. The vast majority of men also exhibit endocrine or exocrine testicular failure including: impotence, gynaescomastia, poor androgenization, azoo- or oligozoospermia, low ejaculate volume, low testosterone and elevated levels of LH and FSH (Assies et al., 1995).

Androgen insensitivity syndrome (AIS) is attributed to a defect in the androgen receptor (AR) gene resulting in quantitative and qualitative receptor binding abnormalities (reviewed in Brinkmann et al., 2001; Mak and Jarvi., 1996). Defects within the AR can prevent the normal development of both internal and external male structures. Increased luteinizing hormone (LH) production results in increased testosterone production by leydig cells, which is converted to oestrogens, resulting in feminisation (reviewed in Thielemans et al., 1998). Several types of AIS have been reported including: Complete Androgen Insensitivity Syndrome (CAIS), Partial Androgen Insensitivity Syndrome (PAIS) and Mild Androgen Insensitivity Syndrome (MAIS).

Individuals with CAIS present with female external genitalia, a short blind ending vagina, absence of any Wollfian derived structures including the epididymis, vas deferens and seminal vesicles, the absence of a prostate and gynaecomastia (reviewed in Quigley et al., 1995). During puberty in these patients testosterone and LH levels are elevated, the higher levels of testosterone within these individuals act as a substrate for aromatase activity, resulting in substantial amounts of estrogens, causing further feminisation of these individuals (reviewed in Brinkmann et al., 2001).

PAIS (also known as Reifenstein syndrome) is characterised by hypospadias, micropenis, with gynaestocomastia, sex reversal or intersex genitalia, the majority of patients are brought up as females (reviewed in Thielemans et al., 1998). The severity of the disease depends on the degree of androgen insensitivity, individuals germ cells display mitotic activity but contain no spermatozoa (reviewed in Brinkmann, 2001).

Spinal and bulbar muscular atrophy (SBMA) / Kennedy disease has a clinical age onset of 20-30 years, presenting with progressive muscular atrophy and weakness, secondary testicular atrophy causing infertility later on in life including defective spermatogenesis, oligo or azoospermia and gynaecomastia. SBMA is associated with a CAG trinucleotide repeat within the androgen receptor, the number of repeats varies in individuals without any phenotypic aberrations. However, if the CAG repeat exceeds 40 (normal situation is 15-31) it can compromise the function of the gene (La Spada et al., 1992).

1.2.1.4 Imprinted genes and uniparental disomy

In some cases the normal pattern of Mendelian inheritance does not occur, in that Mendelian genetics assume that both maternal and paternal copies of a gene are equally

expressed. This however, is not always the case, the discovery of certain genes (imprinted genes), have been shown to carry a specific molecular modification which are specific to the parent from which it originates. This modification causes the "silencing" of either the paternal or maternal gene, therefore, loss of any function of such genes (whether it be due to mutation or deletion) may result in either over- or under expression (reviewed in Meschede and Horst, 1997). This same phenomenon can be caused as a result of uniparental disomy (UPD), whereby at least segments if not entire chromosome homologues are derived from the same parent. A number of UPD and aberrations in imprinted genes have been implicated in infertility.

1.2.1.4.1 Beckwith-Wiedemann syndrome (BWS)

Patients with BWS often present with pre- and post natal overgrowth, renal abnormalities, are prone to develop embryonic tumours and most males also display cryptorchidism (Elliot and Maher, 1994).

1.2.1.4.2 Prader-Willi syndrome (PWS) and Angelman syndrome (AS)

The lack of expression of imprinted genes within the chromosome region of 15q11-q13 result in two clinically distinct syndromes PWS and AS. These syndromes arise through several mechanisms including deletions and UPD. PWS and AS syndrome both result from a genetic deficiency from paternally and maternally inherited chromosome 15 (respectively) (Glenn et al., 1997). An estimated 70-75% of individuals with PWS and AS are found to contain a deletion within the region of 15q11-q13, with UPD and single gene mutations accounting for the remainder (Knoll et al., 1993). PWS individuals are characterised by severe obesity, mental retardation, delayed and incomplete development in puberty and hypogonadism as a result of deficient hypothalamic release

of gonadotrophin-releasing hormone, resulting in low levels of testosterone, LH and FSH. AS individuals often present with severe mental retardation, problems with movement, microcephaly and hypopigmentation (Williams et al., 1995).

1.2.1.5 Infertility syndromes for which the mode of inheritance is not reported or complex

Bioactive LH (hypogonadism due to a mutation in LH). LH is a heterodimer consisting of two subunits α and β, alterations within this gene can cause the abnormal binding activity of LH (reviewed in Thielemans et al., 1998). Hence causes inadequate stimulation of Leydig cells resulting in testosterone deficiency with poor spermatogenesis. Patients homozygous for the mutation do not undergo normal pubertal development and are infertile, however, heterozygous patients undergo normal pubertal development presenting with sub fertility (Weiss et al., 1992).

Kearns-Sayre syndrome predominantly affects endocrine and neuromuscular systems, phenotypes including: pigmentary degeneration of the retina, cardiac conduction defect and cerebellar ataxia. Abnormalities in the reproductive system are reported in 20-30% of affected males, including cryptorchidism, pubertal delay, lower testicular volume and low gonadotrophin levels (Harvey and Barnett, 1992). Prevalence of sub fertility among these individuals has not yet been reported (Meschede and Horst, 1997). Kearns-Sayre syndrome is a multisystemic disorder caused by mutations in the mitochondrial genome (reviewed in Johns, 1995).

Young syndrome patients are characterised by chronic sinopulmonary infections and obstructive azoospermia, however, it should be noted that in these patients

spermatogenesis is normal. Histological studies have revealed the obstructive azoospermia as being caused by thickened secretions, resulting in obstructions within the epididymis (Handelsman et al., 1984).

Table 1.2- List of syndromes caused by single gene defects, frequency, phenotype and inheritance if known

Condition/ syndrome	*Frequency*	*Phenotype*	*Inheritance/genotype*
Cystic fibrosis	1 / 2,500	Wollfian duct maldevelopment, respiratory infections, pancreatic insufficiency	Autosomal recessive CFTR gene chromosome 7q31.2
Congenital absence of the vas deferens	1/60,000	Isolated abnormalities in wollfian duct	CFTR gene
Imotile cilia	1 / 10,000-16,000	Immotile spermatozoa, chronic sinusitis, bronchitis	Autosomal recessive chromosome 1p35.1
Bardet-Biedle	Unknown	Hypogonadism resulting in spermatogenic impairment	Autosomal recessive chromosome 16q21
Persistant mullerian duct syndrome	Unknown	Pseudohermaphroditism and impaired spermatogenesis	Autosomal recessive, two loci MIF 19p13 and MIF receptor 12q13
5α-reductase	Unknown	Pseudohermaphroditism and abnormal external genitalia	Autosomal recessive, located on chromosome 2
Usher's	1 / 30,000	Sperm axonemal dysfunction, hearing loss, retinitis pigmentosa	Autosomal recessive USH1A-E chromosome 11q13.5
Cerebellar ataxia	Unknown	Lack of secondary sexual characteristics, hypothalamic pituitary dysfunction	
3-β hydroxysteroid dehydrogenase deficiency	Unknown	Incomplete masculinization	Autosomal recessive maps to chromosome 1
17-α hydroxylase	Unknown	Defective virilization, resulting from	Autosomal recessive

deficiency		impaired testosterone synthesis.	
17-β hydroxy dehydrogenase	Unknown	Pseudohermaphroditism, with complete /incomplete female genitalia	Autosomal recessive
Myotonic dystrophy	1 / 8,000	Testicular atrophy, muscle wasting, cataract	Autosomal dominant variable penetrence chromosome 19q13.3
Polycystic kidney disease	1 / 800	Multiple cysts in seminal vesicles, epididymis, liver, kidneys	Autosomal dominant, PKD1 chromosome 16p13.3
Noonan	1/1,000-1/2,500	Cryptorchidism, testicular atrophy and delayed puberty	Autosomal dominant, chromosome 12q22
Kallmann	1 / 10,000	Delayed puberty, small testes, anosmia cleft palate	X-linked recessive KAL locus Xp22.3
Androgen insensitvity	1 / 60,000	Different degree of testicular feminisation, (oligo/azoospermia)	X-linked recessive androgen receptor Xq11-12
Aarskog-scott	Unknown	Genital abnormalities, reduced fertility, cryptorchidism, defects in sperm acrosome reaction implicated	X-linked recessive Xp11.21
Adrenomyelopathy	Unknown	Endocrine/exocrine testicular failure: impotence, azoo/oligozoospermia low testosterone levels	X-linked recessive, ADLP gene Xq28
SBMA	Unknown	Testicular atrophy, defective spermatogenesis, oligo- azoospermia and gynaecomastia	Trinucleotide repeat CAG
Bioactive LH	Unknown	Inadequate stimulation of the leydig cells, testosterone deficiency results in poor spermatogenesis	Unknown mode of inheritance 19q13.32
AKAP-82	Unknown	Disturbance of sperm motility	Chromosome Xp11.12
Kearns-Sayre	Unknown	Cryptorchidism, delayed puberty, low gonadotrophin	Unknown mode of inheritance
Youngs Syndrome	Unknown	Obstructive azoospermia, as a result of thickened secretions within the epididymas	Unknown mode of inheritance

Table adapted from Male fertility and infertility, Patrizio, P and Broomfield, D (1999) page 164

1.2.2 Genes implicated in infertility

A vast number of mutations within genes have been reported to impact on fertility, through numerous studies carried out in model organisms including drosophila and mice. A comprehensive review of over 200 mutations associated with reproductive defects in both male and female mice has recently been published (Matzuk and Lamb 2002 www.nature.com/fertility). An adaptation of the table presented in this review listing individual genes, sex affected, reproductive phenotype and the fertility status of individuals affected can be found in appendix A. It is possible that all forms of infertility may have some kind of genetic component, for example, individuals may have a genetic susceptibility to diseases that affect fertility (e.g. chlamydia).

1.2.3 Multifactorial and Polygenic disorders

Several approaches, including linkage analysis and positional cloning in recent years have seen an explosion in the identification of mutations within genes resulting in Mendelian disorders. However, the identification of genes involved in polygenic genetic diseases has been less forthcoming. The detection of Polygenic diseases are considered to be caused as a result of multiple interacting genes and the environment, and are believed to occur at a much higher incidence (Rannala, 2001).

The advent of the Human Genome Mapping Project (HGMP) has provided researchers in the field of human genetics with a powerful resource, that will aid in the identification of the genes involved in polygenic disorders. This will be extremely useful in male and female infertility enabling research to be undertaken in candidate gene screening, homology studies and animal modelling. The majority of male specific genes are located on the Y chromosome (refer to section 1.3.3) and therefore the

complete mapping of the Y chromosome will enable the functional properties of individual genes to be identified through a number of techniques including protein binding studies, expression studies and inhibition studies. The results of the HGMP serve to increase our understanding of the genome and its interactions and as a result aims to elucidate and identify potential polygenic and multifactorial disorders involved in infertility. It has also been proposed that the advent of the HGMP could potentially alter our current thinking in medical care from that of a treatment approach to a prevention based method, dependent on the identification of individual risk (Guyer and Collins, 1993).

1.3 The chromosomal basis of infertility

1.3.1 Meiosis

Meiosis is a specialized form of cell division, its main features are to halve the number of chromosomes in the gametes, and the generation of genetic variation among the population. Meiosis (figure 1.1), unlike mitosis consists of an initial round of DNA replication, which is subsequently followed by two rounds of cell division. In short these are split into; MI which involves the segregation of homologous chromosomes from each other and MII whereby sister chromatids are segregated. This process results in the production of 4 haploid daughter cells from one diploid germ cell. These divisions are, however, preceded by a unique meiotic stage (prophase) in which homologous chromosomes synapse and undergo recombination.

Figure 1.1- schematic representation of meiosis, adapted from genetics principles and analysis, Hartl and Jones, pg90.

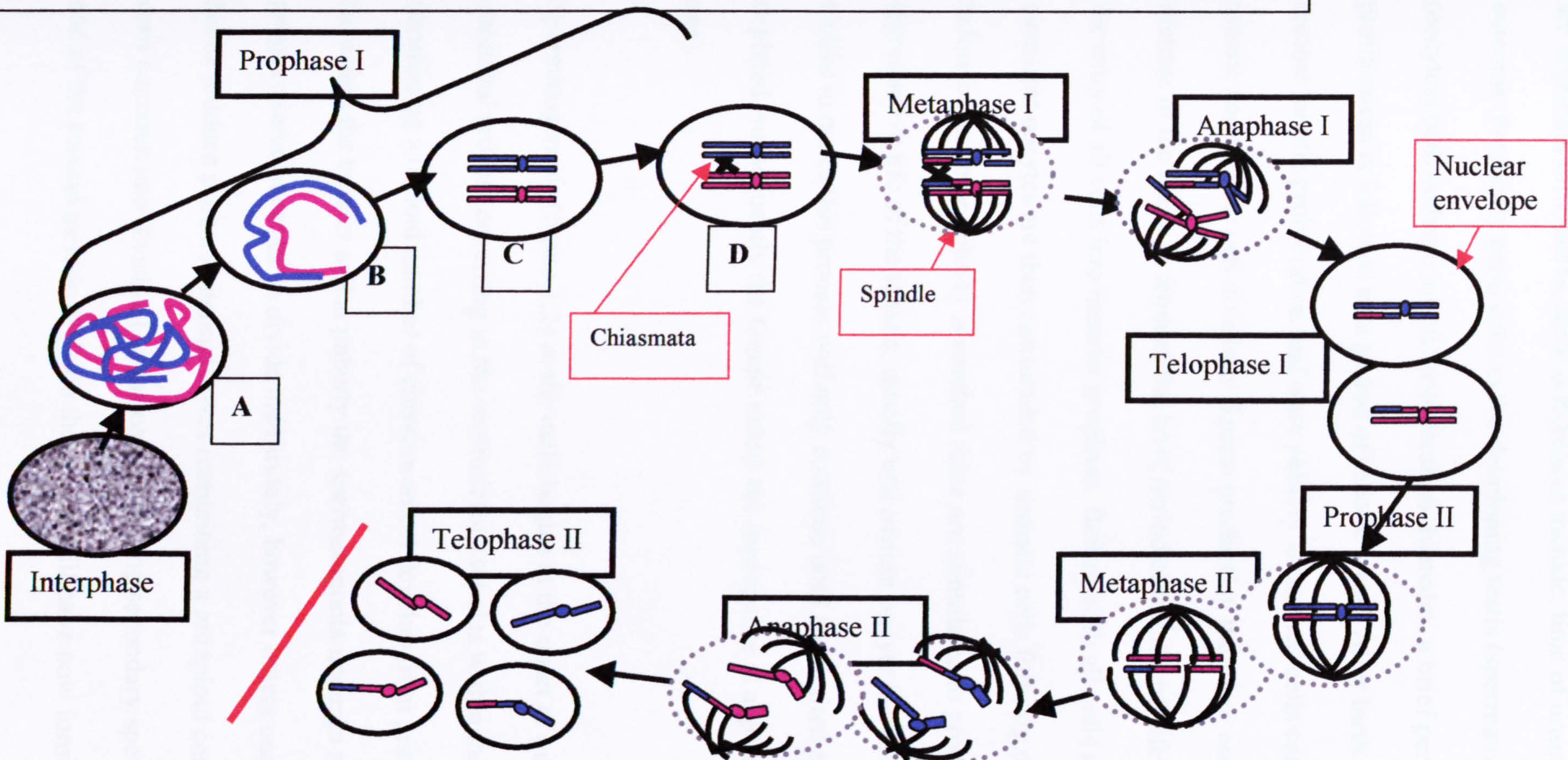


Figure 1.1- illustrates the major features of meiosis, for clarity only the nucleus and one pair of homologous chromosomes are shown. During interphase each chromosome replicates, however chromosomes are not identifiable at this time. Following interphase, prophase I is initiated and consists of a number of stages in which chromosomes start to condense (A) and homologous chromosome pairing begins (b), step sister chromatids become visible (C) and recombination takes place through the formation of chiasmata (illustrated in (D) between pairs of nonsister chromatids). During metaphase I the nuclear envelope disappears and the spindle apparatus assembles and each chromosome migrates to the metaphase plate. During anaphase I the paired centromeres in each bivalent move towards opposite poles of the spindle. In telophase I the spindle breaks down and the nuclear envelope reforms and chromosome condensation is reversed, during prophase II (not shown) the chromosome condense (now consists of one member of each homologous pair), nuclear envelope disappears and spindle reforms in metaphase II, as before chromosomes align in the central plane of the spindle and during anaphase II each centromere splits longitudinally and 2 chromatids move to opposite poles. In telophase II chromosome movement is complete, spindle breaks down and 4 haploid nuclei revert to the interphase state.

The process of meiosis in males and females is fundamentally similar, however there are important differences, these differences include: time of onset, duration and outcome. Prenatally germ cells in the developing testis (spermatogonia) and ovary (oocytes) both undergo mitotic proliferation. In males, a brief period of mitotic proliferation is followed by a period of mitotic arrest. After birth male spermatogonia resume mitotic proliferation and once puberty is reached cells continuously undergo mitotic and meiotic cell divisions. Sperm production therefore continues throughout the lifetime of the male. In females the brief period of mitotic proliferation is followed by the entry of all cells into meiotic prophase. Before birth all cells enter meiotic arrest, quiescent oocytes are then surrounded by somatic cells forming primordial follicles before birth. Once puberty is reached these are stimulated to grow throughout the reproductive life of the female, usually one mature oocyte is ovulated once a month. Unlike in males this process will only continue until the limited pools of oocytes are depleted (subsequently the female enters the menopause at approximately 50 years of age).

Spermatogenesis (figure 1.2) in the male begins at the onset of puberty, and is a continual process occurring in the seminiferous tubules within the testes. Spermatogonia (containing a diploid number of chromosomes) develop from primordial germ cells that lie within the tubules and at puberty the spermatogonia undergo mitosis. Of these progeny some continue to divide indefinitely, however others undergo meiosis and hence produce primary spermatocytes (containing a tetraploid configuration). These cells continue into division I of meiosis producing secondary spermatocytes and by the end of the second meiotic division these cells will have now formed haploid spermatids

(figure 1.2 sperm meiosis), which then undergo a series of morphological differentiation steps in order to become spermatozoans.

Figure 1.2- sperm meiosis

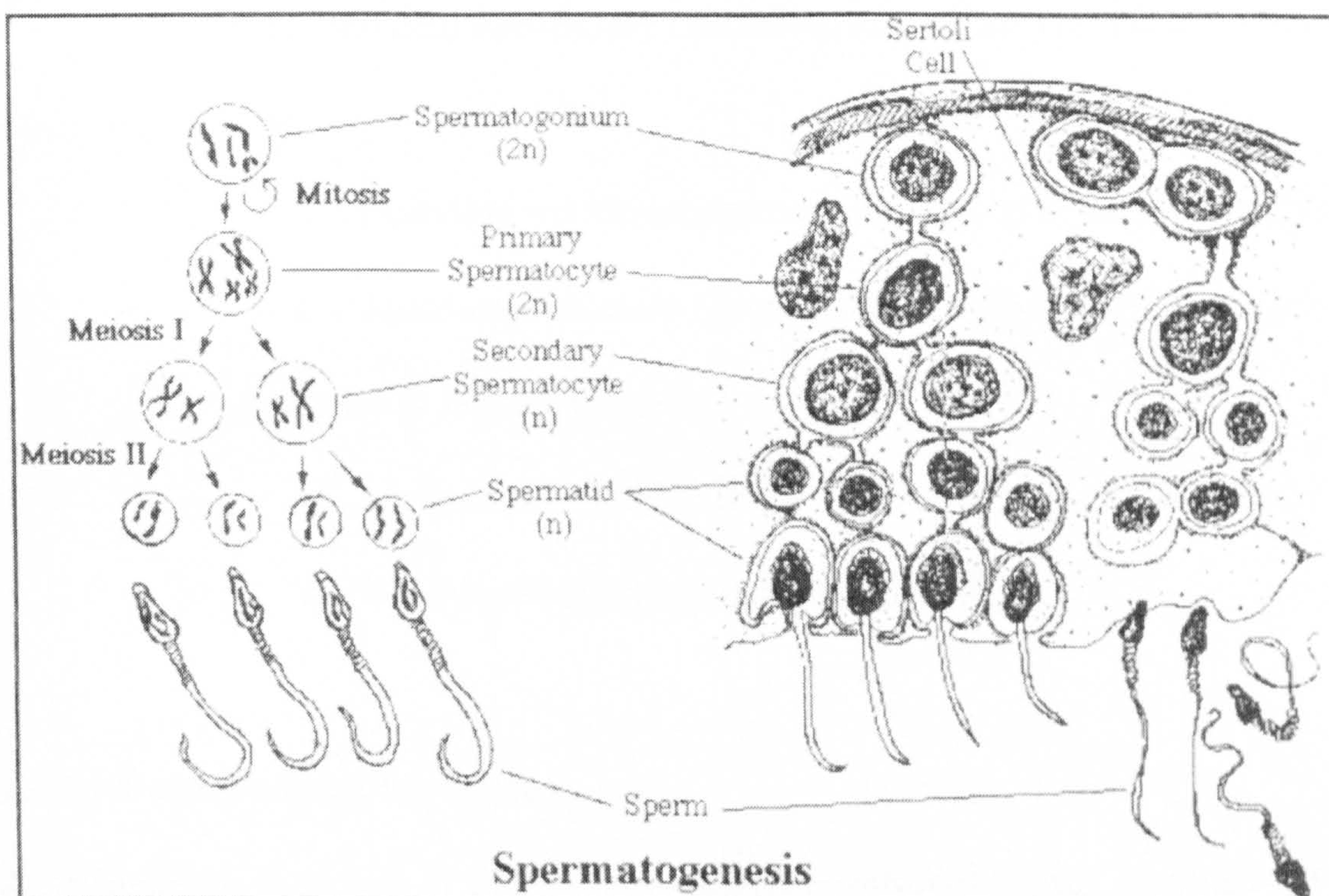


Figure 1.2- The left hand side illustrates the various stages of meiosis during spermatogenesis, on the right hand side is a Sertoli cell, with the lumen of the tubule found at the bottom of the diagram. Various stages of spermatogenesis are illustrated embedded in the plasma membrane of the Sertoli cell. Image taken from www.luc.edu/depts/biology/dev/spermeio.htm

1.3.2 Chromosome aberrations

Given that the formation and movement of chromosomes is involved in pre-meiotic divisions in meiosis and in fertilisation and in subsequent cleavage divisions, it is not surprising that many infertility phenotypes have been associated with identifiable chromosome abnormalities. Structural anomalies are often the result of a breakage that occurs during meiosis and in this case a number of outcomes are possible: loss of material (deletion), gain of material (duplication) and chromosome rearrangements. These can be further divided into the following:

- Constitutional inversions (considered in section 1.3.2.1)
- Constitutional translocations (considered in section 1.3.2.2)
- Y chromosome deletions (considered in section 1.3.3)
- Constitutional aneuploidy (including mosaicism) (considered in section 1.4)
    i. Including sex chromosomes (considered in section 1.4.2)
    ii. Autosomes (mainly chromosome 21) (considered in section 1.4)
- Aneuploidy in the gametes
    i. Maternal age effect for the oocytes (considered in section 1.4.4)
    ii. Sperm aneuploidy (considered in section 1.5)

1.3.2.1 Constitutional Inversions

An inversion is the result of a single chromosome with two breaks, in which the chromosome segment is inverted, subsequently reversing the order of the genes compared to the wildtype. This inversion is problematic for the normal continuation of meiosis, as synapsis is compromised, due to the re-ordering of the genes along the length of the chromosome in inversion heterozygotes. In this case one or the other of the chromosomes twists into a loop (inversion loop) in the inverted region. Within the inversion loop if no crossing over takes place the homologous chromosomes can separate normally, if the crossing over takes place within this loop the chromatids involved become joined, resulting in the formation of chromosomes containing duplications and deletions (figure 1.3 and 1.4). Individuals are hence at risk of producing unbalanced gametes (i.e. may lead to the conception of a foetus with an unbalanced amount of genetic material). Inversions are split into two categories; those not involving the centromere (paracentric); and those involving the centromere

(pericentric). Frequencies of paracentric and pericentric inversions have been found to be 8 times higher in severe oligozoospermic males compared to the normal fertile population (Patrizio and Broomfield, 1999).

There are a number of mechanisms by which inversions result in reduced fertility, including the time constraints and mechanics of the formation of a pairing loop imposed by the meiotic machinery which can hinder meiosis (Forejt et al., 1982). The use of single sperm PCR has also identified a reduced number of cross overs within the pairing loop suggesting reduced recombination within the inverted region which in turn can result in the breakdown of meiosis (Brown et al., 1998). Chandley et al. (1987) observed extensive disturbances of meiotic pairing resulting in failed synapse formation during meiosis I across the inverted region in heterozygote carriers, which in turn can lead to unbalanced gametes. (Patrizio and Broomfield, 1999).

Figure 1.3- Formation of a pairing loop in a pericentric inversion heterozygote, illustrating the results of a crossing over within the pairing loop

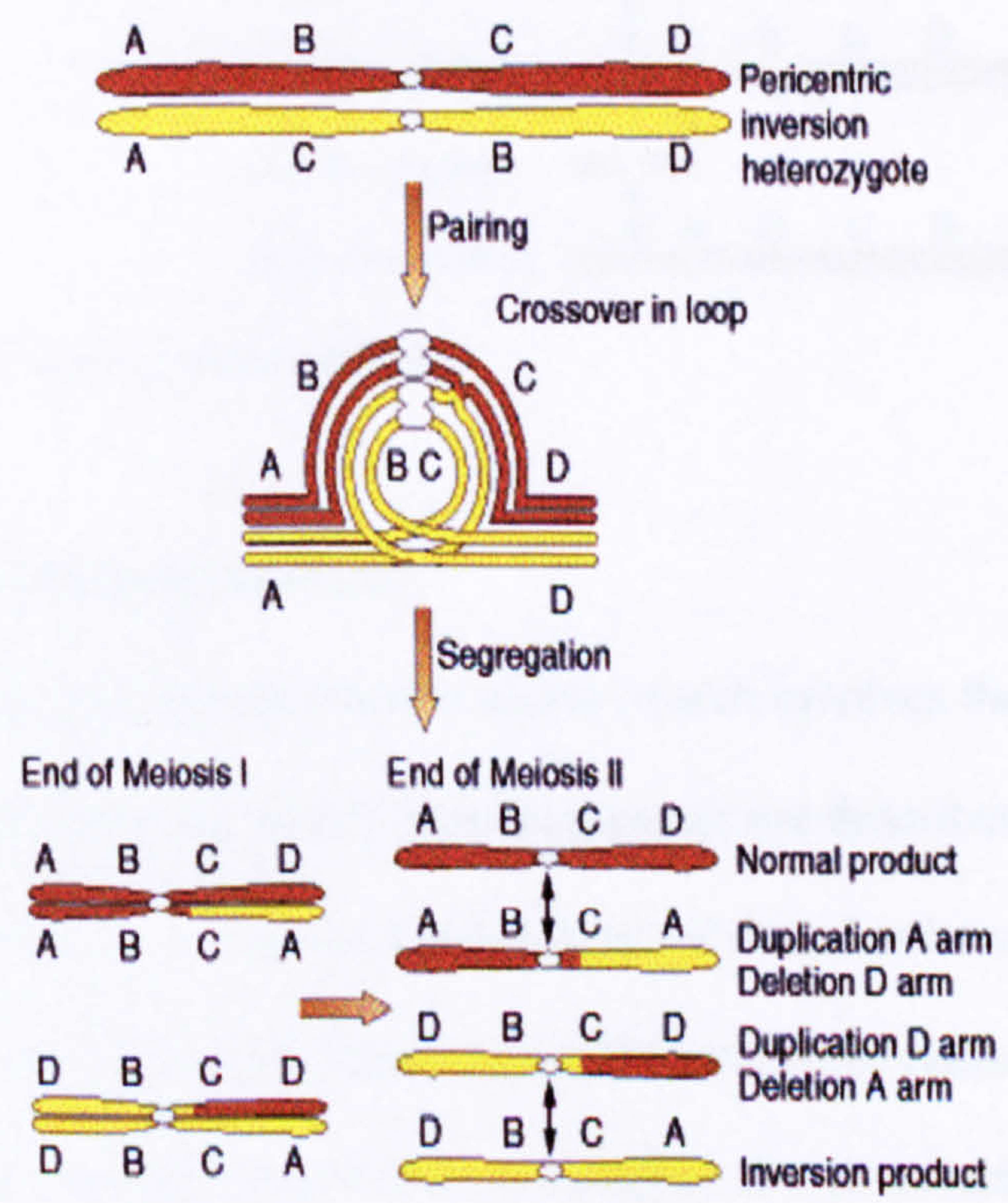
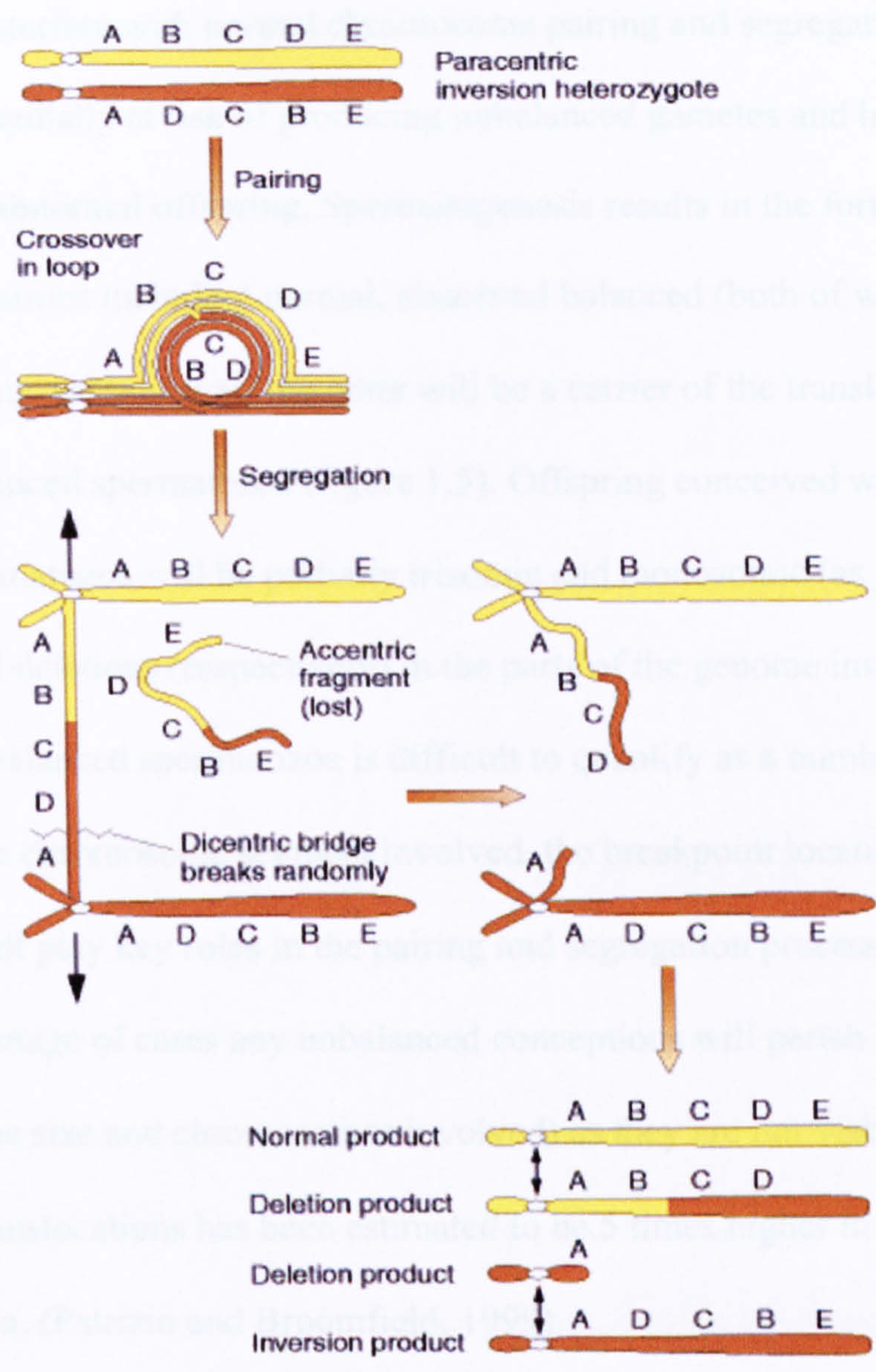


Figure 1.4- Formation of a pairing loop in a paracentric inversion heterozygote, illustrating the results of a crossing over within the pairing loop



Images taken from http://www.med.nyu/Sackler/2003t&i.htm

1.3.2.2 Constitutional translocations

Structural anomalies also include translocations (which involves the exchange of non – homologous chromosome segments). Rearrangements are described as being balanced, (if all genetic information is kept) and unbalanced (if there has been any loss or gain of genetic information) (figure 1.5). Balanced translocations are estimated to occur in 1 in 500 individuals, the majority of which do not display any abnormal phenotype with the

exception of decreased fertility and the risk of chromosome abnormalities in offspring due to the mal-segregation of the translocation.

Translocations interfere with normal chromosome pairing and segregation at MI, and therefore are potentially at risk of producing unbalanced gametes and hence chromosomally abnormal offspring. Spermatogenesis results in the formation of three possible combinations including normal, abnormal balanced (both of which are phenotypically normal however, the latter will be a carrier of the translocation) and abnormal unbalanced spermatozoa (figure 1.5). Offspring conceived with abnormal unbalanced spermatozoa will be partially trisomic and monosomic (as a result of duplications and deletions (respectively) in the parts of the genome involved). The risk of abnormal unbalanced spermatozoa is difficult to quantify as a number of factors such as the size of the chromosome segment involved, the breakpoint location and the type of rearrangement all play key roles in the pairing and segregation process of chromosomes. In a large percentage of cases any unbalanced conceptions will perish in utero (again dependant on the size and chromosomes involved) as they are not viable. The incidence of reciprocal translocations has been estimated to be 5 times higher in males with severe oligozoospermia. (Patrizio and Broomfield, 1999).

1.3.2.2.1 Autosomal translocations

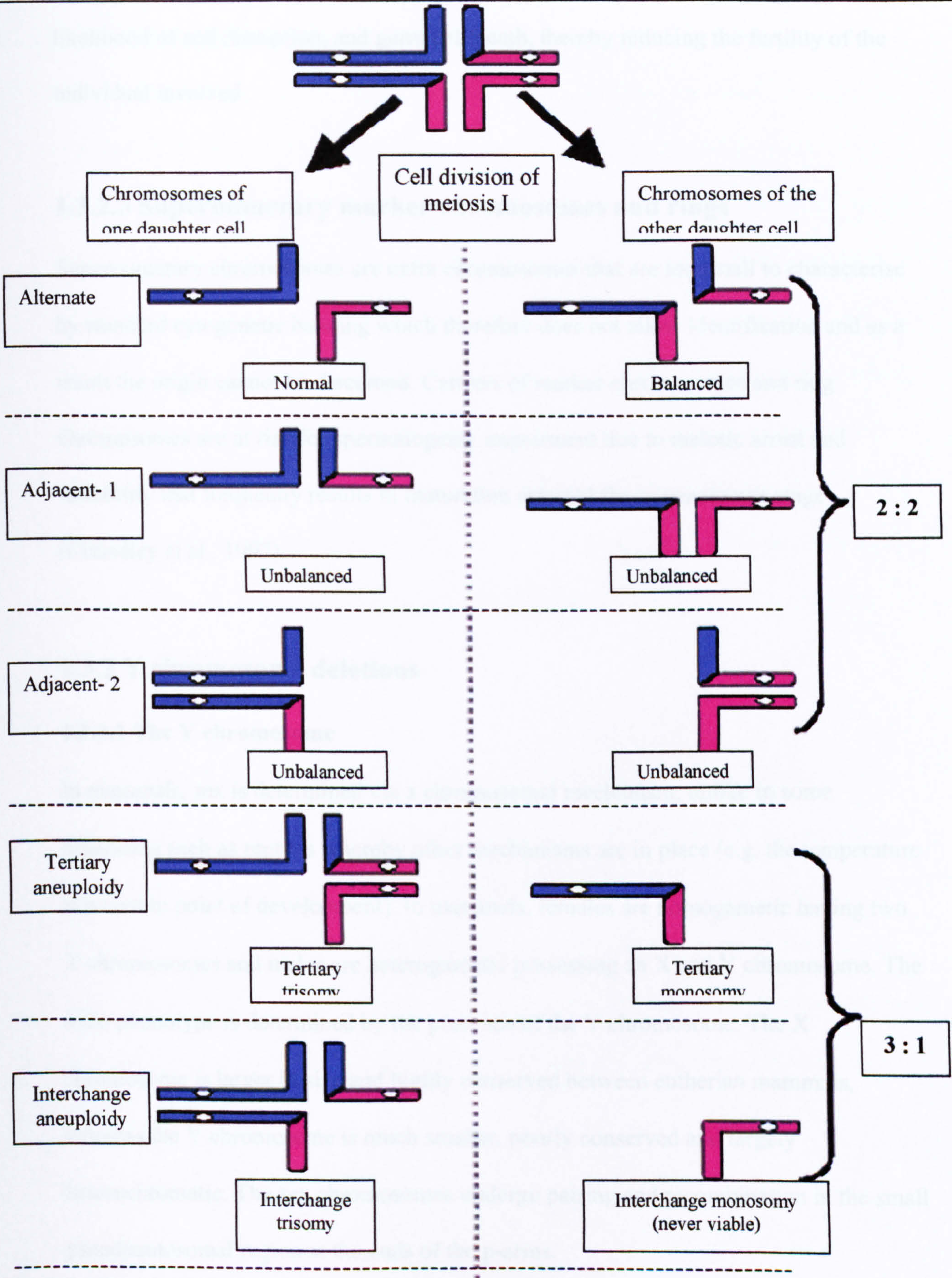
There are a number of reasons as to why autosome-autosome translocations can result in a reduction in fertility. In order for these chromosomes to proceed through meiosis it is essential for the chromosomes to synapse and must therefore form a pairing cross (quadrivalent). The formation of this quadrivalent can affect fertility as the mechanics involved and the time constraints imposed in this formation can impede the meiotic process (Forejt et al., 1982). The disjunction of the quadrivalent is also susceptible to

producing unbalanced gametes, depending on the type of segregation. The gametes will either contain the following: adjacent-1 and adjacent-2 segregation = extra or missing chromosome; alternate segregation = normal and carrier of the translocation (refer to figure 1.5). Failure of meiosis can also occur as a result of asynaptic regions within the quadrivalent, leading to the elimination of germ cells (Miklos et al., 1974). Lyon et al. (1966) have provided evidence of non-homologous pairing between segments of translocated chromosomes and the sex chromosomes (X and Y) during meiosis I. This interaction can interfere with X inactivation resulting in a lethal gene dosage effect on the germ cells (Forejt et al., 1982). Chandley et al. (1984) reported that interactions may occur between the translocated segments and other parts of the nucleus which may result in errors in meiosis and cell death.

1.3.2.2.2 Robertsonian translocations

Robertsonian translocation involves the centromeric regions of two nonhomologous acrocentric chromosomes fusing to form a single centromere. The heterozygous carrier is usually phenotypically normal but there is a high risk of aberrant segregation in meiosis. There is an estimated 10 fold increase in the incidences of robertsonian translocations in infertile men, the most common translocation observed in the male infertility population is that of t(13q14q), followed by t(14q21q). Meiotic studies of infertile carriers of t(13q14q) and t(14q21q) have demonstrated the behaviour of the autosomes involved in meiosis to be abnormal during spermatogenesis resulting in infertility (Luciani et al., 1984; Rosenmann et al., 1985). The heterochromatic short arm of acrocentric chromosomes have been found to contain nucleolar organiser regions (NOR). These NOR are involved with RNA synthesis, however they are also required to associate with the sex chromosome vesicle. Robez et al. (1986) reported that

Figure 1.5 Illustrates the formation of a quadrivalent and the possible segregation patterns in a heterozygous translocation



The categories of 2:2 and 3:1 segregation that may occur in gametogenesis in the translocation heterozygote. In the four 3:1 categories, only one of the two possible combinations in each category is depicted. Adapted from Gardner and Sutherland 1996 pg 63.

Robertsonian translocations in which the NOR has been lost are at an increased likelihood of cell disruption, and germ cell death, thereby reducing the fertility of the individual involved.

1.3.2.3 Supernumerary marker chromosomes and rings

Supernumerary chromosomes are extra chromosomes that are too small to characterise by standard cytogenetic banding which therefore does not allow identification and as a result the origin cannot be discerned. Carriers of marker chromosomes and ring chromosomes are at risk for spermatogenic impairment due to meiotic arrest and instability that frequently results in maturation arrest at the spermatocyte stage (Chandley et al., 1997).

1.3.3 Y chromosome deletions

1.3.3.1 The Y chromosome

In mammals, sex is determined via a chromosomal mechanism, unlike in some organisms such as reptiles whereby other mechanisms are in place (e.g. the temperature at a certain point of development). In mammals, females are homogametic having two X chromosomes and males are heterogametic possessing an X and Y chromosome. The male phenotype is determined by the presence of the Y chromosome. The X chromosome is larger in size and highly conserved between eutherian mammals, whereas the Y chromosome is much smaller, poorly conserved and largely heterochromatic. The sex chromosomes undergo pairing and recombination in the small pseudoautosomal region at the ends of the p-arms.

Genes that have been identified on the Y chromosome have been shown to possess X linked homologues which, together with the pairing at the pseudoautosomal region

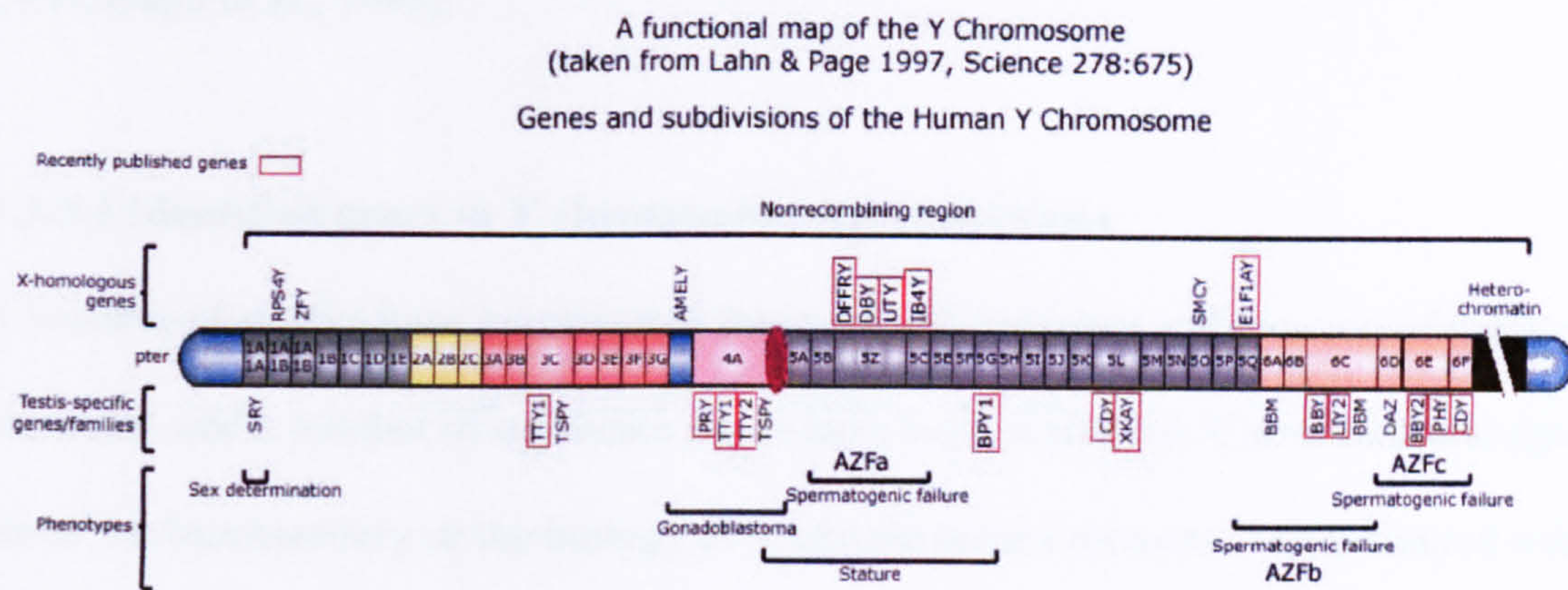
suggests that the X and Y chromosomes may have arisen from an autosomal pair in an ancestral mammal. Differentiation between chromosomes X and Y is thought to have occurred when an allele at a single locus on the proto-Y took over a male determining function from either an ancestral genetic or environmental determining system. Furthermore, the evolution of a second male determining or differentiating locus on the proto-Y may then set up conditions whereby coinheritance of these alleles would be advantageous to males. This situation could have resulted in a suppression of recombination between these loci (Graves, 1998). Restriction of recombination is likely to result in the isolation from the homologue resulting in mutations, inactivation and deletions via either genetic drift or the selection of a new allele (Charlesworth, 1991). These genetic changes have been demonstrated to be present in the Y chromosome, by the comparison of the sequence of genes when compared with their X chromosome counterparts (Whitfield et al., 1993).

It is hypothesised that the formation of the Y chromosome has arisen through various cycles of attrition of the X chromosome and the addition of autosomal regions with genes on the Y chromosome being under constant threat of mutation, deletion, inactivation and degradation. It has been suggested that only favoured genes survive over a long evolutionary time, thus selective forces are in operation in retaining those that perform male specific functions such as male determination and differentiation (Graves, 1995; 1998).

1.3.3.2 Deletions in the Y chromosome and incidence

Until relatively recently, the large majority of infertility was described as idiopathic, with no specific known aetiological factor being established. One genetic factor however, being abnormalities within the Y chromosome, which are now attributed to a significant proportion of infertility phenotypes. The first breakthrough came in 1976 when Tiepolo and Zuffardi reported macrodeletions in the chromosome region Yq in 6 azoospermic males. Since then it has been hypothesised that 1 or more genes within this region may possess important roles in the maintenance of spermatogenesis (Fitch, 1995; Patrizio and Broomfield, 1999). Numerous studies have utilised PCR and STS's, reporting microdeletions within the Y chromosome in severely oligozoospermic males, this has lead to the suggestion of a presence of an azoospermia factor (AZF). The AZF locus located on Yq and has been mapped to interval 6 of the Y chromosome (within the cytological band Yq11.23), at least three deletion intervals have been defined (AZFa, AZFb and AZFc, located in the proximal, central and distal segments of Yq respectively), (Patrizio and Broomfield, 1999; Reijo et al., 1996; Vogt et al., 1996) (figure 1.6). In many cases deletions in the Y chromosome are thought to arise *de-novo* from fertile fathers with intact Y chromosomes and are rarely cytogenetically visible, these deletions represent one of the most frequent structural chromosomal anomalies affecting an estimated 1 in 5,000-10,000 males (Le Bourhis et al., 2000; Kent-First et al., 1996).

Figure 1.6- Map of the Y chromosome



1.3.3.3 Mechanism of Y chromosome deletions

The frequency of *de-novo* Y deletions indicates that the Y chromosome is susceptible to spontaneous loss of genetic material (McElreavey and Krausz, 1999). Instability of the Y chromosome may be related to high levels of repetitive elements clustered along the length of the chromosome, deletions may occur through the aberrant recombination events (either between areas of homologous or similar sequence repeats, between the X and Y chromosomes, Y chromosome unbalanced sister chromatid exchange) or by slippage during DNA replication. It is also possible that particular sequences may promote deletion of the AZF regions and as a consequence individuals may be more susceptible to *de-novo* deletions than others (McElreavey and Krausz, 1999).

The deletions reported so far are, however, variable in extent and location and at present no pattern has been identified and may be attributed to a number of factors including: ethnic variation, geographical variation, patient selection and experimental design. The incidence of deletions in the AZF region is estimated to affect approximately 15% of

men with azoospermia and 10% of men with severe oligozoospermia (Kent-First et al., 1996; Reijo et al., 1995).

1.3.3.4 Identified genes in Y chromosome microdeletions

A number of studies have investigated the incidence presence and location of these deletions, and a number of candidate genes have been identified. Current knowledge about the biochemistry or the biology of Y chromosomes proteins is very limited with the exception of two genes which have been the subject of extensive research: RBM (RNA binding motif) and DAZ (deleted in azoospermia) and will be discussed further in subsequent sections. In recent years a number of other candidate genes have also been identified, but as of yet there is limited information on function and association with infertility, however, their location falls within the AZF region of the Y chromosome. Within the AZFa region genes such as DFFRY (Drosophila fat facets related Y), DBY (dead box Y) and UTY (ubiquitous transcribed tetratricopeptide repeat gene Y) have been identified. Deletions within this region have been implicated in infertility, including oligozoospermia (DFFRY alone) and Sertoli cell only syndrome (DFFRY and DBY together) (McElreavey and Krausz, 1999). These genes are involved in general 'housekeeping' functions and are not specifically expressed in the testes, as are other genes within this region, for this reason they may not be directly involved in spermatogenesis but may be involved in control of protein turnover required for successful spermatogenesis (Affara, 2001). Within the AZFb region PRY (protein-tyrosine phosphatase BAS like (PTP-BL)- related Y) and RBM have been identified. Mapped to the AZFc region, genes CDY (chromodomain Y), BPY (basic proteins Y) and DAZ are found. Stuppia et al, (1998) reported deletions within the AZFc region that do not encompass DAZ genes but may involve other genes located in this region such as

CDY and BPY, suggesting that other loci may contribute to fertility, and this in turn may partially explain the variation seen in phenotype, as these may be associated with different deletions.

1.3.3.5 RBM (RNA binding motif)

RBM (Ma et al, 1993) is part of a family estimated to contain 40 members of genes and pseudogenes, spread across the entire length of the Y chromosome with clustering in the AZFb region (Chandley et al., 1998). RBM has been shown to be expressed specifically within the testes and antibodies to the RBM protein illustrate that localisation is confined to the nucleus of germ cells and is present in all stages of spermatogenesis with the exception of elongating spermatids, (Elliot et al., 1997). The function of RBM has been suggested to be in RNA binding protein and localisation factors associated with splicing factors (Elliot et al., 1998). Comparative analysis of RBM and related genes in different eutherian mammals and marsupials suggest that these genes have an important function as they are conserved on the Y chromosome and is consistent with these genes having an essential function such as spermatogenesis (Affara, 2001). Due to the fact that RBM genes are a multicopy family it is difficult to provide direct genetic and functional evidence that they are critical to spermatogenesis and if this is the case whether one or multiple copies are required for normal spermatogenesis (Ma et al., 2000). Nevertheless there is indirect evidence for the requirement of RBM for normal spermatogenesis these include: the location within the AZFb region, testes specific expression and the protein association with germ cell nuclei. (Elliot et al., 1997).

1.3.3.6 DAZ (deleted in azoospermia)

The second candidate gene "DAZ" was isolated in 1995 (Reijo et al., 1995), and, as in RBM, was found to encode an RNA binding protein specifically expressed in the testes, and is a multi gene family containing at least 7 copies clustered in the distal region of interval 6, located within the AZFc region. Unlike other gene families on the Y chromosome DAZ genes are not dispersed along the Y chromosomes, but are all locatedwithin the commonly- deleted DAZ region (Krostiner et al., 1998). Deletions encompassing the DAZ region of the Y chromosome have been identified as the most common molecularly defined cause of infertility in men. This occurs at an estimated rate of 13% of azoospermic men and 6% of severely oligozoospermic men (<5 million/ml) (Reijo et al., 1995; Stuppia et al., 1996; Foresta et al., 1997; Simoni et al., 1997). The identification of genes critical to fertility has been extensively researched with several putative genes mapping to the DAZ region (Ma et al., 1993; Reijo et al., 1995; Lahn and Page, 1997). No specific point mutations, however have yet been identified, thus evidence linking deletions within the DAZ genes and infertility as in RBM is indirect. Nevertheless, no deletions have so far been identified in men with normal sperm counts, whereas in men with low or complete absence of sperm cells, deletions removing all or most of the DAZ gene cluster are found in 6-13% (Reijo et al., 1995; Pryor et al., 1997; Simoni et al., 1997). Also the fact that DAZ genes are not dispersed along the Y chromosome, suggests that the DAZ region is of importance as deletions of single genes or a subset of genes in this case may lead to phenotypically silent or mild defects in spermatogenesis, whereas anomalies have been reported in severe defects only. DAZ genes have also been highly conserved despite the tendency for degradation of genes on the Y chromosome (Saxena et al., 1996), suggesting the

involvement in an important function. Homologues of the DAZ genes in model organisms have also been identified and disruption of these causes infertility in drosophila by arresting MI at the pachytene stage and in mice no functional copy results in sterility, and one copy in sub fertility (reduced numbers of spermatozoa). Deletions within the DAZ gene cluster in humans have been found to produce similar phenotypes compared to those of the model organisms, sterility and greatly reduced numbers of spermatozoa (Reijo et al., 1995).

1.3.3.7 The future of Y chromosome microdeletions

The function of these genes in the AZF region and their expression during spermatogenesis is still uncertain and knowledge of genetic control remains limited but it is reasonable to assume that the regulation will be through the expression of a number of genes. The pattern of Y chromosome deletions is currently inconclusive, although there is a definite cause and effect relationship between Y chromosome deletions and male infertility. A definite prognosis will only be made when one genotype is strongly correlated with a particular phenotype and there is a known function for the gene and its role in spermatogenesis (Liow et al., 2001). Attempts to correlate genotypes and phenotypes have been largely inconclusive, a number of studies have not included histological findings and those that have, often have not correlated deletion findings with phenotype. Studies that have attempted to do this have found that similar or identical deletions in patients can cause different impairments in spermatogenesis (Affara, 2001).

1.4 Aneuploidy

1.4.1 Aneuploidy as a result of non-disjunction

Aneuploidy arises as the result of the failure of chromosomes to segregate properly at meiosis resulting in a process called non-disjunction. Non-disjunction results in the loss (monosomy) or gain (trisomy) of chromosomes from the normal diploid number, this in turn is the most commonly identified chromosomal abnormality in humans, occurring in at least 5% of all clinically recognized pregnancies (Griffin, 1996a; Hassold et al., 1993). In the majority of cases an aneuploid conceptus will not survive full term, as a result it is the most common genetic cause of pregnancy loss, however, it is also the leading cause of mental retardation in humans in those that do survive full term (Hassold et al., 1993; 1996). Despite the clinical ramifications caused by aneuploidy, relatively little is currently known about how aneuploidy originates in humans.

Analysis of aneuploidy within the female gametes is difficult to undertake in humans, as female gametes are only obtainable through the hyperstimulation of the ovaries and surgical removal of the gametes as occurs in IVF and ICSI treatment (refer to section 1.6). Male gametes on the other hand do not require surgical removal and hence are more amenable for analysis, therefore it is not surprising to find that most studies investigating aneuploidy have been carried out in spermatozoa.

1.4.2 Sex chromosome aneuploidy

1.4.2.1 Turner Syndrome

Turner Syndrome is a sex chromosome abnormality that affects females. These individuals usually present with monosomy of the X chromosome (45,XO) in 55% of

cases with other karyotypes including 46,X,I(Xq), 46,XX/45,X and various structural abnormalities including deletions and ring X chromosomes (Shah et al., 2003). The incidence is estimated to be around 1/5,000-1/10,000 females. Individuals often present with ovarian dysgenesis with hypoplasia and are often short in stature and do not undergo sexual maturation as a result of the haplo-insufficiency of the XY homologous genes critical for gonadal development (Witters et al., 2001). The majority of 45,X individuals are due to paternal origin with the following possible mechanisms acting: meiotic non-disjunction at meiosis I or II, or anaphase lag resulting in a sperm with a missing sex chromosome in an estimated 75% of cases (Hassold et al., 1993). The generation of a 45,X karyotype in a 46,XY or 46,XX zygote may also arise through anaphase lag of the paternal X or Y chromosome (Jacobs et al., 1997).

1.4.2.2 Triple X syndrome (47,XXX)

The incidence of 47,XXX is estimated to occur at an incidence of 1/1,000 females, the additional X chromosome is maternal in origin and associated with increasing maternal age in approximately 95% of cases (Hassold et al., 1996). Individuals often present with normal phenotypes including weight, mental function and pre-pubertal development, individuals are usually fertile but display an early onset of the menopause at around 30 years of age compared to the general population (around 50 years of age) (May et al., 1990). However, individuals with four or more X chromosomes have been reported to present with clinical features and is attributed to the increased dosage of genes that escape X inactivation and as a result the severity of the clinical features reported increase in proportion with the number of X chromosomes (Shah et al., 2003).

1.4.2.3 Klinefelter Syndrome (47,XXY)

In this syndrome affected males, prior to puberty are phenotypically normal, with the exception that they are generally tall with disproportionately long and thin legs. After puberty however manifestations include small atrophic testes that are severely depleted or absent of germ cells, and impaired secondary sexual characteristics, educational disorders especially that of dyslexia, normal intelligence or some mild retardation with the majority of patients being infertile.

There are two main karyotypes found in these cases:

1) Non-mosaic	47,XXY	85%
2) Mosaic	46,XY/47,XXY	15%

(in some rare cases, others variants of the syndrome are found: 48,XXXY, 48,XXYY, 49,XXXXY, and in mosaicism more than two cell lines are found). The incidence of Klinefelters is estimated to be around 1 in 1,000 live male births, 1 in 300 spontaneous abortions, 1-2 in 100 infertile men and 7-13 in 100 azoospermic men (Mak and Jarvi, 1996). Klinefelter syndrome is caused by the meiotic non-disjunction of the XY bivalent at male meiosis I (50%), or of the X chromosome in female meiosis I (33%), with the remainder of cases as a result of errors in female meiosis II or from postzygotic mitotic error leading to mosaicism. The severity of Klinefelter syndrome varies, some tubules may contain Sertoli cells whereas others may be completely devoid. The range of spermatogenesis and the production of mature spermatozoa is sometimes seen, however, this is often when a mosaic karyotype is present. The severity of the syndrome is associated with which karyotype is presented, those with a mosaic karyotype generally have less severe abnormalities than those with the non-mosaic karyotype, also

the increasing number of X chromosomes present the greater the severity of the phenotype.

1.4.2.4 XYY syndrome

47,XYY syndrome, has an estimated incidence of 1-4 in 1,000 of all live male births (Mak and Jarvi, 1996). These men are often phenotypically tall and the origin of this syndrome is as a result of non-disjunction of the Y chromosome at paternal MII résulting in YY disomic spermatozoa. In the majority of cases these men are severely oligozoospermic or azoospermic. The mechanism of reduced sperm production in affected individuals appears to be secondary to spermatogenic arrest of most YY germ cells due to abnormal meiotic pairing during meiosis, (Speed et al., 1991). The severity of the damage to the germ cells has been shown to correlate with higher FSH and LH levels (Mak and Jarvi, 1996).

1.4.2.5 Noonan syndrome

Noonan syndrome is the male equivalent of Turner syndrome, the incidence is found to be around 1 in 1,000-2,500, the phenotype presented is similar to that of Turner's syndrome. Karyotype analysis of these patients reveals a 46,XO/XY mosaicism, in most cases males have compromised testicular function and elevated gonadotrophins, cryptorchidism and testicular atrophy.

1.4.3 Autosomal trisomies

1.4.3.1 Trisomy 21 (Down syndrome)

The incidence of Down syndrome occurs at an estimated rate of 1 in 700, in rare cases, female patients with Down syndrome may become pregnant and bear children. The

majority if not all males, however have impaired spermatogenesis, males are almost exclusively sterile, presenting with spermatogenetic arrest, reduced number of germ cells, and hyalinized tubules. The mechanism by which trisomy 21 affects testicular histology is unclear, the prevailing hypothesis is that there may be a reduced proliferation of the primordial germ cells during their migration to the gonadal ridge, perhaps associated with an accelerated rate of apoptosis. (Patrizio and Broomfield, 1999).

1.4.4 Maternal age effect

The relationship between reduced fertility in women and increasing maternal age is long established (Hassold et al., 1996). Increasing maternal age has been significantly correlated an increased risk of producing trisomic offspring such as Down syndrome, however, increasing age has also been found to be the most common cause of pregnancy loss. An estimate of 25% of first trimester spontaneous abortions have been found to be trisomic. This may be a conservative estimate as it is likely that many other aneuploid conceptuses are spontaneously aborted prior to reaching the stage of clinical recognition (including autosomal monosomies and trisomies of chromosomes 1 and 19) (Shah et al., 2003). Several models have been put forward to explain the effect of maternal age, the first of which is the "production line hypothesis" and is based on the findings of Henderson and Edwards (1968) in rabbit eggs. This model proposes that oocytes that enter meiosis first are the first to be ovulated, with those entering meiosis last being ovulated last, and that these are more prone to non-disjunction. This therefore suggests that the depletion of oocytes within the ovaries results in the remainder being more prone to mal-segregation (Peters and McNatty., 1980). There are however, some difficulties with this particular model, in that mammalian eggs all enter meiosis

prenatally proceeding to diplotene whereby they then arrest until ovulation when the eggs resume meiosis (Griffin, 1996a). Secondly, it has been hypothesised that it is unlikely that such a significant effect on chromosome segregation could occur years later as a result of events initiated prior to birth (Griffin et al., 1996a; Koehler et al., 1996; Speed and Chandley et al., 1983). As a result, the Local factors Hypothesis was proposed (Crowley et al., 1979; Eichenlaub-Ritter et al., 1988; Sugarawa and Mikamo et al., 1983), suggesting that the effect seen may be due to extrinsic factors within the ovary that occur with increasing age that directly affect the eggs. This suggests that the ovarian environment itself becomes compromised as a woman ages and that the eggs in the ovary become more prone to the mal-segregation of chromosomes. A number of changes within the ovary have been associated with increasing age and subsequently affecting chromosome segregation including that of hormone levels, pH and oxygen concentration (Gaulden., 1992). In fact several studies have demonstrated that the spindle apparatus in older women with a lower intracellular pH is less well-formed (Van Blerkom et al., 1997; Van Blerkom., 1998; 2000; Van Blerkom and Davis., 2001). This hypothesis has been backed up further by several studies that have compared the non-disjunction of human chromosome 21 and the Drosophila NOD^{DTW} mutation (Hawley et al., 1994; Koehler et al., 1996). These studies have proposed that older and younger eggs with proximal (or two) chiasmata have fewer problems segregating chromosome bivalents whereas older eggs that have either no or distal chiasmata are more likely to have difficulties with the segregation of the bivalents compared to younger eggs. Secondly it has been suggested that the human homologue of the NOD gene product in Drosophila plays a central role and is a kinesin like protein, which is believed to be involved in maintaining contact between chromosome homologues and between the centromeres and the kinetochores (Shah et al., 2003). At present this seems to be the

prevailing hypothesis, however the lack of human material to experiment with and few comparable mammalian systems have made it difficult to identify whether this is the mechanism involved to explain this phenomenon as being the mechanism involved.

1.4.4.1 Differential aneuploidy rates in human male and female gametes

As discussed previously the process of meiosis (section 1.3.1) is responsible for the generation of haploid gametes and genetic variation among the population. The correct segregation of chromosome homologues requires the formation of reciprocal genetic exchanges a process known as recombination that results in the formation of chiasmata (the physical manifestations of genetic recombination) along the length of the chromosome (reviewed by Smith and Nicolas, 1998). Errors within meiotic chromosome segregation result in aneuploid gametes and have been found to occur at different rates within females and males. The rate of aneuploidy that occurs within female gametes is difficult to estimate, primarily due to the difficulties in obtaining female oocytes. Hassold and Hunt, (2001), reviewed studies investigating the levels of aneuploidy in IVF-derived pre-implantation embryos and that of 'control' oocytes, these studies have indicated that the overall aneuploidy rate may be in excess of 20%. In contrast it has been estimated that male gametes have a much lower incidence of aneuploidy with estimates placing this at around 2% (Hassold, 1998). Errors in meiotic chromosome segregation occur frequently in human females, particularly in the first meiotic division (MI). Maternal MI segregation errors predominate amongst almost all trisomies (reviewed by Hassold and Hunt, 2001). This is not surprising however, when considering the differences in the process of meiosis in males and females (section 1.3.1). In contrast to males, females undergo a protracted arrest of meiosis in which all oocytes enter meiosis prenatally and are arrested at MI prior to ovulation, as a result the

completion of MI could take 40 years or longer (reviewed by Hunt and LeMarie-Adkins, 1998).

1.4.4.2 Possible mechanisms for this disparity in aneuploidy rates

The mechanisms for this disparity between aneuploidy rates in males and females are not yet clear, however the use of genetic mapping techniques used to study the inheritance of DNA polymorphisms in human trisomic conceptions has uncovered an association of aberrant recombination and non-disjunction in humans by comparing the frequency and distribution of meiotic exchanges in trisomy-generating meioses (Hassold et al., 2000). These studies are reviewed by Hassold and Hunt, (2001), the general findings of these studies suggest that all MI-derived trisomies studied to date exhibit significant reductions in recombination and include paternally derived cases of trisomy 21 and 47,XXY, and maternally derived cases of 15, 16, 18, 21 and sex chromosome trisomies. The extent of reduced recombination is variable, with the most marked reduction seen in paternally derived 47,XXYs, in which a reduction of around 5 fold is seen in the genetic map of the XY pairing region between normal meioses and trisomy-generating meioses (around 50 cM compared to 10-15 cM) (Thomas et al., 2000). The effect seen for other chromosomes varies, however it is probable that decreased recombination is associated with all human trisomic conditions (reviewed by Hassold and Hunt, 2001). The reduced map lengths seen in different trisomic conditions are believed to arise through failure of the chromosomes to recombine (hence the chromosome bivalent was achiasmate),or a reduction in the number of chiasmata. In this case it would involve a bivalent that in the normal situation contains 2 or more chiasmata, however only a single sub-optimally placed chiasma was present (reviewed by Hassold and Hunt, 2001). These reports suggest that both decreased and aberrantly

situated recombination events are risk factors for meiotic non-disjunction (Koehler et al., 1996).

The identification of meiotic division checkpoints have also provided possible reasons for the disparity in aneuploidy rates between males and females, in particular a spindle assembly checkpoint mechanism that operates at the metaphase/anaphase transition (Wells and Murray, 1996). This checkpoint ensures that there are no defects in spindle assembly or chromosome alignment at the spindle equator prior to the initiation of anaphase. In the mouse, there is evidence of a gender specific effect of an achiasmate chromosome on meiotic cell division. For example in the male mouse, the existence of a univalent chromosome results in the metaphase arrest and as a consequence the animal is sterile (Sutcliffe et al., 1991). LeMarie-Adkins et al. (1997) however, investigated meiotic cell cycle progression in murine oocytes from XO females and control siblings. The results from this study demonstrated that even though the X chromosome did not align at the metaphase plate in the majority of cases no delay was detected in the onset of anaphase. The results of this study lead LeMarie-Adkins et al. (1997) to suggest that mammalian female meiosis may lack this stringent chromosome-mediated checkpoint control that operates in male meiosis and hence provide an explanation for the disparity in gamete aneuploidy rate between males and females.

1.4.5 Aneuploidy in human sperm

Sperm aneuploidy and its association with male infertility is considered within the following sections on chromosomes in human sperm.

1.5 Chromosomal studies on human sperm

1.5.1 Techniques used to analyse chromosome complement of spermatozoa

1.5.1.1 Hamster oocyte-human sperm fusion assay

Whole, G-banded sperm chromosome complements can be analysed by karyotyping sperm using the hamster oocyte-human (Humster) sperm fusion system, this system has advantages as it generates information on both numerical and structural abnormalities of all the chromosomes. Disadvantages however, include the fact that it is a technically demanding method that requires a considerable amount time and is a costly procedure, hence is only capable of analysing a few cells and therefore is only able to generate a small amount of data. Results using this system are also potentially biased, whereby only spermatozoa that are able to fertilise hamster oocytes can be karyotyped, potentially excluding spermatozoa with morphological disadvantages or genetic mutations (Jacobs et al., 1992; Martin et al., 1993). The Humster assay has however, provided a useful benchmark for which data generated from subsequent assays can be compared against (Downie et al., 1997).

1.5.1.2 In-situ hybridisation (ISH)

The technique in-situ hybridisation enables the hybridisation of a chromosome specific DNA probe to bind to complementary sequences on a target chromosome, following this the bound probe is then detected, and hence the chromosome (Downie et al., 1997). Early studies utilising this technique to study the chromosome complement of spermatozoa used isotopic detection methods, however this technique was quickly replaced with non-isotopic detection methods as long autoradiography times were required and the signals produced were often indistinct (Downie et al., 1997). The major

drawback of early ISH techniques is that they only enabled one colour to be used and as a result could not provide reliable estimates of disomic spermatozoa as it is not possible to distinguish disomic cells from that of diploid cells.

1.5.1.3 Fluorescent in-situ hybridisation (FISH)

Since the use of isotopic ISH in the study of aneuploidy in spermatozoa, the non-isotopic method used in subsequent studies has been fluorescent in-situ hybridisation (FISH) a technique that utilises fluorochromes in the detection of probes (in either red, green, blue or a combination of these). The advent of FISH has revolutionised studies on chromosomes, and the development of this technique has been rapidly adopted in an attempt to identify chromosomal abnormalities in spermatozoa. Two methods of FISH have been used in these studies, including indirectly labelled FISH and directly labelled FISH. Indirectly labelled FISH involves the hybridisation of a DNA probe labelled with either biotin or digoxigenin to the target chromosome(s). Following hybridisation the DNA probe is then detected using a fluorochrome-conjugated protein for example avidin, for biotinylated probes or a fluorochrome-conjugated antibody for the detection of digoxigenin labelled probes. This technique is advantageous as it is a highly sensitive technique and enables the intensity of the signals produced to be increased by the consecutive amplification of the signal using antibodies. This method however, is costly and also produces higher background labelling and is more time consuming compared to directly labelled FISH. Directly labelled FISH technique uses probes in which fluorochromes including Cy3, Cy5, FITC, TRITC and Texas Red have been incorporated within the probe. This is advantageous over indirectly labelled FISH as it does not require post hybridisation detection of the haptens and reduces the non-specific binding of probes.

The use of FISH has many advantages over previous techniques (Humster sperm fusion and ISH) as the technique itself is rapid, taking 2-3 days, and enables the analysis of thousands of spermatozoa to be undertaken for 2-4 chromosomes at any one time. It is imperative that if autosomes are being investigated that a dual colour FISH is carried out and if the gonosomes are being analysed at least one autosome should also be investigated. This enables the distinction of disomic and diploid cells, and is required to provide estimates of accurate disomic frequencies (Griffin et al., 1995; 1996b). Thus enabling a large number of men to be screened for investigated chromosomes, and has proved to be a reliable technique for establishing the frequency of chromosome abnormalities in spermatozoa. The main disadvantage with the use of FISH over previous methods is that unlike the Humster assay it does not allow the detection of structural chromosomal abnormalities.

At least 100 studies have used FISH to examine the chromosome complement in spermatozoa, in order to address a number of issues:

- At what frequency do chromosome abnormalities in spermatozoa occur in the normal fertile population?
- Is there any age effect associated with increases in non-disjunction?
- Are certain chromosome pairs more susceptible to non-disjunction, than others?
- Is there any increased frequency of chromosome abnormalities in the spermatozoa of the infertile population?
- Are there any phenotypes that confer any increased risk of chromosome abnormalities in spermatozoa?

- Is there any increase in the frequency of chromosomally abnormal spermatozoa in men whose karyotype carries a numerical or structural abnormalities?
- Are there lifestyle/environmental factors that may be associated with increases in aneuploid frequencies in spermatozoa (will be considered in section 1.5.9)?
- Are there intrinsic factors such as DNA polymorphisms that may confer increased risks of chromosomally abnormal spermatozoa?

Studies addressing these points will be considered in subsequent sections.

During the early to mid 1990's at least 10 studies investigated the incidence of chromosome aneuploidy within the spermatozoa of normal fertile men using FISH. These studies, taken collectively, have examined the frequency of disomy and diploidy of both the autosomes and the gonosomes. These studies examined the rate of aneuploidy using single colour FISH or dual colour FISH for the sex chromosomes only, therefore disomic cells cannot be distinguished from diploid cells. These studies hence, almost certainly produce inaccurate high estimates of disomy levels as it is impossible to tell if the spermatozoon is truly disomic or if it is in fact a diploid cell. Since these early investigations the majority of studies now use a minimum of two colour FISH when investigating autosomes or three colour FISH when analysing gonosomes, thereby producing more accurate informative results. Studies have only been considered if dual or triple colour FISH has been utilised in the study depending on whether autosomes or gonosomes have been investigated.

1.5.2 FISH studies analysing the spermatozoa of normal fertile males

The identification of estimates of aneuploidy in the spermatozoa is of particular interest as chromosome abnormalities are known to cause infertility and are a leading cause of pregnancy loss and the birth of children with mental and physical handicaps (Hassold et al., 1993; 1996). Studies into aneuploidy frequencies in male gametes have been heavily investigated over the past decade, particularly as female gametes are not so easily accessible. Studies have investigated the rates of aneuploidy of the autosomes and gonosomes in the spermatozoa of normal fertile males and taken collectively all the chromosomes have been analysed by at least one study. Studies vary in the number of subjects analysed and the number of spermatozoa analysed per subject, however the majority of studies have analysed a minimum of 5,000 sperm nuclei per donor and per probe set.

The reported frequencies of disomic sperm found in normal fertile males for both the autosomes and gonosomes vary considerably from study to study for example for chromosome 16, the lowest frequency reported was in the region of 0.05% (Spriggs et al., 1996) with the highest being around 0.54% (Bischoff et al., 1994). A similar degree of variation was reported for the sex chromosomes from study to study, with the lowest reported frequency for XY disomy being around 0.1% (Griffin et al., 1995) and the highest frequency 0.79% (Chevret et al., 1995).

Studies investigating the chromosome complement of spermatozoa within the normal fertile population have been undertaken to answer several questions including an assessment of the baseline frequency of chromosome abnormalities in sperm, the

distribution of disomy in order to establish if non-disjunction occurs at a similar rate identifying if any chromosomes are more prone to non-disjunction.

1.5.2.1 Studies addressing the question as to whether particular chromosomes are more susceptible to non-disjunction

Rives et al. (1998), analysed aneuploidy frequencies for all the autosomes and gonosomes in 4 individuals, reporting all chromosomes were equally susceptible to non-disjunction, with disomy levels for the autosomes to be around 0.24%. Bischoff et al. (1994) investigated chromosomes 3, 4, 6, 7, 8, 10, 12, 16, 17, 18, X and Y in two individuals, one individual showed no significant difference in the rate of non-disjunction for the chromosomes investigated in agreement Rives et al. (1998). A significant difference however, in the rate of non-disjunction was observed for the autosomes in the other subject enrolled in the study. There may be a degree therefore of variability in non-disjunction and some individuals may be predisposed to higher or lower frequencies of non-disjunction (Bischoff et al., 1994). In contrast to Rives et al. (1998) several studies have demonstrated increased rates of non-disjunction for chromosomes investigated. Spriggs et al. (1995) reported significant increases in the disomy rates of chromosome 21 and the sex chromosomes in five subjects compared to chromosomes 1, 2, 4, 9, 12, 15, 16, 18 and 20. Subsequently the same group (Martin et al., 1999a) analysed the same five individuals for chromosomes 13 and 22 to establish if all acrocentric chromosomes have an increased rate of non-disjunction, or whether chromosome 21 is an exception. In this study the frequency of non-disjunction for chromosome 13 (0.19%) was not significantly different to that of the other autosomes investigated previously by Spriggs et al. (1995), however, chromosome 22 showed a significant increase with a disomy rate of 1.21%, suggesting that the G- group

chromosomes are potentially more prone to non-disjunction. In agreement Blanco et al. (1996) analysed the levels of non-disjunction for chromosomes 6 and 21 and found that chromosome 21 demonstrated a significantly higher rate of non-disjunction compared to chromosome 6. Williams et al. (1993) reported the frequency of sex chromosome disomies to be up to 2 fold greater than that of chromosomes 16 and 18. Increased frequency of sex chromosome disomies have also been reported in two studies (Downie et al., 1997; Scarpato et al., 1998). However, Downie et al. (1997) also reported a significant increase in the disomy frequency of chromosome 3.

In summary a number of studies have found evidence that certain chromosomes are more susceptible to non-disjunction than others in the normal fertile population. Including chromosome 3 (Blanco et al., 1996); chromosome 18 (Bischoff et al., 1994); chromosome 21 (Blanco et al., 1996; Spriggs et al., 1996); chromosome 22 (Martin et al., 1999) and the sex chromosomes particularly the XY bivalent (Scarpato et al., 1998; Spriggs et al., 1996; Williams et al., 1993).

1.5.2.2 Gonosome aneuploidy levels within normal fertile males

The vast majority of studies have investigated the frequency of aneuploidy in the sex chromosomes and these studies report the mean frequencies to lie between 0.02% and 0.38% for XX disomy, 0.06% and 0.34% for XY disomy and finally 0.01%- 0.21% for YY disomy (Abruzzo et al., 1996; Bautmanger et al., 1999; Bischoff et al., 1994; Chevret et al., 1995; Downie et al., 1997; Goldman et al., 1993; Griffin et al., 1995; Martin et al., 1995; Rives et al., 1998; Shi et al., 2000; Spriggs et al., 1996; Williams et al., 1993). Five of these studies noted significant increases in the frequencies of XY disomy compared to XX and YY disomy (Chevret et al., 1995; Downie et al., 1997;

Goldman et al., 1993; Shi et al., 2000; Rives et al., 1998) with levels ranging from 0.13%-0.34%. Bischoff et al. (1994) however, reported a significant increase in XX disomy (0.38%) compared to XY (0.12%) and YY disomy (0.09%). Several studies have reported no significant difference between the disomy frequencies of XX, XY or YY, these studies also reported lower incidences of these disomies compared to the studies taken collectively with values ranging from 0.02%-0.07% for XX, 0.06%-0.16% for XY and 0.03%-0.21% for YY disomy (Abruzzo et al., 1996; Bautmanger et al., 1999; Griffin et al., 1995; Martin et al., 1995; Spriggs et al., 1996; Williams et al., 1993).

The following studies: Goldman et al., 1993; Griffin et al., 1995; Martin et al., 1995; Martin et al., 1996; Rives et al., 1998; Spriggs et al., 1995 investigated disomy rates in the autosomes and gonosomes with three groups (Goldman et al., 1993; Martin et al., 1996; Spriggs et al., 1995) reporting the incidence of sex chromosome disomy to be significantly higher than that of autosome disomy.

Griffin et al. (1996b), carried out a study investigating whether there is any evidence that the sex chromosomes preferentially segregate with chromosome 18 or 21, to establish whether this would explain why with trisomy 18 there is an excess of females and with trisomy 21 there is an excess of males. No evidence of a preferential segregation of chromosome 18 with chromosome X was provided, however there was evidence of a preferential segregation with chromosome 21 and chromosome Y. Scarpato et al. (1998) also reported evidence of a preferential segregation in sperm disomic for chromosome 2 and chromosome X. These results suggest that autosomal aneuploidies may be preferentially associated with either chromosome X or Y.

1.5.3 FISH studies on the spermatozoa, investing the effect of donor age

To date there have been a number of studies that have investigated the effect of increasing paternal age and semen parameters. These studies have generally indicated a correlation between increasing paternal age and a reduction in quality of normal semen parameters. In a review study (Kidd et al., 2001) investigating studies published over the last 20 years, a general consensus indicated that increasing age is associated with a decline in semen volume and sperm motility and an increase in morphologically abnormal spermatozoa, and a decrease in sperm motility, however, no reliable effect was identified for sperm concentration. Increasing age may not only have a consequence on semen quality but may also affect the genetic integrity of spermatozoa. Such a relationship between advancing maternal age and significant increases in the generation of aneuploid conceptuses has been well established (refer to section 1.4.4). Studies investigating paternal age have analysed chromosomes 1, 6, 8, 9, 12, 13, 14, 17, 18, 21 and the sex chromosomes.

1.5.3.1 Autosomes investigated for paternal age effect

Martin et al. (1995) conducted a study examining the aneuploidy frequencies for chromosomes 1, 12 and the sex chromosomes in 10 normal men aged 21-52, providing evidence of a significant association between an increase in disomy for chromosome 1 and an increase in donor age. In contrast several other studies have found no such age-related increase for chromosome 1 (Guttenbach et al., 2000; Kinakin et al., 1997; McInnes et al., 1998). Evidence for this association and chromosome 21 was also investigated in several studies, Rousseaux et al. (1998) analysed the chromosome 21 disomy frequencies in 3 men aged over 60 and 8 men aged under 30. In two out of the

three individuals aged over 60 significantly higher levels of disomy 21 were found compared to controls, suggesting there is evidence of a small age effect on the non-disjunction rate of chromosome 21. In contrast Bosch et al. (2001) and McInnes et al. (1998) identified no such age for chromosome 21. The following autosomes have also been investigated including chromosomes 6, (Bosch et al., 2001); 8, (Robbins et al., 1995); 9, (Luetjens et al., 2002); 12, (Asada et al., 2000; Martin et al., 1995); 13, (McInnes et al., 1998) 14, (Rousseaux et al., 1998); 17, (Guttenbach et al., 2000) and chromosome 18 (Asada et al., 2000; Griffin et al., 1995; Guttenbach et al., 2000; Luetjens et al. 2002). These studies have revealed no association between increasing donor age and increasing aneuploidy for these autosomes investigated.

1.5.3.2 Sex chromosomes investigated for paternal age effect

The majority of studies investigating the effect of paternal age on non-disjunction of sperm have analysed sex chromosomes due to the contribution of paternally derived sex chromosomes trisomies these include XYY - 100%, XXY - 50% and XXX 5% (Griffin, 1996b).

Griffin et al. (1995) investigated disomy frequencies for chromosomes 18, X and Y in 24 men aged 18-60. Significant associations with increasing age and XX, XY and YY disomy was identified. Robbins et al. (1995) analysed 4 individuals aged 42-50 and compared these against 10 younger individuals, the chromosomes investigated included 8, X and Y in agreement with Griffin et al. (1995) a significant association was found with XX and YY disomy, with a borderline effect found for XY disomy. Conversely an association with XY disomy was reported in two studies (Asada et al., 2000; Guttenbach et al., 2000) however, these studies found no association for XX or YY

disomy. Kinakin et al. (1997) and Martin et al. (1995) revealed evidence of a paternal age effect and YY disomy, but no evidence for XX or XY disomy. One study (Bosch et al., 2001), however reported no such association in sex chromosome disomy levels and increasing paternal age in 18 individuals aged 24-74. On the contrary a number of studies have demonstrated associations with chromosome 1 (Martin et al., 1995), chromosome 21 (Rousseaux et al., 1998) sex chromosome disomies XX (Griffin et al., 1995; Robbins et al., 1995); XY disomy (Asada et al., 2000; Griffin et al., 1995; Guttenbach et al., 2000) and YY disomy (Griffin et al., 1995; Kinakin et al., 1997; Martin et al., 1995; Robbins et al., 1995).

At present there is no consensus of opinion amongst studies investigating paternal age effects and non-disjunction. There is evidence, however to suggest that older men like older women have increased risks of producing aneuploid offspring , especially for the sex chromosomes.

1.5.4 FISH studies on the spermatozoa of infertile men

The results of the studies investigating aneuploidy frequencies (mean values are presented for patient groups unless stated otherwise) in the sperm of infertile males, aneuploidy frequencies are also presented in table 1.3 (page 70).

1.5.4.1 Chromosomes 1-5 (Groups A and B)

At least ten studies have investigated the incidence of disomy 1 in infertile males. The majority report a significant increase in the frequency of disomy 1 when compared with fertile controls (Finkelstein et al., 1998; Lahdetie et al., 1997; Moosani et al., 1995; Pfeffer et al., 1999; Rives et al., 1999; Viville et al., 2000) with levels ranging from

0.18%- 1.35%. Rives et al. (1999) examined 50 patients with various compromised semen parameters and 10 males with proven fertility. Patient levels (0.34%) were significantly higher than controls (0.22%). Moosani et al. (1995) also reported an approximate two fold increase in 5 males with idiopathic infertility (0.18% in patients compared to 0.09% in controls) however it is noteworthy that the patient levels in the second study were lower than the control levels in the first. In order to distinguish the motile from the immotile fraction (a common procedure in infertility clinics) Pfeffer et al. (1999) examined the pellet and supernatant of "swim-up" preparations in 10 males with severe OAT (as defined by Pang et al. (1999) and four healthy control donors. The patients had significantly higher disomy frequencies in both the pellet and swim up preparation (0.62% and 0.57% respectively) compared to the control individuals (0.28% and 0.17% respectively) but no significant difference between pellet and supernatant. Lahdetie et al. (1997) investigated three groups of individuals: four OAT patients (group 1), eight with normal/intermediate semen parameters (group 2) and 18 healthy fertile males (group 3). A significant increase was found in group 1 (0.22% compared to 0.08% and 0.1% for groups 2 and 3 respectively). Finkelstein et al. (1998) enrolled 12 subjects with poor quality semen as a result of a varicocele; a significant increase (1.35%) was noted, compared to eight controls (0.08%). Viville et al. (2000) concentrated their efforts on four patients exhibiting 100% teratozoospermia. Three had similar levels to controls, however, one (presenting with 100% macrocephalic spermatozoa) presented with a markedly significantly higher rate of disomy 1 (17.7% compared to 0% in controls).

While the majority report an association between increased disomy levels for chromosome 1 and infertility, several studies (Guttenbach et al., 1997; Harkonen et al.,

2001; Miharu et al., 1994) found no evidence of such an increase. Guttenbach et al. (1997), examined 45 males with varying compromised semen parameters (and 8 controls) reporting no significant difference between them (0.13% and 0.12% respectively). Similarly Miharu et al. (1994), analysed 9 patients and 12 controls; reporting frequencies of 0.14% and 0.13% respectively) and Harkonen et al. (2001) (studying 20 teratozoospermic males) found no evidence of an association between disomy 1 and male infertility with control frequencies of 0.07% and infertile frequencies of 0.12%.

As far as we are aware, only one study (Pang et al., 1999) has examined a chromosome apart from 1 in chromosome groups A and B. Disomy analysis of chromosome 4 in nine OAT males and four control donors revealed a significant increase in the patient cohort (1.58% versus 0.15%).

1.5.4.2 Group C autosomes (chromosomes 6-12)

Pang et al. (1999) is unique in having analysed all of the C group chromosomes. Each were found to have significantly higher disomy levels in nine OAT males compared to controls. The mean frequencies obtained follow for patients and controls respectively: chromosome 6 (1.38%, 0.13%); 7 (1.83%, 0.31%); 8 (1.74%, 0.23%); 9 (1.68%, 0.2%); 10 (1.63%, 0.2%); 11 (1.68%, 0.13%); and 12 (0.69%, 0.05%).

In keeping with their results for chromosome 1, Lahdetie et al. (1997) reported a significant increase in disomy 7 levels in OAT individuals (0.13% compared to 0.06%), partially supporting Pang et al. (1999) but suggesting a more modest increase. Similar findings were reported by Harkonen et al. (2001) with a significant increase in disomy

frequencies of teratozoospermic individuals of 0.12% versus 0.06% within the control group. Guttenbach et al. (1997) however, as for chromosome 1, found no such evidence for chromosome 7 (0.12% in patients and 0.15% in controls).

Calogero et al. (2001a) studied chromosome 8 disomy levels in spermatozoa of 3 groups (19 OAT individuals (group 1), 9 teratozoospermic men (group 2) and 13 controls (group 3) groups 1 and 2 displayed significantly higher disomy frequencies with values of 0.49%, 0.46% and 0.15% respectively. Burrello et al. (2002) focussed their attention on azoospermic individuals, extracting spermatozoa from the testes (six donors) or epididymis (ten donors); there were 14 normally fertile controls. Significantly higher frequencies of chromosome 8 disomy were observed in the two azoospermic groups with levels of 0.68% for epididymal spermatozoa and 1.03% for testicular spermatozoa versus 0.18% in the control group. Acar et al. (2000) however reported no such increase within the six infertile males (all of whom had at least one compromised semen parameter) compared to controls with frequencies of 1.33% and 0.69% respectively. Guttenbach et al. (1997), extended their analyses to chromosome 10 and, again, found no significant difference between the infertile and control group (frequencies of 0.12% and 0.14% respectively). In agreement Acar et al. (2000) reported no significant difference between disomy 10 frequencies between the infertile and control groups with frequencies of 1.45% and 0.95% respectively.

Several authors have asked the same question of chromosome 12 (Asada et al., 2000; Burrello et al., 2002; Calogero et al., 2001a; Moosani et al., 1995). In contrast to the findings of Pang et al. (1999) no significant difference was observed between infertile (group 1) and control (group 2) individuals in any of these studies. (Group 1 -0.19%,

group 2 - 0.15% (Moosani et al., 1995). Calogero et al. (2001a) and Burrello et al. (2002) 0% in both groups. Asada et al. (2000) examined three groups of men including; ten men over the age of 39 with idiopathic infertility and normozoospermia (or with a sperm concentration of > 20 million/ml), five males with oligozoospermia (with a sperm concentration of < 20 million/ml) and ten control donors aged 25 or less. No significant difference was observed between the three groups for disomy 12.

1.5.4.3 Group D (chromosomes 13-15)

Group D is a clinically significant group as approximately 10-15% of Patau syndrome cases arise as a result of disomy 13 sperm and because uniparental disomy of paternal chromosome 15 is a cause of Angelman syndrome (Griffin, 1996b). To date at least 10 studies have investigated the incidence of disomy for chromosome 13 and they suggest a significant increase in infertile individuals compared to controls (Hristova et al., 2002; Martin et al., 2003a; McInnes et al., 1998; Pang et al., 1999; Pfeffer et al., 1999; Rives et al., 1999; Templado et al., 2002; Ushijima et al., 2000; Vegetti et al., 2000). Pang et al. (1999) reported levels of 1.86% in OAT men compared to 0.2% for controls and, consistent with these findings, Pfeffer et al. (1999) reported frequencies of 0.88% and 1.03% in OAT men in pellet and swim up preparations respectively and 0.3% in controls. Carrell et al, (2003) investigated 24 patients with a history of recurrent pregnancy loss (RPL) (≥ 3 pregnancy losses) and two control groups 16 males from the general population and 10 fertile donors. Disomy 13 frequencies in the RPL patients were found to be significantly higher than in the two control groups with levels of 1.02%, 0.44% and 0.39% respectively. Martin et al, (2003a) investigated 30 infertile males split into three groups of 10 exhibiting mild, moderate and severe oligozoospermia (10-19 x 10^6 sperm/ml, 1-9 x 10^6 sperm/ml and <10^6 sperm/ml,

respectively) with disomy 13 rates of 0.17%, 0.24% and 0.3% in respective groups. Martin et al. (2003a) report a marginal trend of increased chromosome abnormality with decreased sperm concentration for chromosome 13. McInnes et al. (1998) analysed nine infertile men presenting with either oligozoospermia or teratozoospermia and found a significant increase in both groups compared to 18 controls (0.28% versus 0.13%). Rives et al. (1999) reported levels of 0.32% in patients versus 0.21% in controls and Templado et al. (2002) analysed asthenoteratozoospermic patients and 18 controls finding a significant increase in the patient cohort (0.23% compared to 0.13% in controls. Hristova et al. (2002) reported similar findings in ten infertile patients and controls (0.24% versus 0.13%) while Ushijima et al. (2000) studied eight OAT patients and ten controls finding much lower levels overall but nevertheless a significantly higher rate of chromosome 13 disomy (0.13%) compared to controls (0.09%). Vegetti et al. (2000) analysed four groups of men: group 1 contained 15 individuals with abnormal semen parameters but normal karyotypes; group 2 contained 13 individuals with abnormal karyotypes; group 3 contained 4 karyotypically normal individuals with abnormal semen parameters that had undergone ICSI resulting in a child/ foetus with chromosome abnormalities, and group 4 normal fertile control donors. They established a significantly higher mean frequency of disomy 13 in groups 1 (0.23%) and 2 (0.21%) but not group 3 (control rate was 0.08%).

In contrast to the above, two studies reported no significant association between infertile males and any increase in sperm disomy for chromosome 13. Martin et al. (2000) studied three patients with non-obstructive azoospermia, no significant increase was found with levels of 0.2% compared to 0.13% in the controls. Rubio et al. (2001) reported no significant difference between disomy 13 frequency within infertile males

(0.17%) compared to control frequencies (0.14%). Schultz et al. (2000) also found no significant increase but made use of the alpha-satellite probe that hybridises to both chromosomes 13 and 21 and thus the validity of the results may be called into question when drawing conclusions about chromosome 13 alone.

The remainder of group D chromosomes (14 and 15) have been examined to a lesser extent with only one study investigating chromosome 14 disomy (Rives et al., 1999) and one for chromosome 15 disomy (Finkelstein et al., 1998). Rives et al. (1999), observed significantly higher frequencies of chromosome 14 disomy (0.43%) compared to that of controls (0.22%). Finkelstein et al. (1998) reported highly significant increases in disomy 15 in their infertile population (1.67%) compared to controls (0.09%).

1.5.4.4 Chromosomes 16-20 (groups E and F)

In these groups, chromosomes 16 and 18 are the most clinically significant. Trisomy 16 is the most common trisomy among spontaneous abortions however we are not aware of a convincing report of a trisomy 16 that has arisen as a result of a disomy 16 sperm. Approximately 10% of trisomy 18 conceptuses (including those that go to term and lead to Edwards syndrome babies) arise as a result of disomy 18 sperm. Miharu et al. (1994) analysed disomy rates of chromosome 16, no significant differences were observed between infertile and control groups with values of 0.16% and 0.17% respectively.

Chromosome 17 disomy and its association with infertility has been investigated by two sets of authors (Guttenbach et al., 1997; Pang et al., 1999). As with the other chromosomes studied, Pang et al. (1999) reported a significant increase in OAT men (1.62% versus 0.2% in controls). Similarly Guttenbach et al. (1997) were consistent in

their findings for other chromosomes recording no significant difference in patients and controls (0.13% and 0.16% respectively).

Chromosome 18 is a commonly studied chromosome partly because of its clinical significance and partly because of the early availability of the reliable alpha satellite probe. The vast majority of studies investigating the incidence of chromosome 18 disomy within selected infertile populations and control donors found statistically significant increases in infertile males compared to controls. Pang et al. (1999) reported a significant increase in the frequency of disomy in OAT patients compared to controls (1.67% versus 0.11%). Colombero et al. (1999) suggested significant increases in infertile patients with chromosome disomy 18 levels of 0.6%, however, they do not give control frequencies. Carrell et al, (2003) reported increased frequencies of disomy 18 in RPL (recurrent pregnancy loss) patients compared to fertile control donors with rates of 0.51% and 0.25% respectively. Rives et al. (1999) also found an increase in disomy rate with 0.51% compared to control levels of 0.24%. Pfeffer et al. (1999) reported rates of 0.77% and 0.96% (pellet and swim up preparation respectively) versus 0.18% and 0.17% in OAT males and controls respectively. Finkelstein et al. (1998) and Calogero et al. (2001a) (2 groups) reported increased disomy levels within the infertile cohort with infertile male rates of 0.85%, 0.64% and 0.39% with control levels of 0.12% and 0.2% respectively. Vegetti et al. (2000), found significantly increased levels compared to controls (0.06%) in two infertile groups (including males with abnormal semen parameters and normal karyotypes and males with abnormal karyotypes) (frequencies of 0.22% and 0.19% respectively). Harkonen et al. (2001) and Rubio et al. (2001) reported a significant increase in disomy 18 in infertile males with frequencies of 0.1% and 0.05% compared to control frequencies of 0.05% and 0.02% respectively. A significant

increase in disomy 18 was also reported in spermatozoa surgically extracted from the testes and epididymis of patients with levels of 1.23% and 0.37% respectively versus 0.2% in the control group. (Burrello et al., 2002). Mateizel et al. (2002) investigated the rates of disomy 18 in testicular sperm derived from 17 patients with spermatogenic failure and 26 patients with normal spermatogenesis (considered as the control group). A significantly higher frequency of disomy 18 was reported in patients with spermatogenic failure (1.3% versus 0.3% within patients with normal spermatogenesis). Levron et al. (2001) reported a significant increase in disomy 18 in three groups of infertile males compared to controls, nonobstructive azoospermia, obstructive azoospermia and severe oligozoospermia with frequencies of 0.65%, 0.55% and 0.86% respectively. Control frequencies were not given for individual chromosomes however the total frequency for all the investigated chromosomes was reported to be 1.5%. In contrast, the following studies found no evidence of a significant increase in the disomy levels of chromosome 18 within the spermatozoa of infertile males. Aran et al. (1999) investigated the frequencies of disomy 18 in the spermatozoa of nineteen infertile patients and five control donors, reporting a rate of disomy of 0.05% and 0.09% respectively. Damri et al. (2000) investigated twelve individuals undergoing ICSI and three fertile controls, the rates of 0.06% versus 0.11% in the control group were obtained. Ohashi et al. (2001) reported disomy frequencies in ten severely oligozoospermic males (0.2%), ten oligozoospermic males (0.13%) and seven control males (0.17%). Nishikawa et al. (2000) reported frequencies of 0.15% within the ten infertile individuals analysed.

To the best of our knowledge no studies published to date have analysed the F group chromosomes (19 and 20) in this context.

1.5.4.5 Group G chromosomes (21-22)

Chromosome 21 is perhaps the most clinically significant of the chromosomes. Although predominantly of maternal origin, trisomy 21 arises ~7% of the time as a result of disomic sperm (Griffin, 1996b). Thus approximately 1 in 10,000 babies are paternally derived Down Syndrome cases and, despite the absence of a reliable alpha-satellite probe to detect the chromosome, a large number of studies (at least eleven) have examined chromosome 21 sperm disomy in this context. The majority noted a significant increase in the rate of disomy 21 in the infertile populations analysed compared to that of the controls enrolled in their studies. Pang et al. (1999) reported a mean frequency of 1.94% in OAT men and 0.18% in controls. Rives et al. (1999) noted higher levels of disomy in the infertile population analysed (0.5% compared to 0.22%), similarly Pfeffer et al. (1999) reported comparable frequencies of 0.48% and 0.46% in infertile males and control levels of 0.18% and 0.17% (in the pellet and swim up preparation respectively). Carrell et al, (2003) reported increased rates of chromosome 21 disomy in RPL patients compared to the general population and fertile control donors with levels of 0.47%, 0.28% and 0.24% respectively. Martin et al, (2003a) reported a marginal trend of increased chromosome 21 disomy frequencies with decreased sperm concentration 0.22% (mild oligozoospermia), 0.44% (moderate oligozoospermia) and 0.58% (severe oligozoospermia). Ushijima et al. (2000) reported a significant increase in the rate of disomy in infertile individuals (0.24%) compared to 0.19% observed in the controls while McInnes et al. (1998) recorded frequencies of 0.48% compared to controls (0.37%). Vegetti et al. (2000) identified a significant increase in disomy for chromosome 21 in two infertile groups (including males with abnormal semen parameters and normal karyotypes, and males with abnormal karyotypes) with frequencies of 0.29% and 0.22% respectively compared to control

frequencies of 0.07%. Rubio et al. (2001) also reported a significant increase in disomy 21 with frequencies of 0.24% and 0.17% in infertile males and controls respectively. Finally Colombero et al. (1999) reported a significant increase of 1.2% in patients compared to control levels.

As with most autosomes, several studies found no such evidence of an increase. Martin et al. (2000) observed frequencies of 0.46% in sperm extracted from the testes of azoospermic men, however, this was not significantly different to control levels (0.37%). Consistent with these findings Hristova et al. (2002) found frequencies of disomy to be similar to that of Martin et al. (2000) with infertile levels of 0.37% and control levels of 0.36%. Templado et al. (2002) reported frequencies of disomy 21 in infertile males to be 0.23% versus 0.36% within the control group. Guttenbach et al. (1997) reported lower frequencies of disomy although authors were in agreement finding no evidence of any difference between the infertile and control groups (0.14% and 0.12% respectively).

Not surprisingly, studies for chromosome 22 are less common in the literature and we are aware of only one. Rives et al. (1999) reported a significant increase in infertile men with a frequency of 0.42% compared to 0.25% observed in the controls.

1.5.4.6 Sex chromosomes

While chromosome 21 is perhaps the most clinically significant because of the severity of Down Syndrome, it is in the sex chromosomes where an increase in sperm disomy is more likely to have a effect on the frequency of trisomic conceptuses. Sex chromosome trisomies present with far milder clinical features than those of the autosomes,

nevertheless, they are, collectively more frequent among liveborns (0.45% of live births compared to 0.3% for Down Syndrome) and, unlike the autosomes, arise approximately 50% of the time in the sperm. That is nearly 50% of XXY, 5% of XXX, 25% of XO and 100% of XYY cases arise as a result of an aneuploid sperm (Griffin et al., 1995; 1996a). This, coupled with the availability of reliable FISH probes has made them the most studied chromosomes in this context. A further advantage of analysing the sex chromosomes is that it is possible to identify whether the disomic sperm has arisen as a result of a meiosis I (XY) or meiosis II error (XX or YY) (Griffin et al., 1995; 1996a). This is not usually possible in autosomes except where DNA polymorphisms have been identified (O'Keefe et al., 1997). Studies can be split into those that specify individual disomies (XX disomy, XY disomy and YY disomy) and those that do not specify the individual disomy results but report on the sex chromosome disomies as a whole.

These studies will be split into several groups those that found increases in XY disomy, XX disomy and YY disomy and those that do not specify the individual disomy results but report on the sex chromosome disomies as a whole. All the studies within these sections have been described previously in earlier sections as at least one autosome has also been analysed in conjunction with the sex chromosomes to enable disomic sperm to be distinguished from that of diploid.

1.5.4.6.1 XY Disomy

The XY bivalent is thought to be most prone to non-disjunction in the male gametes. Moreover most studies found statistically significant increases in the rate of XY disomy within infertile individuals analysed compared to levels obtained from control males. Pang et al. (1999) report a mean rate of 1.05% in OAT men versus 0.08% in controls.

Finkelstein et al. (1998) reported rates of 0.74% in the infertile males and 0.1% in control donors. Martin et al, (2003a) reported significantly increased rates of XY disomy coincident with decreasing sperm concentration with disomy rates of 0.25% (mild oligozoospermia), 1.04% (moderate oligozoospermia) and 0.68% (severe oligozoospermia). Three studies (Martin et al., 2000; Rives et al., 1999; Templado et al., 2002) established similar XY disomy frequencies for cohorts of infertile males investigated, with disomy levels of 0.62%, 0.54% and 0.5% respectively, compared to control levels of 0.3%, 0.35% and 0.3% respectively. Nishikawa et al. (2000) reported significantly higher frequencies of XY disomy in infertile males (0.36%) compared to controls (0.14%). Studies carried out by Moosani et al. (1995), Ushijima et al. (2000) and Vegetti et al. (2000) recorded the disomy frequency of the infertile individuals to be lower than that of the previous studies mentioned, nevertheless a significant increase was observed between the infertile and control males. That is 0.34%, 0.23%, and 0.2% compared to 0.16%, 0.14% and 0.05% respectively in their control groups. Harkonen et al. (2001) reported lower frequencies of 0.13% and 0.05% in infertile and control males respectively, nonetheless the rates were significantly increased within the infertile group. Ohashi et al. (2001) divided infertile males into two groups: severe oligozoospermic men (<5 million/ml) and males with oligozoospermia (5-20 million/ml). They found a significant increase in XY disomy only in the males with severe oligozoospermia (0.41%) whereas the frequency of disomy for the oligozoospermic males was found to be comparable to the control frequency (0.16% and 0.18% respectively). Levron et al. (2001) reported significant increases in the frequency of XY disomy compared to controls with frequencies of 3.27% (nonobstructive azoospermia), 2.18% (obstructive azoospermia), 0.17% (severe oligospermia), 1.5% (control frequency for all investigated chromosomes).

In contrast several studies have not demonstrated significantly increased levels of XY disomy in infertile males compared to controls (Calogero et al., 2001a, Hristova et al., 2002; Schultz et al., 1998; Acar et al., 2000; Burrello et al., 2002), 0% and 0.15% patients versus 0% controls (Calogero et al., 2001a), 0.41% patients versus 0.3% controls (Hristova et al., 2002); 0.44% patients versus 0.36 % controls (Schultz et al., 1998); 0.94% patients versus controls (Acar et al., 2000) and 0.5% and 0% patients versus 0% controls (Burrello et al., 2002). The latter study examined azoospermic males.

1.5.4.6.2 XX and YY Disomy

The following studies have all reported a significant increase in XX disomy in infertile males versus control populations: Pang et al. (1999) reported 0.7% disomy in OAT men versus 0.04% in controls. Calogero et al. (2001a) - 0.33% and 0.48% versus 0.2% in controls; Ushijima et al. (2000) and Finkelstein et al. (1998)- 0.16% versus 0.12% in controls, Templado et al. (2002) - 0.13% versus 0.05% in controls; Hristova et al. (2002) - 1% and 0.05% in controls. Vegetti et al. (2000) reported a significant increase in two groups of infertile men, namely those with abnormal semen parameters and normal karyotypes and those with abnormal karyotypes. XX disomy frequencies were 0.17% and 0.09% respectively compared to a control rate of 0.03%. Martin et al, (2003a) reported a significant increase in XX disomy in correlation with decreased sperm concentration, with rates of 0.04% (mild oligozoospermia), 0.08% (moderate oligozoospermia) and 0.10% (severe oligozoospermia). Burrello et al. (2002) reported a significant increase in XX disomy in spermatozoa extracted from the testes and epididymis of azoospermic men compared to controls (1.23%, 0.56% and 0.2% respectively). Frequencies of 2.61% (nonobstructive azoospermia), 1.63% (obstructive

azoospermia), 2.0% (severe oligospermia), 1.5% (control frequency for all investigated chromosomes) were reported in the study carried out by Levron et al. (2001).

XX disomy is the only one where the majority of studies have not found an increase in the infertile patient cohort compared to controls. Ohashi et al. (2001) examined males presenting with severe oligozoospermia and oligozoospermia; the rates for both these groups were reported as 0.07%, not significantly different from that of the control (0.11%). In the following list, the reported frequencies of infertile patients, fertile controls followed by the reference are given: 0.79%, 0.44% (Acar et al., 2000), 0.15%, 0.07% (Schultz et al., 2000), 0.1%, 0.15% (Nishikawa et al., 1999), 0.09%, 0.08% (Moosani et al., 1995); 0.06%, 0.05% (Martin et al., 2000), 0.22%, 0.2% (Rives et al., 1999) and 0.3%, 0.3% (Harkonen et al., 2001),

At least seven studies have provided evidence for an effect for YY disomy. Pang et al. (1999) recorded the most dramatic increase with levels in OAT men of 0.77% versus 0% within the controls. The remainder found lower but comparable frequencies. That is Calogero et al. (2001a) recorded 0.48% and 0.33% YY disomy in the infertile groups versus 0.16% controls. Rives et al. (1999) recorded 0.24% versus 0.17%, Ushijima et al. (2000) reported infertile levels of 0.21% versus 0.12%, Finkelstein et al. (1998) reported infertile frequencies of 0.18% versus 0.12%, Harkonen et al. (2001) reported infertile frequencies of 0.15% compared to 0.05% (all figures, infertile patients versus fertile controls (usually sperm donors). Vegetti et al. (2000) reported a significant increase in two groups (including males with abnormal semen parameters and normal karyotypes and males with abnormal karyotypes) (0.18% and 0.12% respectively) compared to controls (0.05%). Martin et al, (2003a) reported a significant increase in

YY disomy coincident with decreased sperm concentration with rates of 0.04% (mild oligozoospermia), 0.06% (moderate oligozoospermia) and 0.09% (severe oligozoospermia). Burrello et al. (2002) recorded a significant increase in the rate of YY disomy in both testicular and epididymal spermatozoa with an incidence of 0.77% and 0.59% versus control levels of 0.16%. Levron et al. (2001) reported significantly increased frequencies of YY disomy in males with nonobstructive azoospermia (1.31%), obstructive azoospermia (0.55%), severe oligospermia (1.6%), compared to the control frequency of 1.5% (total for all investigated chromosomes).

Equally there have been a number of studies whereby no difference was observed between the rate of YY disomy in the spermatozoa of infertile men compared to that of control donors. Asada et al. (2000); Hristova et al. (2002); Martin et al. (2000); Moosani et al. (1995); Nishikawa et al. (2000); Ohashi et al. (2001); Schultz et al. (2000) and Templado et al. (2002) collectively all report values of disomy within a similar range (0%-0.18%) for both patients and controls with no significant difference between the groups. Acar et al. (2000), are in agreement with these studies in not identifying any significant increase in YY disomy between infertile males and controls, however report a higher incidence with frequencies of 0.98% versus 0.41% respectively, although in this case only 400 sperm were analysed.

1.5.4.6.3 Studies that have considered sex chromosome disomy as a whole

A number of studies that have investigated the sex chromosome disomy collectively i.e. have not presented the results of the disomy rates for the individual chromosomes (i.e. XX, XY and YY disomy). These studies are clearly less informative but nevertheless significant. The majority have indicated increases in sex chromosome disomy in

infertile patients compared to fertile controls (Aran et al., 1999; Carrell et al., 2003; Colombero et al., 1999; Pfeffer at al., 1999). Carrell et al, (2003) reported a significant increase in sex chromosome disomy (0.77%) compared to the general population (0.40%) and fertile control donors (0.31%). Colombero et al. (1999) observed the disomy frequency of the infertile population studied to be 0.64% with control frequencies of 0.46%. Aran et al. (1999) reported similar sex chromosome disomy levels within the infertile males analysed to be 0.53% versus 0.37% in the control subjects. Pfeffer et al. (1999) analysed spermatozoa of infertile males prior to and after swim up preparation. This study concluded no significant difference in the rate of disomy prior to or post swim up preparation, however, they did note a significant increase in disomy of the sex chromosomes in both preparations (1.08% and 1.13% respectively) compared to that of the control (0.48% and 0.25% respectively). Mateizel et al. (2002) reported no significant difference in sex chromosome disomy in the infertile males and control males analysed with levels of 2.4% and 2.2% respectively. Damri et al. (2000), report sex chromosome frequencies of 0.18% in infertile and 0.28% in the control.

Miharu et al. (1994) and Guttenbach et al. (1997) report the disomy frequency of the sex chromosomes in two groups (X and Y), but do not as far as we can determine distinguish XX, YY and XY disomy, these studies found no statistical difference between the rates of disomy in the infertile and control individuals for chromosome X (0.16% versus 0.13%) (Miharu et al., 1994), (0.14% versus 0.12%) (Guttenbach et al., 1997) or chromosome Y (0.11% versus 0.08%) (Miharu et al., 1994), (0.1% versus 0.11%) (Guttenbach et al., 1997).

Table 1.3- The incidence of sperm disomy within control and infertile populations within the literature. Control values are displayed in the first row, with infertile frequencies highlighted in blue

Author	Year	See Key	1	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	21	22	XX	XY	YY	Sex	Statistical Test
Miharu	1994		0.14													0.17								0.11	
			0.13													**0.16**								**0.14**	
																									ANOVA
Moosani	1995		0.09									0.15									0.08	0.16	0.18		
			0.18									**0.19**									**0.09**	**0.34**	**0.09**		
p value			<0.001																			<0.001			Chi squared
Guttenbach	1997		0.12				0.15			0.14							0.16		0.12					0.12	
			0.13				**0.12**			**0.12**							**0.13**		**0.14**					**0.12**	Not specified
Lahdetie	1997		0.1				0.06																		
			0.22				**0.13**																		
p value			<.0001				<.0001																		Relative risks
Finkelstein	1998		0.08												0.09			0.12			0.12	0.1	0.12		
			1.35												**1.67**			**0.85**			**0.16**	**0.74**	**0.18**		
p value			0.004												0.004			0.004			0.001	0.05	0.221		Mann-Whitney U-test
p value			<0.001												<0.001			<0.001			<0.001	<0.001	>0.05		Chi squared
McInnes	1998												0.13						0.37						
													0.28						**0.48**						
p value													<.0001						<.0001						two-tailed Z statistic
Aran	1999																	0.09						**0.37**	
																		0.05						**0.53**	
p value																								**<0.05**	Chi squared
Colombero	1999	A																0.63	0.63					0.46	
																		0.84	**0.84**					**0.64**	
p value																									
Pang	1999			0.15		0.13	0.31	0.23	0.2	0.2	0.13	0.05	0.2				0.2	0.11	0.18		0.04	0.08	0		
				1.58		**1.38**	**1.83**	**1.74**	**1.68**	**1.63**	**1.68**	**0.69**	**1.86**				**1.62**	**1.67**	**1.94**		**0.7**	**1.05**	**0.77**		Chi squared,
p value				<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05				<0.05	<0.05	<0.05		<0.05	<0.05	<0.05		Fisher's exact tests
Pfeffer	1999	B	0.28										0.3					0.18	0.15					**0.48**	
			0.62										**0.88**					**0.77**	**0.48**					**1.08**	Chi squared,

Author	Year	See Key	1	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	21	22	XX	XY	YY	Sex	Statistical Test
p value			<0.05										<0.05					<0.05	<0.05					<0.05	Fisher's exact tests
Rives	1999		0.22										0.21	0.22				0.24	0.22	0.25	0.22	0.35	0.17		
			0.34										0.32	0.43				0.51	0.5	0.42	0.2	0.54	0.24		
p value			0.002										0.002	0.002				0.002	0.002	0.002		<.0002	0.018		Analysis of variance
Acar	2000							0.69		0.75											0.44	0.5	0.41		
								1.33		1.45											0.79	0.94	0.98		
p value																									Fisher's exact tests
Damri	2000																		0.11					0.28	
																			0.06					0.18	
p value																									
Martin	2000												0.13						0.37		0.05	0.3	0.06		
													0.2						0.46		0.06	0.62	0		
p value																							<0.001		two-tailed Z statistic
Nishikawa	2000																	0.14			0.15	0.14	0.14		
																		0.15			0.1	0.36	0.13		
p value																						<0.01			Chi squared
Shultz	2000	C											0.26					0.22			0.07	0.36	0.09		
													0.1					0.3			0.15	0.44	0.18		
p value																									Poisson distribution
Ushijima	2000												0.09						0.19		0.12	0.14	0.12		
													0.13						0.24		0.16	0.23	0.21		
p value													<0.001						<0.05					<0.001	Students t-test
Vegetti	2000												0.08					0.06	0.26		0.03	0.05	0.05		
		D											0.23*					0.22*	0.29*		0.17*	0.23*	0.18*		
		E											0.21*					0.19*	0.22*		0.09*	0.16*	0.12*		
		F											0.11					0.07	0.21		0.04	0.15	0.25		
p value													0.023					0.006	0.003		0.007	0.002	0.007		Wilcoxon score
Calogero	2001							0.15				0						0.2			0.2	0	0.16		
		G						0.49				0						0.64			0.33	0.15	0.33		
		H						0.46				0						0.39			0.48	0	0.48		
p value								<0.001										<0.001			0.009		<0.001		Mann-Whitney
Harkonen	2001		0.07				0.06											0.05			0.03	0.05	0.05		
			0.12				0.12											0.1			0.03	0.13	0.15		

Author	Year	See Key	1	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	21	22	XX	XY	YY	Sex	Statistical Test
p value							0.004											0.04				0.02	0.006		Poisson regression
Levron	2001																	1.5^			1.5^	1.5^	1.5^		
		I																0.65			2.61	3.27	1.31		
		J																0.55			1.63	2.18	0.55		
		K																0.86			2	0.17	1.6		
p value																		<0.001			<0.001	<0.001	<0.001		Chi squared
Ohashi	2001																	0.17			0.11	0.18	0.11		
		L																0.17			0.07	0.41	0.08		
p value																									Mann-Whitney U-test
Rubio	2001												0.14					0.02	0.17					0.44	
													0.17					0.05	0.24					0.68	Chi squared,
p value																		0.037	0.042					<.0001	Fisher's exact tests
Burrello	2002							0.18				0						0.2			0.2	0	0.16		
		M						0.68				0						0.37			0.56	0	0.59		
		N						1.03				0						1.23			1.23	0	0.77		
p value								<0.05										<0.05			<0.05		<0.05		Mann-Whitney U-test
Hristova	2002												0.13						0.36		0.05	0.3	0.06		
													0.24						0.37		0.1	0.41	0.04		
p value													<.0001								<0.003				two-tailed Z statistic
Mateizel	2002																	0.3						2.2	
																		1.3						2.4	
p value																		0.05							Chi squared
Templado	2002												0.13						0.37		0.05	0.3	0.06		
													0.23						0.23		0.13	0.5	0.07		
p value													<.0001								<.0001	<.0001			two-tailed Z statistic
Carrell	2003												0.39					0.25	0.24					0.31	
													1.02					0.51	0.47					0.77	
p value													<0.005					<0.05	<0.05					<0.05	Analysis of variance
Martin	2003a	O											0.17						0.22		0.04	0.25	0.04		
		P											0.24						0.44		0.08	1.04	0.06		
		Q											0.3						0.58		0.1	0.68	0.09		
p value		R											0.1						0.009		0.006	0.009	0.03		Spearman correlation

Key for table 1.3- A- incidence of disomy 18 and 21 presented together (therefore values presented are not of individual disomies); B- data included on pellet fraction only; C- could not distinguish between chromosome 13 and 21; D- abnormal semen parameters, normal karyotype; E- abnormal karyotypes and abnormal/normal semen; F- abnormal semen, normal karyotype but aneuploid conceptus; G- OAT individuals; H- teratozoospermic individuals; I- non-obstructive azoospermia; J- obstructive azoospermia; K- severe oligozoospermia; L- severe OAT data only included on table; M-epididymal sperm; N- testicular sperm; O- mild oligozoospermia; P- moderate oligozoospermia; Q- severe oligozoospermia; R- correlation between increased disomy and reduced sperm count/ml. * denotes aneuploidy levels significantly increased to controls (lowest level of significance quoted); ^ denotes total aneuploidy levels for 18, X and Y.

1.5.5 Studies that have investigated the rates of disomy in recurrent miscarriages/ implantation failure

Several studies have addressed the question as to whether patients with a history of recurrent miscarriages or implantation failure after ICSI are at risk of having a higher incidence of chromosomal abnormalities within the spermatozoa of the male partners (Rubio et al., 1999; Rubio et al., 2001). In the study carried out by Rubio et al. (1999) semen samples were obtained from twelve couples undergoing IVF, all of which previously had two or more spontaneous abortions within the first trimester. The findings of this study suggest that there is evidence of increased sperm chromosome aberrations in couples with a history of recurrent spontaneous abortions particularly for the sex chromosomes with frequencies two-three fold higher within the population analysed compared to control frequencies. Blanco et al. (2001) subsequently carried out a larger study, investigating nineteen couples undergoing ICSI that had experienced repeated implantation failure and forty couples whom had repeated miscarriages of unknown aetiology. These patients were further split into groups according to semen parameters, a significant increase in the frequency of sex chromosome disomy was found in the spermatozoa of the males in both the repeated miscarriage and ICSI implantation failure groups compared to controls. However, individuals within these groups for which semen parameters were classified as oligoasthenoteratozoospermic also demonstrated a significant increase in the frequency of disomy for not only the sex chromosomes but that of chromosomes 18 and 21 compared to normozoospermic individuals. Several studies suggest that individuals with a history of recurrent miscarriages (Rubio et al., 1999; Rubio et al., 2001) and those with repeated ICSI implantation failures (Rubio et al., 2001) have a significantly increased rate of sperm disomy particularly for the sex chromosomes (Rubio et al., 1999; Rubio et al., 2001).

Individuals identified as having OAT semen parameters were also reported to have much higher incidences of sperm disomy incidences for chromosomes 18, 21, X and Y compared to normozoospermic individuals. These results suggest that cases couples with a history of pregnancy failures in some cases are likely to in part be due to the increased frequency of chromosomal abnormalities found within the spermatozoa of these individuals.

1.5.6 Conclusion for all the FISH studies on infertile males

Despite some significant differences between studies, there is a general consensus that suggests there is evidence of an increased risk of aneuploidy within the spermatozoa of infertile males with the significant majority of studies reporting an increase in the rate of disomy in at least one chromosome with the exception of a few studies (Guttenbach et al., 1997; Martin et al., 2000; Miharu et al., 1994; Shultz et al., 2000). It is also clear that particular chromosomes may be more prone to non-disjunction including chromosome 21 and the sex chromosomes particularly XY disomy. The differences in the rates of disomy and whether or not a significant increase was observed between infertile males and controls between studies are vast as can be seen from this section. A number of factors/ differences exist between studies, which may account for some of the differences observed between studies and these are now considered.

1.5.6.1 Patient Differences

There are a number of obvious differences that may affect results when comparing studies, for example the number of patients enrolled in studies differs, with this varying from 1-50 individuals. Patient number is an important factor as if too few patients are analysed in one study the results may be skewed or biased by one or two individuals

with particularly higher aneuploid frequencies within their sperm. Certainly one key factor involved is differences in the criteria used in patient selection. Firstly the age ranges of patients vary significantly between studies and may contribute towards the differences observed. Secondly there are studies that give no indication as to whether the controls were age-matched, which given the reports of the presence of a paternal age effect may alter the results depending on the age ranges of individuals (Asada et al., 2000; Griffin et al., 1995; Guttenbach et al., 2000; Kinakin et al., 1997; Martin et al., 1995; Robbins et al., 1995; Rousseaux et al., 1998). If both age and infertility phenotypes are affecting sperm disomy then it is clear that there is a complex relationship between them.

Infertile males are classified (based on analysis of semen parameters, refer to section 1.1) as having defects in count, motility, morphology or any combination of the three with OAT males being the severest affected (with all three parameters impaired). It is difficult to draw direct comparisons between studies as often more than one type of infertility is analysed within studies and the results are not presented for each type. Also even in studies investigating the same infertility the severity varies from individual to individual. It is possible or even likely that specific infertility phenotypes are more likely to confer an increased risk for aneuploidy than others and at levels of severity. Many studies do not clearly define the semen parameters of individuals or in some cases even specify the impairment involved reporting no further details other than infertile. It is therefore feasible that in some cases the differences seen are a reflection of the different andrological phenotypes of the males studied and that certain criteria may correlate more closely with chromosome abnormalities of certain chromosome pairs. Clearly there are other problems even if studies have reported patient details there are

potentially differences in the classification of semen parameters either individual or the use of a different classification system.

Several other variables amongst the patients analysed within studies include differences in days of abstinence prior to assessment, which may result in differences in disomy levels. Differences in the type of spermatozoa utilised including immature (testicular extracted spermatozoa) or mature spermatozoa (ejaculated). There is much concern over the use of testicular spermatozoa in ART. This may be justified as these are not yet fully formed differentiated and the risk is hard to establish as only a handful of cells can be analysed. Similarly lifestyle differences are not always considered. It is well established that sperm counts can vary according to exercise, alcohol consumption, caffeine intake, smoking etc and some of these factors (e.g. smoking) have been associated with increased levels of sperm aneuploidy (Robbins et al., 1997; Rubes et al., 1998; Shi et al., 2001). Indeed the effect of extrinsic factors is an area that requires further investigation, for instance few papers have reported the association between pollution and sperm disomy and the area of endocrine disruption is one that has yet to be investigated in this context.

1.5.6.2 Technical Differences

FISH on human sperm is a more involved procedure than on metaphase preparations. The major difference lies in the fact that it is necessary to decondense the sperm nuclei prior to denaturation and probe hybridisation. This step is critical in that the DNA in sperm nuclei is tightly packed through interprotamine disulphide bridges. The method and quality of the decondensation treatment between studies is thus critical, as it is likely to effect the hybridization and interpretation of the results (Rives et al., 1998).

Inadequate or absent decondensation of sperm nuclei will result in reduced access of the probes to the nucleus and thus may result in an underestimate of disomy levels (Miharu et al., 1994). Excessive decondensation results in a highly diffuse signal as a result of the over decondensation of the chromatin within the sperm nucleus, potentially resulting in an over estimate of disomy levels through "split spots" (Rives et al., 1998). Between studies there are considerable differences in the type of decondensation procedure used. To date there are three main types of incubations used in the decondensation procedures these include: NaOH, Dithiothreitol (DTT) and finally DTT subsequently followed by an incubation in Lithium salt (LIS). The differences between studies also extend to formamide concentration, pH, duration and temperature of the denaturation solution and stringency washes. For the most part studies utilise the DTT only or DTT and LIS combined.

The type of probe used for the experiments is also critical. The most commonly used probes for sperm FISH studies are those that bind to the peri-centromeric regions of chromosomes (usually alpha-satellite probes) or, in the cases of chromosomes 1, 9, 16 and Y to the dark C-bands (Downie et al., 1997). With the use of these particular probes it is especially important to ensure that adequate decondensation is carried out as the centromeres are more condensed within the nuclei than the p and q arms (Rives et al., 1998). Clearly the smaller the signal from the probe, the more likely it is to detect disomy as larger probes may give overlapping signals in disomic cells. Signals vary in size in proportion to the size of the tandem repeat array (indeed alpha satellite and this is particularly apparent for the Y chromosome. Most studies investigating the Y chromosome use a satellite III probe that recognises a large proportion of the long arm (Griffin et al., 1995; 1996b). There is a probe that recognises the centromere of the Y

chromosome that gives a much more punctate signal but, as the Y chromosome alphoid array is the smallest in the human karyotype, signals can often be unreliable. For chromosome 21, as the alpha satellite probe also recognises chromosome 13, it is necessary to use single locus probes (e.g. BACs). These probes frequently give bright signals and, for chromosome 21, a number of studies have made good use of it. A further point of note is that, when establishing the levels of XY disomy, it is much more easy to tell apart two signals of different colour than two of the same. XY disomy and 21 disomy are often quoted as the most frequent among human sperm (Griffin et al., 1996b; Spriggs et al., 1995; 1996) in our opinion, the extent to which this reflects the fact that the small size of the probe (for chromosome 21) and the use of two differently coloured probes (for XY) remains to be determined.

Differences are likely to arise between studies dependent on the stringency applied to the scoring criteria. It is clear that in order to produce results that are comparable between studies the same strict scoring criteria should be utilised between studies. Sperm nuclei are scored as normal if they contain 1 signal for each of the chromosome pair investigated, as disomic if they contain two signals for the same chromosome, and diploid if they contain two signals for all chromosomes investigated. Griffin et al. (1995; 1996a) established scoring criteria that include: Only scoring sperm of similar size (approximately 1.5-2 times normal size after decondensation), not scoring overlapping sperm nuclei, in order to score a cell as disomic the signal intensity and size most be similar, the distance between the two signals must be separated by at least 1 signal domain and not scoring signals that appear outside the cell nucleus. While the majority of studies have adhered to such criteria, the stringency to which they are applied may well vary from lab to lab and from individual to individual. Thus we

cannot rule out the possibility of a small but potentially significant difference in disomy frequencies between studies as a result of observer bias.

Given the complexities of sperm preparation techniques, scoring criteria, intrinsic inter-individual differences and the involvement of extrinsic factors it is perhaps not surprising that results have varied from study to study. Nevertheless, it is clear that with a few exceptions (Acar et al., 2000; Damri et al., 2000; Guttenbach et al., 1997; Martin et al., 2000; Miharu et al., 1994; Schultz et al., 2000) a general agreement in favour of significant increases in sperm disomy has been established for all chromosomes analysed. As with many studies however more questions are raised than are answered, for instance, if the inter-laboratory differences are real then what is the cause of them? Are particular andrological phenotypes more associated with mal-segregation of specific chromosome pairs than others? In an ideal world therefore future studies would use a range of males, each with one, two or three perturbations in their andrological criteria and each with at least three repeat samples. The problem of differing signal sizes could be circumvented by use of equally sized BACs for all of the chromosomes analysed. Scoring of very large numbers of sperm (e.g. more than 20,000) would be necessary in order to minimise statistical anomalies and a standard protocol for scoring criteria would have to be established. Moreover, a consensus needs to be reached among chromosome researchers and statisticians as to the most appropriate test to use. It is likely that such demands are unlikely to be fulfilled without the use of automation. Fortunately automated dot counters (after many false dawns) are now becoming commonplace and reliable; their use and implementation will enable the generation of a large volume of data on chromosome disomy. It is also likely that the use of these automated counters will reduce observer bias.

As there is so much variability between all the studies, it is an difficult and time consuming task to directly compare studies against each other. Ideally detailed patient accounts must be included in all studies, specifically the parameters mentioned earlier and technical differences mentioned above need to be discussed and specific levels/ parameters should be agreed for future studies. Future studies should therefore be required to adhere to all the parameters set although this will be difficult to accomplish it is only then that studies will be directly comparable in order to elucidate potential increased risks of higher disomy levels in infertile men and any particular subsets that are predisposed to an increased rate of disomy. There also needs to be further studies investigating chromosomes including 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 15, 16, 19, 20, 22 where few or no studies have analysed these chromosomes. Also it would be advantageous to study large numbers of infertile males, studies such as these are likely to be published in the near future as a result of the development of automated dot counters.

1.5.7 FISH studies on the spermatozoa of men carrying structural or numerical abnormalities within their karyotype

As discussed in section 1.3 a number of chromosome numerical and structural abnormalities have been associated with infertility including: numerical abnormalities such as Klinefelter syndrome in which the majority of patients are found to be infertile and XYY syndrome in which there is a variable phenotype ranging from fertility to severe infertility. Structural abnormalities including chromosome translocations and inversions, which again are variable in phenotype depending on the chromosomes involved and the size of the affected region. However, the recent advent of intracytoplasmic sperm injection (ICSI) has now given some of these patients who were

previously deemed infertile the opportunity to conceive. The use of ICSI in the treatment of infertility in these patients has been questioned due to the concern of the genetic composition of the spermatozoa to be used in this procedure. In order to assess whether these patients are at any increased risk of producing children with chromosome abnormalities a number of studies have used FISH to establish the rates of chromosomal abnormalities within the spermatozoa of these patients.

1.5.7.1 FISH studies in the spermatozoa of translocations carriers

Translocations and their involvement in infertility have previously been discussed in section 1.3.2.2. Reciprocal translocations often form quadrivalents at meiosis I (figure 1.5 section 1.3.2.2), which segregate in a number of ways resulting in gametes that are either normal, abnormal balanced and abnormal unbalanced. FISH has been utilised to identify the frequency at which these types of segregation occur in the spermatozoa of a number of translocation carriers involving various chromosomes (table 1.4).

These studies are of importance for identifying the mechanisms of meiotic segregation of such translocations and are hence able to estimate the risk of pregnancy loss and birth defects within these individuals (Van Hummelen et al., 1997). It is important to note that the rates of balanced and unbalanced spermatozoa between translocation carriers may not be equal. The segregation pattern of the quadrivalent is different dependent on a number of factors including; the chromosomes involved, the size of the translocated segments, the likelihood of crossing-over within the translocated region and the position of the chromosome breakpoints (Oliver-Bonet, 2001). It is interesting to note (table 1.4) the few cases for which translocations involve the same chromosomes, the frequency of the different types of segregation are similar e.g. t(3;9)(p25;q32) and t(3;9)(q26.2;q32)

(Honda et al., 1999). When the segregation frequencies however are compared when only one chromosome is the same involved the translocation e.g. chromosome 8 in t(7;8)(q11.21;cen) and t(8;9)(q24;q32) the segregation patterns have different frequencies. Hence the risk of chromosomally imbalanced gametes and hence the potential risks to a foetus and chance of survival is very much dependant on the chromosomes involved.

Several studies have investigated whether there is an interchromosomal effect within these individuals by assessing aneuploidy frequencies for other chromosomes not involved in the translocations and comparing them to karyotypically normal control males. To date several studies have suggested evidence of an interchromosomal effect for several chromosomes including that of chromosome 18 (Mercier et al., 1998; Oliver-Bonet et al., 2001; Rousseaux et al., 1995), evidence for such an effect involving the sex chromosomes has also been documented (Cifuentes et al., 1999; Oliver-Bonet et al., 2001). The study carried out by Cifuentes et al. (1999) investigated the interchromosomal effect on chromosomes 1, 8, 6, 12, 15, 17, 21, X and Y in up to 29 reciprocal translocation carriers. Over 50% of the translocation carriers demonstrated a significant increase for at least one chromosome, particularly that of chromosome 1, 21 but also that of the sex chromosomes, no significant difference was observed for the other chromosomes investigated. These studies currently suggest that the non-disjunction of chromosomes 1, 18, 21, X and Y may be more frequently affected by the presence of a translocation. It is clear however, that more studies investigating this finding are required.

Table 1.4 Segregation patterns of chromosomes in the spermatozoa of heterozygote translocation carriers as determined by FISH

Translocation	*Normal*	*Alternate*	*Adjacent 1*	*Adjacent 2*	*3:1*	*4:0*	*Reference*
t(1;10)(p22.1;q22.3)		90.5%[b]		4.9%	3.9%	0%	Van Hummelen et al., 1997
t(1;11)(p36.3;q13.1)	82.5%			8.3%	9.2%		Spriggs and Martin 1994
t(2;14)(p23.1;q31)	88%[a]			5.5%	6.5%	0%	Rousseaux et al., 1995
t(2;18)(p21;q11.2)	43.6%[a]		29.8%	10.5%	12.8%		Estop et al., 1998a
t(3;9)(q26.2;q32)		88.35%[b]		5.44%	5.94%		Honda et al., 1999
t(3;9)(p25;q32)		89.23%[b]		6.02%	4.48%		Honda et al., 1999
t(3;11)(q27.3;q24.3)	44.3%[a]		15.9%	6.5%	28.9%	0.8%	Martini et al., 1998
t(4;8)(q28;p23)		30.5%	28.5%	20.5%	19.5%		Oliver-Bonet et al., 2001
t(5;7)(q21;q32)	49.7%		32.4%	16.2%	1.7%		Cifuentes et al., 1999
t(5;8)(q33;q13)	28%	21.7%	38.31%	6.97%	6.56%	1.23%[c]	Blanco et al., 1998a
t(6;11)(q14;p14)	88%[a]			9.0%	3.1%	0.5%	Rousseaux et al., 1995
t(6;11)(q14;p14)	86%[a]			10.5%	3.5%	0.1%	Rousseaux et al., 1995
t(7;8)(q11.21;cen)	30.4%	26.3%	25.1%	11.1%	7.06%	0.04%	Mercier et al., 1998
t(8;9)(q24;q32)	44.4%[a]		41%	3.1%	9.4%		Estop et al., 1998a
t(10;12)(q26.1;p13.3)		84.25%[b]		10.95%	4.42%		Estop et al., 1997
t(11;22)(q23;q11)		27.4%	17.6%	12.5%	40.1%		Estop et al., 1998b
t(11;22)(q25;q22)	29.1%		21.2%	15.2%	34.6%		Van Assche et al., 1999
t(17;22)(q11;q12)	19%[a]		12.9%	5.8%	46.8%		Geneix et al., 2002
t(Y;16)(q11.21;q24)	51%[a]		36%		12%		Giltay et al., 1999

N.B. not all studies distinguished individual segregation patterns those that have included more than one segregation type in the value have been designated a letter:
a= normal/alternate **b**= alternate or adjacent 1 **c**= diploid or 4:0 segregation

1.5.7.2 Aneuploid frequencies in the spermatozoa of Klinefelter syndrome males

Studies have analysed the frequencies of aneuploidy in the spermatozoa of non-mosaic and mosaic Klinefelter syndrome for the sex chromosomes and autosomes including: 1, 8, 12, 18, 21 (Hennebicq et al., 2001; Bielanska et al., 2000; Hennebicq et al., 2000; Rives et al., 2000; Lim et al., 1999; Kruse et al., 1998; Foresta et al., 1998; Morel et al., 2000) to establish whether these patients through the use of ICSI are at any increased risk of producing aneuploid progeny specifically for the sex chromosomes.

1.5.7.3 Aneuploid frequencies in mosaic Klinefelter males

The majority of studies investigating aneuploidy rates in the spermatozoa of Klinefelter individuals have done so in mosaic individuals exhibiting a 47,XXY/46,XY karyotype (Bielanska et al., 2000; Lim et al., 1999; Morel et al., 2000; Rives et al., 2000) and one study a 47XXY/48,XXXY/46,XY individual (Kruse et al., 1998). All studies investigated aneuploidy levels for the sex chromosomes and at least one autosome with the exception of the study carried out by Kruse et al. (1998) who utilised a dual-colour approach. This particular study reported a high frequency of XX, XY and XXY aneuploidy in the spermatozoa prior to ICSI, although, it should be noted that only 202 spermatozoa were available for analysis and that disomy frequencies reported are unlikely to be accurate as only chromosomes X and Y were investigated (which does not enable disomic cells to be distinguished from diploid cells). Morel et al. (2000) reported a significant increase in XX, XY and YY disomy in one of the two Klinefelter individuals involved in this study, in agreement with these results significant increases for these disomies were also reported by other studies (Bielanska et al., 2000; Rives et al., 2000). In some cases studies have found no such evidence of an increase in YY

disomy, however, document significant increases in both XX and XY disomy (Lim et al., 1999; Morel et al., 2000).

1.5.7.4 Aneuploid frequencies in non-mosaic Klinefelter individuals

To date there have been a handful of studies that have investigated the chromosome complement of spermatozoa from non-mosaic Klinefelter individuals (47,XXY) (Foresta et al., 1998; Hennebicq et al., 2000; Hennebicq et al., 2001; Rives et al., 2000). Each study has reported significant increases in hyperhaploid levels in the Klinefelter patients investigated for at least one of the sex chromosomes. All studies demonstrated significant increases in XY disomy (Foresta et al., 1998; Hennebicq et al., 2000; Hennebicq et al., 2001; Rives et al., 2000) with up to a ten fold increase compared to controls (Hennebicq et al., 2000). Of these four studies, two studies also reported significant increases in XX disomy (Hennebicq et al., 2001; Rives et al., 2000) however, none of these studies reported any increases in YY disomy.

All authors suggest that it is possible for at least some 47,XXY cells to proceed through meiosis to produce mature hyperhaploid spermatozoa (Bielanska et al., 2000; Foresta et al., 1998; Hennebicq et al., 2000; Lim et al., 1999; Morel et al., 2000). Foresta et al. (1998) have suggested there is evidence of this as the frequency of 24,XY disomic sperm was significantly higher in the absence of the 22,0 hypohaploidy that would be expected in the event of meiosis I non-disjunction in a normal 46,XY cell. It is clear from these studies that Klinefelter syndrome patients have been shown to be at an increased risk of producing offspring aneuploid for the sex chromosomes (Bielanska et al., 2000; Foresta et al., 1998; Hennebicq et al., 2000; Hennebicq et al., 2001; Kruse et al., 1998; Lim et al., 1999; Morel et al., 2000; Rives et al., 2000) through ICSI,

compared to fertile controls and infertile males with normal karyotypes (Rives et al., 2000). A number of these studies however, have revealed the possibility that Klinefelter syndrome may also be associated with an increased risk in producing offspring aneuploid for autosomes. At present the existence of this association needs further investigation, however, reports suggest a significant increase in the following autosomes chromosomes 12 (Rives et al., 2000), chromosome 18 (Lim et al., 1999) and chromosome 21 (Hennebicq et al., 2001). Hennebicq et al. (2001) hypothesised that the increase seen in chromosome 21 disomy is likely to be due to the abnormal sex chromosome content, resulting in the disturbance of the meiotic segregation of some chromosomal pairs. This may be attributed to the presence of an abnormal or absent sex chromosome vesicle. Disturbance of the segregation of chromosome 21 may arise as a result of a close association with one or both sex chromosomes during meiosis, which in turn could result in abnormal segregation. Some studies have found no association with significant increases in autosome aneuploidy in the spermatozoa of Klinefelter individuals for chromosomes 1 (Hennebicq et al., 2001; 2000), 8 (Foresta et al., 1998; Morel et al., 2000), 15 (Morel et al., 2000) or 18 (Bielanska et al., 2000; Morel et al., 2000).

It has also been suggested that if Klinefelter individuals decide to undertake ART that FISH should be used to determine the risk of transmission of sex chromosome (Hennebicq et al., 2000; Hennebicq et al., 2001; Kruse et al., 1998; Lim et al., 1999; Rives et al., 2000) and autosomal aneuploidy particularly for chromosome 21 (Hennebicq et al., 2001) to future children in order to provide additional information for genetic counselling and also indicating if preimplantation or prenatal genetic diagnosis may be advisable depending on the potential risk.

1.5.7.5 Aneuploid frequencies in the spermatozoa of XYY syndrome males

Males with 47,XYY phenotypes have varying semen parameters ranging from normozoospermia to severe oligozoospermia (Johnson, 1998), at present it is unclear why such different effects are seen in these individuals although these facts suggest that this condition may have a deleterious effect in some individuals but not others. Early studies suggested that the extra Y chromosome in these men was lost during the premeiotic stage, thereby leaving an XY bivalent to proceed with normal meiosis (Chandley et al., 1976; Evans et al., 1970; Melnyk et al., 1969; Thompson et al., 1967). More recent reports however, have suggested that there is increasing evidence that XYY cells can enter, and eventually complete meiosis (Blanco et al., 1997; Blanco et al., 2001; Burgoyne., 1979; Burgoyne and Biddle., 1980; Speed et al., 1991). Support for this theory has been produced utilising FISH, indicating that some 47,XYY individuals have increased frequency of aneuploid spermatozoa compared to controls (Blanco et al., 1997; Han et al., 1994; Lim et al., 1999; Martin et al., 1999; Wang et al., 2000).

The rate of aneuploidy in the spermatozoa of males with mosaic and non-mosaic XYY karyotypes has been investigated in a series of studies. The study carried out by Han et al., 1994 utilised two colour FISH for the sex chromosomes to establish the aneuploid frequencies in one XYY male, a significant increase in XY (0.35%) an YY (0.43%) disomy was found compared to control levels (0.11% and 0.16% respectively). These results may be overestimated due to the usage of a dual probe for the sex chromosomes only. The remainder of the studies have utilised three colour FISH; two studies have investigated the effect of an XYY mosaic karyotype and the chromosome complement of spermatozoa in these individuals. In both studies mosaic individuals were identified through the karyotyping of peripheral lymphocytes, in total three XYY mosaic patients

have been investigated by these studies. Of the three patients investigated two patients had similar levels of mosaicism of 19% and 20% with the third having a substantially higher level of 90%. The two patients exhibiting similar frequencies of mosaicism both demonstrated significant increases for XY disomy alone. Specifically, the patient exhibiting the 47,XYY[20%]/46,XY[80%] karyotype had reported levels of 0.61% compared to control the control frequency of 0.22% (Wang et al., 2000); with frequencies of 0.23% identified for the 47,XYY[19%]/46,XY[79%] patient compared to 0.2% recorded from the control (Lim et al., 1999). However, the patient with the higher level of mosaicism 47,XYY[90%]/46,XY[10%] was demonstrated to have a significant increase in both XY and YY disomy with levels of 1.02 and 0.44 respectively compared to the control levels of 0.1% found for both types of disomy (Lim et al., 1999).

Significantly higher frequencies of sex chromosome disomies have been reported within studies of 47,XYY non-mosaic patients. Five studies have investigated the chromosome constitution of spermatozoa from six 47,XYY individuals. However two of these studies analysed two individuals with dual-colour FISH (hence frequencies found may be inaccurate) only one study reported an increase in aneuploid sperm for YY and XY (Mercier et al., 1996), whereas Han et al. (1994) found no evidence of an increase. Studies identified a significant increase in XY disomy for both patients with frequencies of 0.55% (Martin et al., 1999) and 0.3% (Blanco et al., 1997). Blanco et al. (1997) and Chevret et al. (1997) also reported a significantly higher level of YY disomy associated in a 47,XYY patient compared to controls. In contrast Mennicke et al. (1997) found no such evidence of an increase in disomy rate for any of the chromosomes investigated in three 47,XYY individuals.

At present there is limited information available on the effect of an XYY karyotype on the germ cells of these individuals. Investigations though are contradictory, although recent evidence would suggest that in mosaic XYY patients the higher the percentage of the XYY karyotype the higher the levels of disomy. Increased rates of YY disomy as found in three papers (Blanco et al., 1997; Chevret et al., 1997; Lim et al., 1999) provide evidence that some of these hyperdiploid cells found in men with XYY syndrome can indeed undergo meiotic division. The majority of studies have provided evidence that XYY cells appear to be able to complete meiosis. These studies however, suggest that a large proportion of abnormal cells are arrested at various stages of spermatogenesis, thereby resulting in the continuous elimination of abnormal cells, resulting in a small but significant increase in aneuploid spermatozoa (Blanco et al., 1997; Blanco et al., 2001; Lim et al., 1999; Martin et al., 1999; Martini et al., 1996). A number of investigations have suggested that due to this increase in sex chromosome disomies within these patients, and that assessment of the frequencies of chromosome abnormalities within the spermatozoa of these patients may be warranted prior to the commencement of ART. This enables specific individual risks of transmitting the extra sex chromosome and pre-implantation diagnosis to be recommended in patients with increased frequencies compared to the normal population (Lim et al., 1999; Martin et al., 1999; Martini et al., 1996; Wang et al., 2000).

1.5.8 FISH studies on the spermatozoa of karyotypically normal males who have fathered karyotypically abnormal children

1.5.8.1 Fathers with Klinefelter syndrome offspring

Lowe et al. (2001) analysed the frequency of disomy 21 and the sex chromosomes in thirty-eight fathers of boys with Klinefelters syndrome (47,XXY). This study provided

evidence of a significant correlation between the increased incidence of XY disomy within the spermatozoa of fathers and increasing paternal age. Increasing age (measured in this study by decades) demonstrated a significant increase of around 10% in XY disomy in fathers in their thirties, over a 30% increase was found in those men within their forties and an increase of 160% in fathers aged over fifty compared to those within their twenties. The results suggest that men fathering children latter on in life are at a substantially increased risk of producing offspring with Klinefelter syndrome.

1.5.8.2 Fathers with Turner syndrome offspring

Martinez-Pasarell et al. (1999), investigated the sperm disomy rates for chromosomes 6, X and Y in four fathers of Turner syndrome individuals, (in all these cases monosomy X was paternal in origin). The mean frequency of XX disomy (0.04%) and YY disomy (0.07%) in these individuals was not significantly different from levels obtained from the control (0.04% and 0.06% respectively). Nevertheless, a significant increase in the rate of XY disomy was recorded between these patients, (0.22%) versus control levels (0.11%). Based on these results, Martinez-Pasarell et al. (1999) suggested an increase of non-disjunction of the sex chromosomes occurs as a result of errors in meiosis I, thereby producing more XY bearing and as a result an increase of spermatozoa nullisomic for the sex chromosomes. In a further study carried out by the same group Soares et al. (2001a) investigated the disomy frequency for chromosomes 4, 13, 18, 21 and 22 in the same four Turner syndrome fathers. In order to establish whether these individuals had a general increase in chromosome disomy within the spermatozoa or whether this increase was restricted to the sex chromosomes only. This study revealed no significant difference between the disomy rates for chromosomes 4 (0.06%) and 18 (0.05%) in the Turner syndrome fathers compared to that of the control levels with levels of 0.06% and

0.04% respectively. A significant increase in the rate of disomy however, was found for chromosomes 13, 21 and 22, with levels of 0.14%, 0.14% and 0.12% respectively versus the control frequencies of 0.10%, 0.07% and 0.06% respectively. Soares et al. (2001a) suggest an association between the fathering of aneuploid offspring and an increased incidence of disomic spermatozoa, and that this increase does not appear to be restricted to the chromosome pairs involved in the production of the aneuploid offspring. Soares et al. (2001a; 2001b) hypothesised that this may affect the acrocentric chromosomes and sex chromosomes, due to having only one chiasma during meiosis therefore more susceptible to malsegregation.

1.5.8.3 Fathers with Down syndrome offspring

Blanco et al. (1998b) investigated sperm disomy rates for chromosomes 6 and 21 in fifteen fathers with children affected with Down syndrome and compared the rates against those found in nine control males. In ten out of the fifteen cases the extra chromosome 21 was maternal in origin with only two cases being paternal in origin (the origin of the remainder were mitotic, with one case due to a translocation). In both the control group and the patient group a significant increase in the rate of disomy for chromosome 21 was observed compared to that of chromosome 6. No significant difference was observed between the two groups for the two chromosomes investigated. Comparison of each individual with the controls revealed a significant increase in the rate of disomy for chromosome 21 in the two fathers (DP-4 and DP-5) for which the extra chromosome 21 in their offspring was paternal in origin (compared to the controls and the remaining individuals in this group), 0.75% and 0.78% versus 0.37% in the controls. In light of this data Blanco et al. (1998b) also investigated the disomy frequencies of chromosomes 18, X and Y in the same two individuals DP-4 and DP-5 in

an attempt to establish if the increase of disomy 21 was due to disruptions in meiosis. The disomy frequencies of these chromosomes in DP-4 did not show any significant increase compared to the control levels. In patient DP-5 however, a significantly higher frequency of sex chromosome disomy, particularly XY disomy was recorded which may suggest the existence of a generalised susceptibility to the disruption of the synaptic process resulting in an increased incidence of chromosomal anomalies (Blanco et al., 1998b). Soares et al. (2001b) carried out a follow up study on these two patients DP-4 and DP-5 investigating the disomy frequencies of chromosomes 4, 13 and 22, using the same nine control donors in the previous study. A significant increase in the rate of disomy 13 and 22 but not chromosome 4 was found in both of these patients compared with the controls. Soares et al. (2001b) suggested that it is possible that both of these individuals may have a susceptibility to meiotic non-disjunction and that acrocentric chromosomes may be more susceptible to non-disjoin than other chromosomes. In contrast Rubio et al. (2001) analysed the disomy frequency for chromosomes 13, 18, 21, X and Y in the spermatozoa of three fathers, all of which had pregnancies that were affected by Down syndrome. This study found no evidence of any significant difference between the study group and the control donors for any of the investigated chromosomes, however, the origin of the extra chromosome 21 was not determined. The determination of parental origin is of importance as Blanco et al. (1998b) only found evidence of an increase in sperm disomy in individuals for which the extra chromosome was paternal in origin. It is therefore possible that no significant difference was identified by Rubio et al. (2001) as the origin of the extra chromosome 21 may not have been paternal in origin. However, more studies for which the parental origin of the extra chromosome 21 is known needs to be undertaken to determine if this

is the case. Therefore more studies need to be carried out in individuals in which the extra chromosome 21 is determined to be paternal in origin.

1.5.9 Studies investigating environmental and lifestyle factors on aneuploidy frequencies in spermatozoa

To date studies have investigated a wide range of lifestyle and environmental factors in order to examine whether any of these may contribute to increased frequencies of aneuploidy within spermatozoa and include some of the following:

- caffeine
- alcohol
- Smoking tobacco
- environmental exposures
- chemotherapy

1.5.9.1 Lifestyle effects

In modern society there are a number of stresses that are placed on our bodies, these include lifestyle choices such as alcohol and caffeine consumption, smoking tobacco and recreational drug use. These lifestyle choices have often been associated with health problems, however, what remains unclear is what effect these lifestyle habits are having on the reproductive health of individuals. The excessive consumption of alcohol has been implicated as a possible cause of a reduction in the quality of semen parameters, chronic alcoholics have been shown to have a decrease in sperm concentration and forward motility and an increase in abnormal morphology (Goverde et al., 1995; Nagy et al., 1986). Chronic alcoholics have also been found to have decreased serum

concentrations of testosterone and increased levels of oestrodiol (Gomanthi et al., 1993).

Smoking has been demonstrated to have damaging effects on semen parameters including sperm concentration, morphology and motility (Little and Vainio., 1994; Stillman et al., 1986; Vine et al., 1994; Vine., 1996; Vogt et al., 1986). Electron microscopy has shown that smokers have an increased incidence of abnormalities involving the sperm axoneme, including changes in number and arrangement of axoneme microtubules in smokers. These abnormalities could result in significant effects on the progressive motility of spermatozoa (Zavos et al., 1998). Smoking could also potentially bring about significantly higher numbers of genetically abnormal spermatozoa, as smoke has been shown to contain at least 30 chemical agents known to be mutagens, clastogens, aneugens or carcinogens (Claxton et al., 1989; Lofroth., 1989).

A handful of studies have investigated the relationship between aneuploid frequencies in spermatozoa and a number of lifestyle factors including smoking, alcohol and caffeine consumption, chromosomes analysed include 1, 8, 13, 18, 21, X and Y (Robbins et al., 1997a; Rubes et al., 1998; Shi et al., 2001). For the most part studies have analysed more than one lifestyle factor per study. Shi et al. (2001) analysed chromosome 1, 13, X and Y in 31 Chinese men who were split into 3 groups, non-smokers; light smokers (<20 cigarettes per day) and heavy smokers (>20 cigarettes a day). Both light and heavy smokers were found to have significantly higher levels of disomy 13 compared to the group of non-smokers, but no significant increase was found for any of the other chromosomes investigated. The authors however, comment on a significant amount of inter-donor heterogeneity for all chromosomes analysed in both

the light and heavy smokers groups. Rubes et al. (1998) investigated aneuploidy for chromosomes 8, X and Y in 25 men all aged 18, these were split into two groups, those that have smoked 20 cigarettes a day for at least two years and a group of non-smokers. The smoking group exhibited reduced semen quality for count and displayed a significantly higher percentage of round-headed sperm compared to the control group, and a significant increase in YY disomy. The group of smokers however, was also recorded to consume a greater quantity of alcohol and twice the amount of caffeine compared to the control group. Hence the causative agent for increase in YY disomy cannot be attributed to smoking alone but also in conjunction with the consumption of alcohol. Robbins et al. (1997a) carried out a study in 45 men investigating the effect of smoking, consumption of alcohol and caffeine on the incidence of chromosomal aneuploidy for chromosomes 18, X and Y. This study has reportedly controlled for age and the consumption of the two factors alcohol, caffeine or smoking by using poisson regression statistical analyses. After controlling for these effects the following associations were identified between increases in disomy and the three exposures investigated including caffeine consumption and XX and XY disomy, alcohol consumption and XX disomy and an unstable association with smoking and XX disomy. This unstable association between smoking and sperm aneuploidy was also reported by Shi et al. (2001), suggesting that smoking cigarettes potentially increases the risk of aneuploidy for specific chromosomes, but due to the inter- donor differences that some individuals may be more susceptible than others to increases in aneuploidy.

Several studies have investigated the effect of common lifestyle exposures including smoking, consumption of alcohol and caffeine and the presence of any causal effect between such lifestyle factors and sperm aneuploidy and have found a significant

adverse effect on sperm aneuploidy. The results have however, been largely inconclusive in producing direct evidence of a relationship between individual factors and sperm aneuploidy. Understandably this has been a difficult task as there are a number of potential confounding factors that may be involved including smoking, alcohol and caffeine consumption, diet, exercise, stress, geographic differences, environmental and occupational exposures. Occupational exposures have also been implicated in a reduction in semen quality. In the study carried out by Figa-Talamanca et al. (1996) the effects of prolonged automobile driving in taxi drivers was investigated. Evidence of prolonged automobile driving as a risk factor for reduced semen quality, especially for morphology was described. It is possible that any one of these lifestyle factors mentioned above may exert an individual effect or that any of these may act in a synergistic manner. Authors attempting to correlate the effect of individual lifestyle factors and sperm aneuploidy are in a difficult position, as it is difficult to isolate normal healthy individuals that only have exposure to one type of lifestyle exposure such as smoking. This is especially true for smokers, which have been reported to consume greater quantities of alcohol and caffeine compared to non-smokers (Rubes et al., 1998). Studies investigating this phenomenon ideally need to have age matched individuals who are from the same region, and for which samples are taken at similar time periods in order to minimise the risk of different environmental exposures, and comprehensive information is required about lifestyle, diet, exercise and potential occupational and environmental exposures such as pesticides. This is an enormous task as studies ideally require a large number of individuals and can often only rely on the details patients have given with regards to lifestyle exposures and cannot take into account environmental exposures for which individuals may have no knowledge. Previous reports have however, indicated that a number of these lifestyle factors have

been associated with a decline in semen quality and an increase in sperm aneuploidy. Future work will hopefully identify potential effects of individual types of exposures and levels capable of causing adverse effects on the reproductive health of individuals and elucidate the mechanisms involved.

1.5.9.2 Environmental exposure

A number of questions have been posed as to whether environment contaminants induce any genetic damage in humans especially within the gametes, this has far reaching public health perspectives. Within recent years there have been several controversial reports of a significant reduction in sperm counts over the past 50 years or so (Auger et al., 1995; Carlsen et al., 1992; De Mouzon and Thonneau, 1996; Irvine et al., 1996). The debate as to whether this observation is in fact the case still rages on however studies have been undertaken in order to detect any such association between environmental contaminant exposures and alterations in germ cells.

Robbins et al. (1999) analysed the aneuploidy frequencies in the spermatozoa of 32 healthy 18 year olds for chromosomes 8, X and Y due to reports of poor conception rates, high rates of congenital malformations reportedly related to air pollution in the Czech region of Teplice. This study used individuals previously recruited in another study investigating air pollution and health (Sram et al., 1996). All individuals gave information on exposure data for both occupations and hobbies as well as lifestyle information including reproductive health, alcohol and cigarette consumption, passive smoking and medication. This study only considered males with normal semen parameters and non-smoking individuals. Robbins et al. (1999) analysed semen samples collected from the same region at two different times of year, one which had record high

levels of air pollution with the other having low level of air pollution. This study concluded that there was a significant increase of approximately five fold in the levels of YY disomy in the individuals tested in the season with high air pollution compared to those in tested when air pollution levels were low. The authors checked for associations between semen parameters, the period of abstinence and lifestyle factors including: caffeine and alcohol consumption, no effect was found. This provides evidence of a possible association between increased aneuploidy and high levels of air pollution, nevertheless it is a near impossible task to determine if this is the cause of the increase in disomy found, as there are potentially many confounding factors.

Padungtod et al. (1999), analysed aneuploidy frequencies for chromosomes 18, X and Y in 13 individuals working in a pesticide-manufacturing plant in China, the aneuploidy frequencies were compared with 16 individuals working in a textile factory that was not exposed to any pesticides. Those that worked within the pesticide plant were exposed to ethyl parathion or methamidophos (both of which are potent organophosphate pesticides) were found to have significant increases in chromosome disomy levels compared to those working in the textile factory.

There is preliminary evidence to support the hypothesis that exposures to environmental contaminants, whether it is through everyday exposure such as air pollution or whether it is through occupational activities, can cause significant increases in genetic abnormalities in the spermatozoa of men.

1.5.9.3 Chemotherapy

Chemotherapy is widely used in the treatment of cancer, during such treatment fertility is reduced within these patients, there is also concern raised by both patients and specialists on the potential of causing detrimental genetic damage after chemotherapy. In male rodents it has been well documented that treatment with chemo- and radio-therapeutic drugs prior to mating can cause genetic defects in the germ line (Miestrich et al., 1982; Robbins et al 1997). Recently laboratories have addressed the risks of genetic damage in the gametes of human males treated by chemotherapy and the possibility of passing on abnormalities to future offspring. A number of studies have investigated the use of chemotherapy regimes used in the treatment of testicular cancer and Hodgkins disease (both of which are often diagnosed during the patients reproductive years). The genetic effects on the gametes of men undergoing these treatments, have been investigated for the following chromosomes: 1, 2, 4, 7, 8, 9, 12, 13, 15, 16, 18, 20, 21, X and Y (De Mas et al., 2001; Frias et al., 2003; Martin et al., 1997; Martin et al., 1999; Robbins et al., 1997).

1.5.9.3.1 Testicular cancer

Several studies have investigated the genetic composition of spermatozoa in patients with testicular cancer before and after chemotherapy treatment with bleomycin, etoposide and cisplatin. Following an azoospermic period, sperm cell recovery is generally observed after treatment delivery, in order that the genetic consequences on new spermatozoa could be assessed, De Mas et al. (2001) analysed aneuploidy frequencies in 5 patients for chromosomes 7, 16, 18, X and Y prior to treatment and at 6-18 months after treatment. In this study 20,000 spermatozoa were scored per patient, moderate differences in the sperm count before and after treatment were noted and a

significant increase in disomy for chromosomes 16, 18, XY and diploidy was found. However Martin et al. (1997) analysed the frequency of aneuploidy for chromosomes 1, 12, X and Y in four patients treated with the same chemotherapy agents 2-13 years after treatment. In this particular study no such increase in disomy or diploidy was reported for the investigated chromosomes. This study also used sperm karyotyping to check for both numerical and structural abnormalities for all chromosomes. Concluding that there was no significant increased risk of structural or numerical abnormalities in the spermatozoa of patients undergoing chemotherapy for testicular cancer two or more years after treatment. Subsequent work however, (Martin et al., 1999) analysing sperm chromosome complements before during and after treatment reported a significantly increase in aneuploidy particularly for XY disomy and that of diploid cells in one patient within 18 months after treatment. At present results suggest that the use of chemotherapy agents in the treatment of testicular cancer causes a significant increase in aneuploidy in the newly generated spermatozoa after treatment, however these increased frequencies may fall to levels found prior to treatment after 2 or more years.

1.5.9.3.2 Hodgkins Disease

Hodgkins Disease is often diagnosed within the reproductive years of patients, as a result there is interest in the effect of the treatment on the reproductive system of male patients. Several studies have analysed samples before, during and after treatment (Frias et al., 2003; Robbins et al., 1997). In an effort to establish whether any effects seen in aneuploidy levels occur only in those cells undergoing spermatogenesis (transient defect) during treatment or whether any persistent defects are induced are affecting the germ line stem cells. Robbins et al. (1997) analysed the frequency of aneuploidy for chromosomes 8, X and Y in eight patients, before, during and after treatment with

Novanthrone, Oncovin, Vinblastine, Prednisone (NOVP) chemotherapy. A significant increase in aneuploid frequencies of approximately five fold, including disomy, diploidy and hyperhaploidy for all chromosomes investigated during treatment compared to pre-treatment levels, with the exception of YY disomy. Samples analysed after treatment suggest that the increased level of aneuploidy in the spermatozoa of these patients declined to levels comparable to the pre-treatment samples around 100 days after treatment finished. Frias et al. (2003), examined the use of NOVP in the treatment of eight male patients, analysing the aneuploidy levels in the spermatozoa for chromosomes 18, 21, X and Y. This study analysed patients before during and 1-2 years after treatment, aneuploidy frequencies were found to be significantly elevated for all chromosomes investigated during treatment. Levels of disomy 18 increased 7 fold, with disomy 21 rising by 3 fold. Sex chromosome disomy levels increased two fold for XX and YY disomy, with XY disomy having the largest increase of all chromosomes investigated (14 fold). In contrast Robbins et al. (1997) reported no increase for YY disomy however in agreement with Robbins et al. (1997) patients tested post treatment no longer demonstrated these increases in aneuploidy for the chromosomes tested.

1.5.9.4 Conclusions of investigations into the effect of chemotherapy and sperm aneuploidy

It is certain that more studies need to be carried out, as only a handful of studies have investigated the potential association between chemotherapy and aneuploidy. This information is of importance as it will enable accurate information to be passed on to the patients about the genetic risks to potential offspring. There are many types of cancer and modes of treatment, until studies are carried out it will remain unknown as to whether these treatments cause transient chromosomal defects or persistent defects

(Robbins et al., 1997). Therefore it is important that future studies look at patients with the same type of cancer and treatment and that these patients should be followed prior to, during, and at regular intervals for the following 0-2 years after treatment. This would enable the identification of any adverse effects on cells actively undergoing spermatogenesis and the stem cell population of individual treatments for specific cancers (Martin et al., 1997). Studies to date suggest that the chemotherapy regimes analysed including bleomycin, etoposide and cisplatin and NOVP in the treatment of testicular cancer and Hodgkins disease appear to result in the production of transient (non-persistent) chromosomal defects (De Mas et al., 2001; Frias et al., 2003; Martin et al., 1999; Robbins et al., 1997). In these cases there is evidence for an increase in aneuploidy levels therefore increasing the risk of producing aneuploid offspring for a period for an amount of time after treatment with chemotherapy (De Mas et al., 2001; Frias et al., 2003; Robbins et al., 1997). It has therefore been suggested that an appropriate period of contraception should be recommended to patients (established by studies such as these to determine the time required for aneuploid frequencies to return to pre-treatment levels). It is also suggested that patients should also undertake genetic counselling if a pregnancy does occur within this time (De Mas et al., 2001; Frias et al., 2003; Robbins et al., 1997).

1.6 Assisted reproductive techniques (ART)

A large volume of research has gone into assisted reproductive techniques (ART), the first major development came with the advent of in-vitro fertilisation (IVF), the first baby born using the technique of IVF came in 1978 (Steptoe and Edwards 1978). IVF has subsequently become a widespread technique, primarily used for treating female infertility. Until recently however, it was rarely possible for couples to reproduce in the

case of male factor infertility. Within the last decade the advent of intracytoplasmic sperm injection (ICSI) has enabled such couples to procreate. ICSI has been an effective method used to assist fertilisation with sperm from an infertile male and has been heralded as the biggest advancement in ART since IVF enabling couples previously unable to conceive due to male factor infertility to now do so. The birth of the first ICSI baby came in 1992 (Palermo *et al.*, 1992). Recent estimates suggest that IVF accounts for just over half of ART treatment cycles with ICSI being undertaken in approximately 46% of ART cycles (Fraser., 2002 www.nature.com/fertility).

1.6.1 IVF treatment cycle

In brief the most common IVF technique used involves the following:

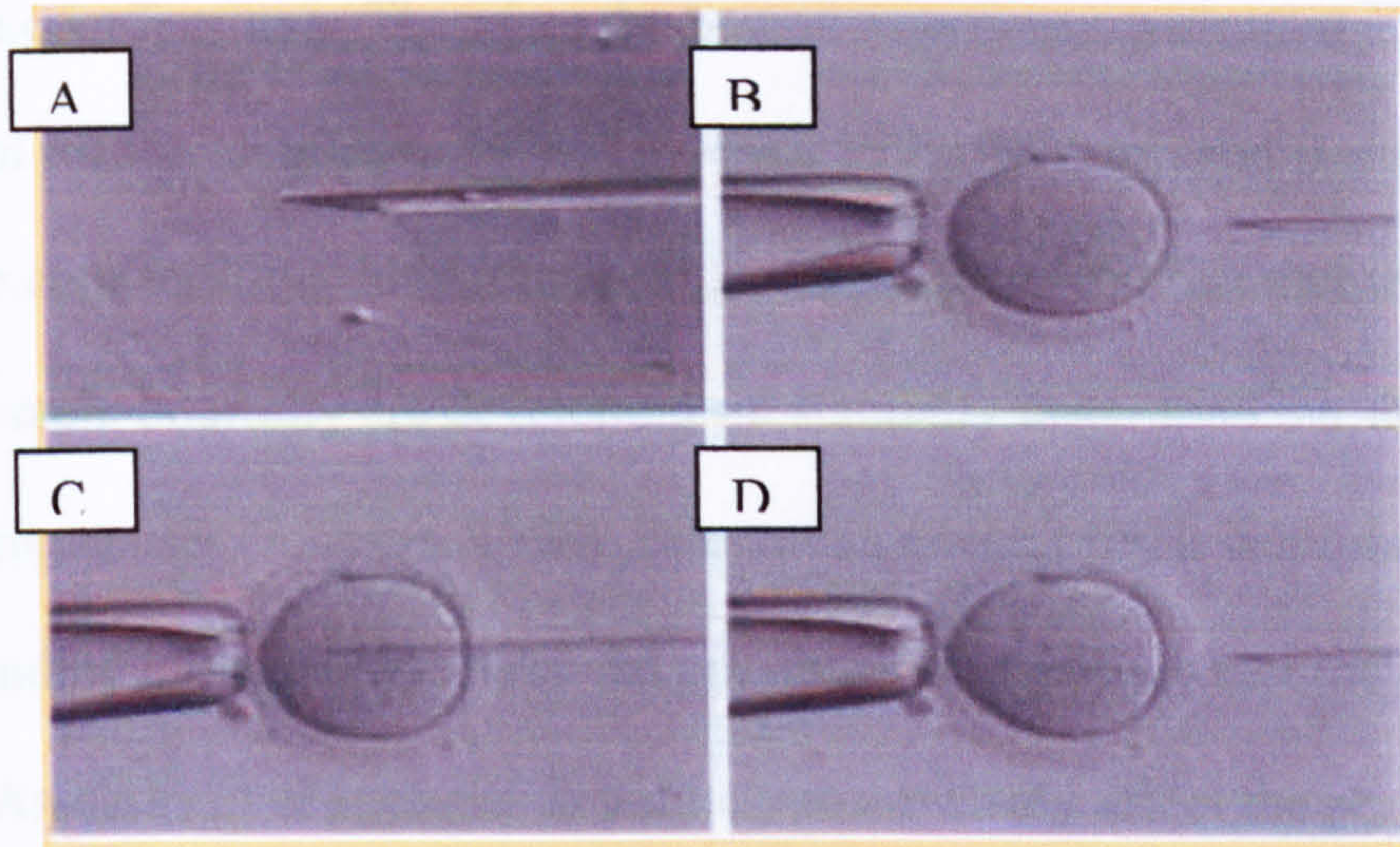
The female partner is administered fertility drugs to hyperstimulate the ovaries to produce multiple eggs, (including a GnRH agonist used in conjunction with hMG, or FSH to produce multiple mature ova). The female is then regularly monitored by ultrasounds and blood tests enabling the appropriate time to harvest the ova to be determined. Prior to this, an injection of hCG is administered to induce the final maturation of the ova, following this oocyte retrieval is performed, most commonly through the use of ultrasound guided aspiration where a needle is guided through the vaginal wall into the ovary where the ova can then be aspirated. Embryologists will then identify and grade the maturity of the ovum. Ova are then transferred to a culture dish with medium and are placed into an incubator. Spermatozoa from the ejaculate of the male is then placed in the culture dish containing the ovum and left to fertilise (http://eastcoastivf.com). Much like unassisted conception, spermatozoa are in competition with each other, the hurdles that sperm have to overcome that are in place in natural conception are similar to those encountered by sperm used in IVF. In both

these cases the spermatozoa that fertilise the eggs are those that swim well, can undergo the acrosome reaction and fuse with the plasma membrane of the egg. The following day the ova are examined for evidence of fertilisation and are incubated further to enable the development of an embryo following this period the embryo is then transferred to the uterus.

1.6.2 ICSI treatment cycle

In the case of ICSI, the female partner is treated in much the same way as in IVF, ovaries are hyperstimulated and the eggs are surgically removed. Sperm is collected from the male partner either through ejaculation or surgically. Oocytes are then cleansed of all surrounding cells and the spermatozoa are prepared separately. The egg is held in place with a glass pipette while a sharp hollow needle is used to immobilise and capture a single sperm. Unlike in IVF and natural conception there is no selective pressure on the sperm as the spermatozoa selected for fertilisation is chosen by the embryologist. The needle containing the spermatozoa is then carefully inserted through the zona pelucida (taking care not to disturb the meiotic spindle) into the cytoplasm of the oocyte (figure 1.7). As in IVF the oocyte is then incubated and checked the following day for evidence of fertilisation. Any oocytes that have been successfully fertilised and appear to be dividing normally are then transferred to the females uterus either 2 days later (at the two-cell stage) or after five days (at the blastocyst stage) (Winston and Hardy., 2002 www.nature.com/fertility).

Figure 1.7- shows the process of ICSI, panel A, sharp hollow needle picks up a single sperm, B- egg is held in place and the needle carrying the sperm is injected into the egg, C- the needle is clearly seen in the cytoplasm, D- the needle is withdrawn and the egg is left to see if fertilisation has taken place. Image taken from www.acrm.com/ART/ART.htm



1.6.3 How safe is ICSI? What are the risks involved with this technique?

The development of ICSI has revolutionised the management of male factor infertility and because of enormous patient demand has been rapidly adopted by infertility clinics all over the world with little regard for the potential genetic problems for future generations (Bhasin et al., 1994; Johnson, 1998). Many have criticised the procedure of ICSI, in the treatment of male infertility, as it is believed that the use of ICSI theoretically increases the risk of propagating disorders associated with male infertility, thereby increasing the fitness of these infertile genotypes (Johnson., 1998). These views on ICSI may well have good standing as the procedure itself bypasses the biological mechanisms of sperm selection, which are in place in natural conception. The use of ICSI has been criticised, as it has not undergone proper cell culture experiments or extensive research trials in other mammals prior to being used in a clinical setting. The concern is that pressure to treat male infertility has meant that vital information that

could be gleamed from these types of experiments has been bypassed. Although in recent years ICSI has been tested in rhesus monkeys, however, this has been many years after the use of ICSI in the treatment of humans (Winston and Hardy., 2002 www.nature.com/fertility). Therefore the human experience with ICSI is the experimental record, (Johnson., 1998; Tornaye., 1999; Winston and Hardy., 2002 www.nature.com/fertility). In part the problem stems from the fact that technological solutions to male infertility are developing substantially faster than our understanding of the underlying causes (Johnson, 1998). Due to this uncertainty it is important to develop an understanding of the genetic risks and possible consequences that may be associated with ICSI. Assessment of such risk is unlikely to adversely affect the practice of ICSI, but will enable couples to be fully aware of any risks associated with the procedure (Fraser., 2002 www.nature.com/fertility; Griffin et al., 2003). Moreover, investigations of the genetic causes of the infertility may lead to subsequent treatment regimes that are alternative to ICSI without the potential risks involved.

With the development of IVF came major concerns about the safety of this technique. Despite this, the few controlled studies have been performed, that do suggest that the frequency of congenital malformations compared to naturally conceived babies is not at any increased rate. Research currently suggests that ICSI has similar fertilisation and conception rates as IVF (Van Steirteghem., 2002). The advent of ICSI brought about similar concerns, although the IVF situation does not seem to cause any perturbations, the use of ICSI is more invasive as the sperm cell is injected directly into the egg. These include concerns that selective mechanisms against physiologically or genetically abnormal spermatozoa are bypassed; immature spermatozoa that previously would not of been able to be used in fertility treatment can now be used including sperm extracted

from the epididymas and testes, abnormal oocytes may be fertilised; altered environment, mechanical or chemical damage to the oocyte may lead to perturbations of meiosis and mitosis; various chemical or environmental exposures may also lead to point mutations, resulting in genetic diseases visible at birth or later in life, (Bonduelle et al, 1996).

1.6.3.1 Chromosomal aberrations within couples undergoing ICSI

Concerns have arisen as several studies have published evidence that infertile couples specifically infertile males have a significant increase in chromosomal aberrations compared with their fertile counterparts.

1.6.3.2 Reports on the rates of congenital malformations and chromosomal defects in children born with the use of ICSI

At present only a handful of studies have been published, looking at the outcomes of ICSI, in an attempt to identify the risks of congenital malformations, abnormal karyotypes, growth parameters and developmental outcome in children born through ICSI, (Aboulghar et al., 2001; Bonduelle et al, 2002a 2002b; 1999; 1998; 1996; Hansen et al, 2002; Loft et al, 1999; Schieve et al, 2002; Zech et al, 2000). The Belgian group that pioneered the technique of ICSI has carried out the largest studies to date (Bonduelle et al., 1996; 1998; 1999; 2002a and 2002b). An assessment of the risks involved with ICSI is difficult to estimate from the results of these studies as in many cases this procedure is often combined with others that may also exert an effect including that of cryopreservation and pre-implantation genetic diagnosis (PGD) (Winston and Hardy., 2002 www.nature.com/fertility). Nevertheless, these studies have

noted some negative effects on the children born by ICSI when compared to IVF and naturally conceived children, these studies will now be considered.

The first studies to be considered will be those carried out by Bonduelle et al. (1996; 1998; 1999; 2002a 2002b) as these are the largest studies to date. Bonduelle et al. (1996) reported no significant increased incidence of major or minor congenital malformations in live births, reporting around 2.6% compared to the incidence in IVF or the naturally conceived population. They do however suggest significantly increased risk in the transmission of paternal chromosome aberrations (including translocations and inversions), with 1.2% of children (out of 877), all of which were transmitted by the fathers and also that of *de-novo* aberrations, mainly that of trisomy involving the sex chromosomes the incidence of which was 1% in 877 children. This increased incidence in trisomy of the sex chromosomes was approximately five times higher than observed within the general population (Jacobs et al, 1992; Hook and Hamerton, 1977; Nielson and Wohlert et al, 1991). This finding may be attributed to the use of gametes from men with infertility as a large number of studies have reported a high proportion of aneuploidy in the spermatozoa of these individuals compared to their fertile counterparts (refer to section 1.5.4) rather than problems with the actual technique (Bonduelle et al., 1996).

Bonduelle et al. (1999) investigated a total of 1,987 conceived by ICSI. Prenatal diagnosis was undertaken in 1,082 cases, revealing abnormal karyotypes in twenty eight individuals (2.6%) ten were inherited structural aberrations, the remainder were *de-novo* with chromosomal aberrations affecting the autosomes in nine cases and the gonosomes in nine cases. The rate of major congenital malformations were found to be 2.3% at

birth however, this rate increased to 2.9% when stillbirths and those picked up by prenatal ultrasound were included.

The largest study carried out to date is that reported by Bonduelle et al. (2002a), this study collected data on 2889 children born after ICSI and 2995 children born after IVF. This study presented data on birthweight, still birth rate and prematurity amongst others, the rates obtained were comparable between both the ICSI and IVF groups. The rate of major malformations in liveborn children (defined as needing a surgical correction or causing a functional impairment) was estimated to be 3.4% in the ICSI population and 3.8% in the IVF group. This rate of major malformations within the ICSI population is significantly higher than that found within the general population, however the rates found were similar to those obtained with IVF pregnancies.

Bonduelle et al. (2002b) also reported data collected on the prenatal testing of a total of 1586 ICSI pregnancies over the past decade, this study presents the findings and their relation to the semen parameters in the male partner. In an attempt to determine whether utilising the gametes of chromosomally normal parents leads to an increased risk of chromosomal aberrations in their children and whether these are as a result of the technique itself or the gametes used in the technique. In total 47 abnormal karyotypes (3%) were found out of the 1586 foetuses tested. These included 25 *de-novo* abnormalities (1.6%) (10 sex chromosome abnormalities, 8 numerical autosome anomalies and 7 structural autosome aberrations), 22 abnormal karyotypes (1.4%) were transmitted to the offspring by the parents. These inherited abnormalities comprised of 1 unbalanced karyotype and 21 balanced, it is of interest to note that 17 of these 22 were paternally inherited. Of the 47 abnormal foetuses 11 were aborted, 2 miscarried as a

result of the prenatal test and 3 *de novo* abnormalities were stillborn, a total of 31 children were born. The maternal age was taken into account and *de-novo* abnormalities were shown to occur in 1.2% when the maternal age was ≤35, significantly higher than the general population (Bonduelle et al., 2002b). Approximately 50% of the abnormalities found were transmitted (mainly paternally derived sex chromosome or structural abnormalities), with a total frequency of 1.4% this is significantly higher than that found within the general population (Hook and Hamerton, 1977). Bonduelle et al. (2000b) presented evidence of an association particularly with poor sperm concentration and also poor motility, therefore males presenting with reduced semen quality for these particular semen parameters may be at an increased risk of producing a chromosomally abnormal child through ICSI.

Loft et al. (1999) investigated the outcome of 730 individuals born after ICSI from thirteen infertility clinics in Denmark. This study sent out questionnaires to the couples involved enquiring about pregnancy outcome, prenatal diagnosis, mode of delivery and the health of the child, a total of 665 returned the questionnaire and allowed further contact to the departments where the children were treated, hence not all abnormalities may have been reported within this study. Similar levels of abnormalities were found within this population compared to previous studies with a rate of major malformations estimated to be 2.2%. Prenatal karyotyping was carried out in 209 cases, 2.9% were found to have a major chromosome aberration including: trisomy 18, trisomy 21, triploidy and an unbalanced translocation, however Loft et al. (1999) suggest that up to $^{2}/_{3}$ of these may be attributed to maternal age as the mothers were aged over 35. One individual (0.5%) was found to have inherited a chromosome structural abnormality.

Six postnatal karyotypes were carried out revealing one deletion 13 and one case of trisomy 21.

Aboulghar et al. (2001) analysed the karyotypes postnatally of a total of 430 children conceived via ICSI and 430 children conceived naturally, the rate of chromosome abnormalities within the ICSI population was reported as 3.4% (15 individuals). This included six individuals with sex chromosome abnormalities, eight individuals with autosomal abnormalities and one individual with a combination of a sex chromosome and autosome abnormality. The control population of naturally conceived children had no observed cytogenetic abnormality within their karyotype and as a result a statistically significant increase in the rate of chromosomal abnormalities was reported within the ICSI population analysed when compared to individuals conceived naturally and the risk appears to be equal between the gonosomes and autosomes.

In contrast the recent study carried out by Hansen et al. (2002) reported a two fold increase in the incidence of major birth defects within their ICSI (301 individuals) and IVF (837 individuals) groups compared to individuals conceived naturally (reporting frequencies of around 8.6%, 9% and 4.2% respectively).

1.6.3.3 Studies looking at testicular extracted sperm (TESE) and cryopreserved embryos

Bonduelle et al. (1998b) also analysed three groups of children born with the assistance of ICSI, these groups were split as follows: group 1, 57 individuals whereby epididymal spermatozoa was utilised; group 2, 50 individuals whereby testicular spermatozoa was used and group 3, 58 individuals in which cryopreserved embryos were used. Prenatal

diagnosis identified 5.8% of the cryopreserved embryos had a 47,XXY karyotype. The rate of major malformations was estimated to be around 2.4% for all groups, with group 1 recorded as having a rate of 3.6%, group 2 rates of 2% were recorded and 1.8% for group 3, minor malformations were also recorded in 8.7% of all groups. Major malformations included ureteral dialation, leg and hip malformation and cleft lip and palate; minor malformations included facial, cardiac and dermatological abnormalities. However approximately $^1/_3$ of individuals were lost for follow-up at the age of two months, therefore these values may be under-reported.

A small case study analysing the ICSI outcome of four pregnancies utilising elongated spermatozoa obtained from TESE (Zech et al., 2000), reported two pregnancies out of the four resulted in major malformations. The ultrasound diagnosed a hydrocephalus in one pregnancy that was terminated, cytogenetic analysis of the foetal tissue revealed a male with trisomy 9 (47,XY + 9), the foetus also exhibited spina bifida and diaphragmatocele. In the second case the malformation was not picked up prior birth but was found to have a neural tube defect. It is not yet clear whether the malformations that occurred arose as a result of the injection of the immature testicular cells, or the procedure itself or if these events were unrelated. Zech et al. (2000) have reported to have at present stopped carrying out such treatment due to the potentially high rate of malformation and the poor success rate that has been observed even within this small number of individuals, and suggest any such treatment should be managed carefully and any resultant pregnancy should have an amniocentesis.

1.6.3.4 Studies investigating parameters including mental and growth parameters

Concerns over the use of ICSI were raised when the study carried out by Bowen et al. (1998) who analysed the medical and development outcome of children at the age of one born with the assistance of ICSI (84 children) and IVF (80 children). No significant difference was observed between the two treatments for congenital malformation and major health problems. This study did observe a significant risk of mild delays in development for children born with the assistance of ICSI when compared to IVF and naturally conceived children. Following this report, several studies have since investigated the development of children conceived by ICSI (Bonduelle et al., 1998b, 2003; Sutcliffe, 2001, 2003). These studies have each investigated at least 200 individuals with mean ages of 15 months to 28 months and have found no evidence of any significant differences in the development of children conceived by ICSI compared to controls. The studies, at present, are reassuring as there is little evidence of any difference in the developmental outcome of ICSI children, although few individuals have been analysed. However, Bower et al. (1998) have provided evidence that ICSI individuals may carry a small risk of developmental abnormalities and in light of this information it would seem pertinent to continue to observe many more ICSI individuals, and possibly into adulthood to ensure that these individuals are not at risk of intellectual impairment or learning difficulties throughout school (Bower et al., 1998; Fraser., 2002 www.nature.com/fertility).

1.6.3.5 Genomic imprinting and ICSI

Recent studies have indicated that possible adverse effects may arise form cryopreservation and the culturing of embryos that may lead to epigenetic alterations of the nuclear genome, including DNA integrity, gene expression and genomic imprinting

(Schatten., 2002 www.nature.com/fertility; Winston and Hardy., 2002 www.nature.com/fertility). Concerns have been raised with the use of testicular extracted spermatozoa in ICSI, as this may result in less complete imprinting than if ejaculated spermatozoa had been utilised (Van Steirteghem et al., 2002). Imprinting defects are unlikely to affect fertilisation but abnormalities may be found at birth or later on in life (Van Steirteghem et al., 2002).

1.6.4 Conclusions on the safety of ICSI

It is still not apparent as to whether any increased risk of abnormalities with ICSI is due to the use of genetically abnormal spermatozoa or whether the procedure itself is the cause. It is clear that the actual technique itself may lead to a number of problems including the injection of aneuploid spermatozoa but also incorporation of harmful contaminants within the egg, leakage of the cytoplasm and membrane damage as well as damage to the meiotic spindle, resulting in aneuploidy as a result of mitotic errors in early cleavage divisions (Bonduelle et al., 2002b; Winston and Hardy., 2002 www.nature.com/fertility). This increase is likely to be related to the increase in chromosomal abnormalities found within the males presenting with severe infertility (Meschede et al., 1998; Peschka et al., 1999; Scholtes et al., 1998; Tuerlings et al., 1998; Van Assche et al., 1996). Indeed Bonduelle et al (1996) reported that at least two of the semen parameters in the fathers of offspring trisomic for the sex chromosomes were found to be abnormal. Preliminary results confirm that the higher frequency of chromosomal aberrations reported in the spermatozoa of infertile men especially OAT males and those with abnormal karyotypes may confer an increased risk of chromosomal abnormalities in ICSI offspring.

Results in the follow-up studies of ICSI children are reassuring with the number of major congenital malformations found being higher than found in natural conceptions but comparable to those of IVF pregnancies. Comparisons between individuals conceived with the assistance of ART techniques and control populations are difficult to undertake as any increase in congenital malformations may be as a result of over-reporting as naturally conceived children are unlikely to be analysed as thoroughly as ICSI children in follow-up studies, differences in the definition of congenital malformations between studies may account for some discrepancies. The rate of abnormalities may however be lower if neonates are analysed by individuals untrained in this area, this can be the case when follow-up studies are augmented by the examination of subsequent hospital records or through telephone contact (Van Steirteghem et al., 2002), as in the study carried out by Loft et al. (1999). It is important to note that the figures reported may not represent the total rate of malformations as these are not only reported at birth but also in early childhood up to six years old, (Loft et al., 1999) and therefore may result in under-reporting. The recent study carried out by Hansen et al. (2002) reported a significant increase in the rate of major congenital abnormalities within ICSI and IVF conceived children, with an approximate two-fold increase compared to the normal population. It is clear that from the studies published to date that there is a increase in the risk of major malformations compared to offspring conceived naturally. However, data collected to date indicate that this increase is comparable to that found within IVF populations.

The use of ICSI has been shown to increase the risk of transmission of chromosomal abnormalities particularly that of paternal origin, mainly that of sex chromosome trisomy and autosomal rearrangements. Studies have revealed that in approximately

50% of cases chromosomes aberrations are *de-novo* in origin, with the remainder being parentally inherited, of these a significant majority are paternal in origin. Although the small body of literature available implies that these approaches are relatively safe (the incidence of chromosomal anomalies has been reported as ranging from 2.6% to 3.6%, higher than that of IVF pregnancies 2.1% (Bonduelle et al., 1996; 1999; 2002). It is apparent that there is an increased risk of *de-novo* aberrations involving the sex chromosomes anomalies among the offspring conceived by ICSI (Bonduelle et al., 1996; 1999; 2002b) but also potentially that of the autosomes also (Aboulghar et al., 2001). At present the detection of any increases in autosome trisomy within ICSI conceived children has not been reported, however this is to be expected as too few individuals have been analysed in follow-up studies. This is evident when considering Trisomy 21 for example, which has an occurrence of 1 in 800, however 90% of cases are maternally derived.

The fact that the significant increase in aneuploid spermatozoa in infertile males does not seem to be reflected in pregnancies established with ICSI may be due to the sample size analysed. Millions of spermatozoa have been analysed for aneuploidy but in comparison only a few thousand children born from ICSI have been analysed. It is also difficult to obtain adequate controls to compare the rates of abnormalities.

In the majority of studies, parameters such as postnatal growth and development have all been shown to be within normal ranges although some studies have revealed differences requiring further investigation. These findings are reassuring although it is important to realise that only a small number of patients have been analysed and not all ICSI individuals have extensive clinical follow up. Therefore the reported figures of

abnormalities in children conceived with ICSI may not reflect the true frequency of abnormalities within this population. The full risks and effects of ICSI are still unknown, primarily as none of these children have yet to reach reproductive age therefore there is still the question of the long-term reproductive health of these individuals. The limited studies carried out to date on the risks of ICSI has clearly identified a high risk of transmitting chromosomal anomalies, and producing de-novo chromosome aberrations mainly involving the sex chromosomes and imprinting defects and are therefore at risk of transmitting infertility. Further investigation needs to be continued in this area in order to fully ascertain the safety of this procedure with much larger patient cohorts being analysed (if not all) children conceived by ICSI and all findings documented.

1.7 What are the perceived risks of ICSI according to infertility specialists?

Given the information presented within sections 1.5 and 1.6, there is clearly a concern about the genetic risks and invasiveness involved in the procedure of ICSI in the treatment of male infertility. There has been considerable research analysing sperm aneuploidy levels in infertile males and the outcome of ICSI pregnancies. Despite this there are a number of questions that have so far been poorly investigated: firstly how these results are perceived and its relevance to ICSI by both infertile couples and the infertility clinics treating them. Secondly whether there is any consensus of opinion regarding the safety of ICSI in infertility clinics across the UK, and thirdly whether patients attending different infertility clinics are provided with similar information on the risks of ICSI (if this is not the case it may be that stricter guidelines should be put in place to ensure that patients receive the same information).

In an attempt to address some of these unanswered questions, P. Hyland, an MSc student undertaking her dissertation within our group designed a questionnaire based study that was sent out to all the infertility clinics around the UK, with the aim of establishing the following:

- What, if any, genetic tests do clinics regularly undertake in male patients prior to ICSI?
- Do all clinics offer genetic counselling prior to ICSI?
- Is aneuploidy in the spermatozoa of infertile men undergoing ICSI a concern in infertility clinics?
- Do infertility clinics currently screen the spermatozoa of these men for aneuploidy levels prior to ICSI?
- Do infertility clinics feel there is any merit in doing such aneuploidy studies prior to ICSI?

The results of this study have been published in Human Reproduction (Griffin et al., 2003) a copy of this paper can be found in Appendix A. The results of this questionnaire suggest that this uncertainty surrounding the safety of ICSI is not just of concern to the scientific community, but is also mirrored within the infertility clinics that are directly involved in treating infertile couples. A significant majority of infertility clinics acknowledge the benefits of screening spermatozoa for the frequency of aneuploidy prior to ICSI. This opinion appears to stem from the belief that specialists believe there to be an increased risk of congenital abnormalities associated with ICSI compared to IVF, which was mainly felt to be due to sperm aneuploidy, despite this, no participants felt that the risks of ICSI outweighed the benefits.

The results generated from this study indicate that the majority of infertility clinics do not screen spermatozoa for aneuploidy levels prior to ICSI and those that do may do so for research purposes only. At least twice as many infertility clinics however, feel that there is merit in pre-screening spermatozoa, compared to those that do not. Screening sperm will also initiate a much larger research study to evaluate the risks of ICSI, in particular the risk of transmitting Down syndrome and trisomy of the sex chromosomes. Given the results of this questionnaire the majority of participants agree that an aneuploidy screening test should be implemented prior to embarking on ICSI treatment along with the existing genetic tests. With this in mind we proceeded to develop such a test.

1.8 General conclusions

It is clear from the data presented within this chapter that there is a relationship between sperm aneuploidy and infertility, and that this affects the safety of current regimes used to treat it. The review of the literature has raised a number of questions that remain unanswered. Firstly, the majority of studies investigating sperm aneuploidy in infertile males have reported increased disomy frequencies, however significant inter-laboratory differences have been reported. It is unclear as to whether these are "true" differences or whether these are due to the patient and technical specific differences between studies identified in section 1.5.6. Secondly, what has been hitherto poorly investigated is whether particular infertility phenotypes are associated with the mal-segregation of specific chromosomes. Thirdly, the use of ICSI has been shown to result in the transmission of chromosome abnormalities but also that of *de-novo* aberrations including increased incidences of sex chromosome trisomies. Questions remain, as to whether this is the case for autosomal trisomies, and also regarding the future

reproductive health of ICSI conceived children. These questions can only be answered in years to come with more detailed monitoring of much larger numbers of individuals. Fourthly, given the evidence of the transmission of chromosomal abnormalities and the increase in sex chromosome trisomy in ICSI conceptuses, should infertility clinics offer a screening test for sperm aneuploidy frequencies prior to undertaking ICSI? Finally, ICSI is currently the only treatment currently available for male infertility, however it is a radical and invasive technique that has raised the concern of many individuals within the scientific community. To the best of our knowledge little research has been carried out investigating other forms of treatment that are less radical and invasive without the potential risks involved with ICSI. Only through a clearer understanding of the mechanisms involved in the aetiology of infertility and non-disjunction can safe pharmocogenomic strategies be implemented.

1.9 Objectives

This thesis is primarily concerned with the questions surrounding male factor infertility. Specifically, investigations have been undertaken examining the issue of whether the aneuploidy frequencies in men should be screened prior to ICSI. With regard to the aetiology of this phenomenon this study has been particularly interested in the relationship between individual semen parameters and sperm aneuploidy of specific chromosome pairs. In addition, preliminary evidence as to the role of genome organisation in the aetiology of sperm aneuploidy is presented. Finally, to the best of our knowledge, there are no reports of any medical intervention improving the rates of sperm aneuploidy. With this in mind, this study has investigated the role of a particular type of alternative therapy and its influence on sperm aneuploidy. Therefore this thesis had the following aims:

1) To development of a reliable protocol for the screening of sperm aneuploidy for chromosomes 21, X and Y prior to ICSI, for use within infertility clinics (chapter 2).
2) To determine the levels of chromosome aneuploidy for chromosomes 21, X and Y in infertile males to establish if particular infertility phenotypes were correlated were with the non-disjunction of the investigated chromosomes (chapter 3).
3) To investigate an alternative treatment to ICSI for male factor infertility, in particular the effect of traditional Chinese herbal medicine (TCM) on the aneuploidy rates for chromosomes 21, X and Y in six males (chapter 4).
4) To investigate the biological activity of TCM herbs used in the treatment of male infertility, specifically that of endocrine activity (oestrogenic, anti-oestrogenic, androgenic and anti-androgenic) and anti-oxidant activity (chapter 5).
5) To analyse genome organisation for chromosomes 18, X and Y within the nucleus of spermatozoa from control and infertile males to determine if the elevated frequency of sperm aneuploidy is related, in part, to changes in genome organisation (chapter 6).

Chapter 2

Development of a sperm aneuploidy screening kit for establishing levels of aneuploidy in ICSI men

Results of this chapter have attracted the interest of Stretton Scientific Ltd who wish to market a pre-ICSI sperm aneuploidy kit.

Chapter 2: Development of a sperm aneuploidy screening kit for establishing levels of aneuploidy in ICSI men

2.1 Introduction

Taking into consideration the results of the study produced by Griffin et al. (2003) located in Appendix A, the aim of this study was to develop a FISH sperm aneuploidy screening kit that could ultimately be made commercially available to infertility clinics, enabling the implementation of such screening. The key features of the screening kit method developed are that it would have to be: reliable, robust, reproducible, able to generate data rapidly, simple to use and that it should contain all the components required. That is any screening kit developed with a view to being used in a clinical setting would have to be reliable ensuring that it works every time (as it will be carried out in patient samples), the kit would also have to be robust ensuring that the technique works in a range of patient samples (including poor quality semen) and that it will work in a range of conditions. The other main features of the kit are that it has to be highly reproducible in that it would be transferable between labs. The technique should also be rapid enabling infertility clinics to carry out the technique and analysis in as short as time as possible so that couples can receive the results as soon as possible. The kit will also have to be made as simple as possible, so that individuals without previous training in FISH techniques would be able to carry out the technique.

2.2 Objectives

The aim of this study was to develop a screening kit ensuring that the requirements of the method were met; for the purpose of this study the chromosomes used in the development of the screening kit were chromosomes 21, X and Y. These specific

chromosomes were chosen as they are clinically significant, hence aneuploid conceptuses involving any of these chromosomes can survive full term giving rise to various syndromes including Down syndrome, Klinefelters syndrome, XYY amongst others (refer to section 1.4).

FISH involves the denaturation of chromosomes and fluorescent labelled chromosome probes (carrying a specific target sequence of the chromosome of interest), these are then hybridised in situ. Once hybridised the chromosomes are then stained with a total DNA counterstain for relocation purposes (Griffin, 1994). The critical steps in sperm FISH include: sperm nuclei decondensation, denaturation of the sperm nuclei and post hybridisation washes. As previously stated in section 1.5.6.2 decondensation of sperm nuclei is critical as it allows demembranation and decondensation of the DNA and protamines in the tightly packaged sperm nuclei. Absent or inadequate decondensation of sperm nuclei will result in reduced access of the probe to the sperm head, however excessive decondensation will result in highly diffuse probe signals. The denaturation of the chromosomal DNA and probe DNA is also of importance as under denaturation will result in the probe not being incorporated in to the chromosome and excessive denaturation will result in "fuzzy" looking chromosomes due to the spooling out of DNA (Bridger et al., 1997). The stringency of the post hybridisation washes are also important as if washes are not stringent enough excess probe will remain resulting in high background levels, however, if washes are over stringent it will result in probes being removed. When comparing reports published investigating sperm disomy levels, these have often report many technical differences with reference to the key factors required for successful FISH (refer to section 1.5.6.2). Section 8.2.3 within the materials and methods reports the sperm FISH procedure developed and utilised, however this

was the result of testing various methods, incubation times and temperatures within previously published work (refer to section 1.5), to determine the best method, that also incorporates all of the requirements of the kit. The adjustments in the sperm FISH technique will now be considered, with the optimised conditions being tested on semen samples from individuals with semen quality varying from normal to severely compromised semen parameters.

2.2.1 Probe protocol methods tested within the sperm screening kit

Two methods in these studies have been utilised, the first of which was a four probe approach (probe protocol 1) that used two probes labelled with two different fluorochromes. With the third probe produced by a combination of two probes spanning the same region (chromosome X centromere) labelled in two different fluorochromes to produce a third colour (figures 2.1, 2.2 and 2.3). This four probe (probe protocol 1) approach was used for the majority of the tests (98 out of the 128 cases tested), primarily due to the lack of appropriate filter sets on the Leica microscope utilised for the majority of the studies. This microscope system only enabled the detection of three different fluorochromes including that of the counterstain, therefore a different fluorochrome for each of the 3 probes and that of the counterstain was not possible. Latterly we were able to utilise a second probe protocol (protocol 2) that utilised a three probe approach (figure 2.4), in which the three probes and the DNA counterstain for the investigated chromosomes were labelled with four different fluorochromes. The use of this probe approach eliminated the need for a combinatorial approach that was made possible with the acquisition of a multi-colour FISH microscope system.

Specifically probe protocol 1 utilises a locus-specific probe for chromosome 21, spanning 21q22.13-q22.2 region, directly labelled with Spectrum Orange (LSI 21, Spectrum Orange) (figure 2.1). The probe for chromosome Y utilised encompassed the Yq12 region and was labelled in Spectrum Green (CEP Y, satellite III) (figure 2.2). For chromosome X a combination of a two different colour probes with Spectrum Green and Spectrum Orange spanning the Xp11.1-q11.1 region (CEP X, Spectrum Green and CEP X, Spectrum Orange) (figure 2.3).

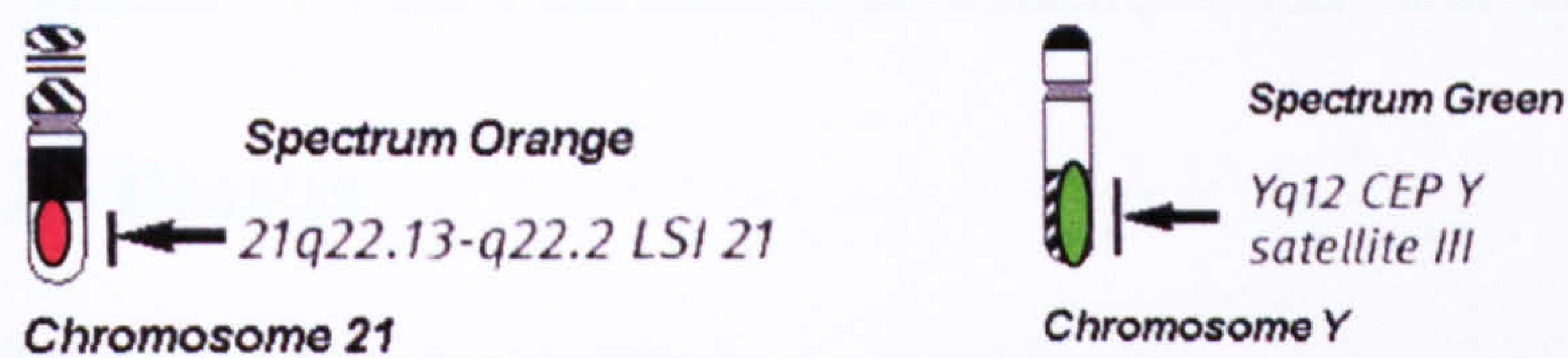


Figure 2.1 illustrates the ideogram for chromosome 21, the region the LSI 21 Spectrum Orange probe hybridises to used in protocol 1 (adapted from www.vysis.com).

Figure 2.2 illustrates the ideogram for chromosome Y, the region the CEP Y Spectrum Green probe hybridises to used in protocol 1 (adapted from www.vysis.com).

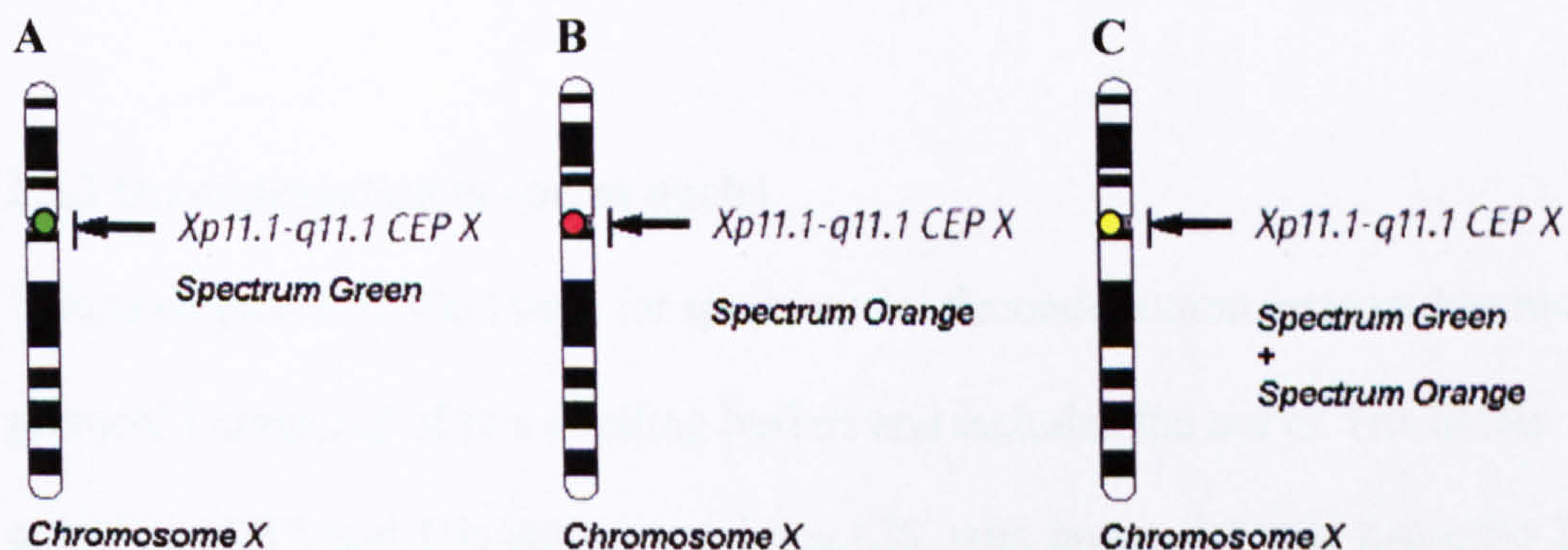


Figure 2.3 image A, B and C each show the ideogram for chromosome X and the region that the CEP X probe spans, image A illustrates the CEP X probe labelled in Spectrum Green and image B illustrates the CEP X probe labelled in Spectrum Orange. In probe protocol 1both of these probes (A and B) are utilised resulting in a CEP X probe that is yellow in colour (image C) (adapted from www.vysis.com).

Probe protocol 2 utilised the same probe for chromosome 21 (LSI, Spectrum Orange) and chromosome X (CEP X Spectrum Green only) as in protocol 1 and a CEP Y probe labelled in Spectrum Aqua spanning the region Yq12 was utilised for chromosome Y (figure 2.4).

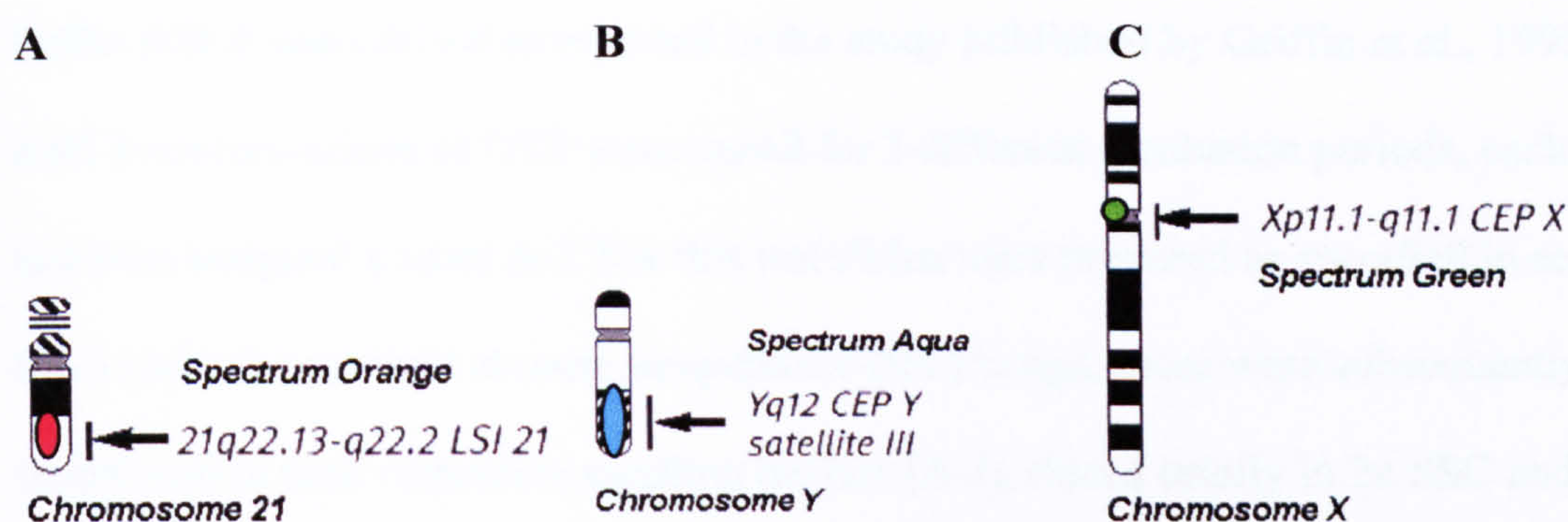


Figure 2.4 illustrates the ideograms for chromosomes 21, Y and X, along with the region to which each of the probes hybridise too and fluorescent label that each of these are labelled with. Image A: LSI 21 Spectrum Orange, image B: CEP Y Spectrum Aqua and image C: CEP X Spectrum Green (adapted from www.vysis.com).

2.3 Results

2.3.1 Sample preparation

Semen samples and slides were prepared as specified in the materials and methods section 8.2.3 and section 8.2.3.1 respectively.

2.3.2 Decondensation of sperm nuclei

Two main protocols were used for sperm nuclei decondensation prior to denaturation, protocol 1 consisted of two swelling buffers and included the use of Tris buffer containing DTT and Tris buffer containing LIS, with protocol 2 only using the Tris buffer containing DTT (refer to section 8.2.3.3).

2.3.2.1 DTT decondensation of sperm nuclei

The first stage was to determine the required concentration of the first swelling buffer containing DTT for adequate decondensation, reported to be approximately 1.5 – 2.5 times the original size of the sperm nuclei (Wyrobek et al., 1990). To determine the appropriate concentration of DTT to use in the decondensation step we tested the effect of a range of concentrations of DTT and incubation times, (in all cases a 0.1M Tris

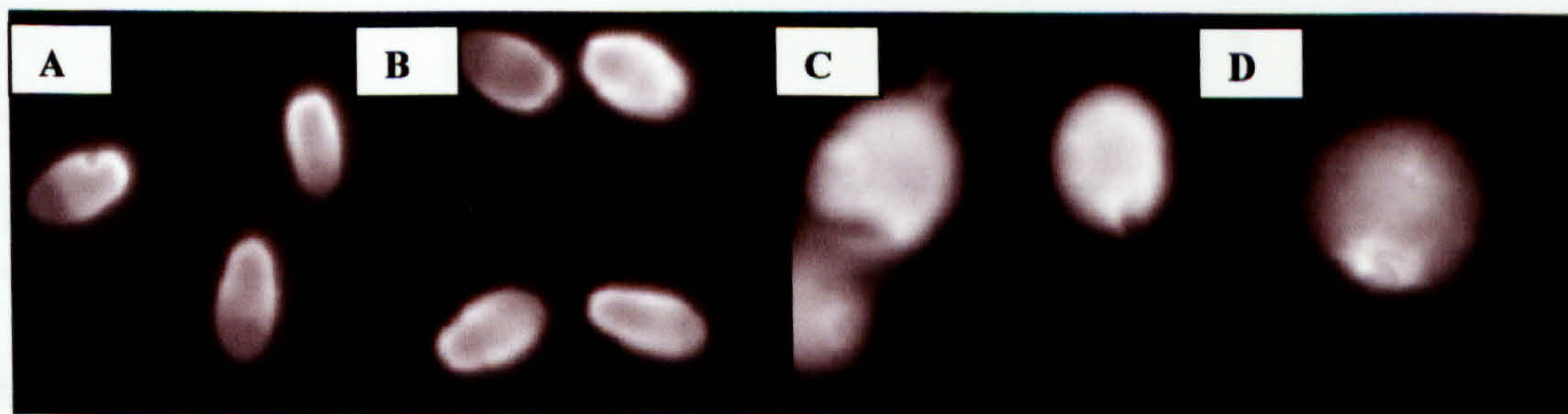
buffer pH8.0 was utilised as reported in the study published by Griffin et al., 1995). In total 3 concentrations of DTT were tested for 3 different incubation periods, each test has been assigned a letter A-I. For this test slides were prepared as specified in section 8.2.3 and left overnight at room temperature (RT) to age, these were subsequently transferred to their respective swelling buffers (A-I), rinsed briefly in 2x SSC and dehydrated through an ethanol row. Slides were then air dried and counterstained with DAPI to enable the degree of decondensation to be visualised, several samples of varying semen quality were tested in each, table 2.1 displays the concentration and incubation times of DTT, and figure 2.5 show the result of each type of treatment.

Table 2.1- Concentration and incubation times tested for the DTT swelling buffer

	A	B	C	D	E	F	G	H	I
C	5mM	10mM	20mM	5mM	10mM	20mM	5mM	10mM	20mM
IT	15	15	15	30	30	30	45	45	45

C = concentration, IT = incubation time

Figure 2.5- provides examples of the effect of the several different types of DTT treatment on the decondensation of sperm nuclei.



Panel A- sperm with no decondensation treatment, Panel B- sperm incubated in 5mM DTT, Panel C- sperm incubated in 10mM DTT and Panel D- sperm incubated in 20mM DTT, (all incubations were carried out for 30 minutes).

From the results presented in figure 2.1 it was deduced that the following methods (table 2.1) A, B, D and G produced inadequate decondensation with the sperm nuclei remaining similar to pre-decondensation size with methods C, F, H and I producing

excess decondensation with the nuclei approximately 2-3 times larger than nuclei not decondensed. Method E (figure 2.5-C) repeatedly gave the appropriate decondensation of approximately 1.5 – 2 fold size increase, irrespective of the sample quality decondensation of the sperm heads were similar in each patient (for the same methods).

2.3.2.2 LIS decondensation of sperm nuclei

The 10mM DTT (30 minute incubation) that produced the best results was then used in conjunction with a second swelling buffer containing LIS, to determine the effect of LIS. As with the DTT swelling buffer previous publications have specified a variety of concentrations and incubation times for LIS swelling buffers, as a result we tested several concentrations and incubation times (table 2.2). The same procedure as carried out for the DTT swelling buffer test was used however with the slide being transferred straight from the DTT swelling buffer into the LIS buffer prior to being rinsed and counterstained with DAPI.

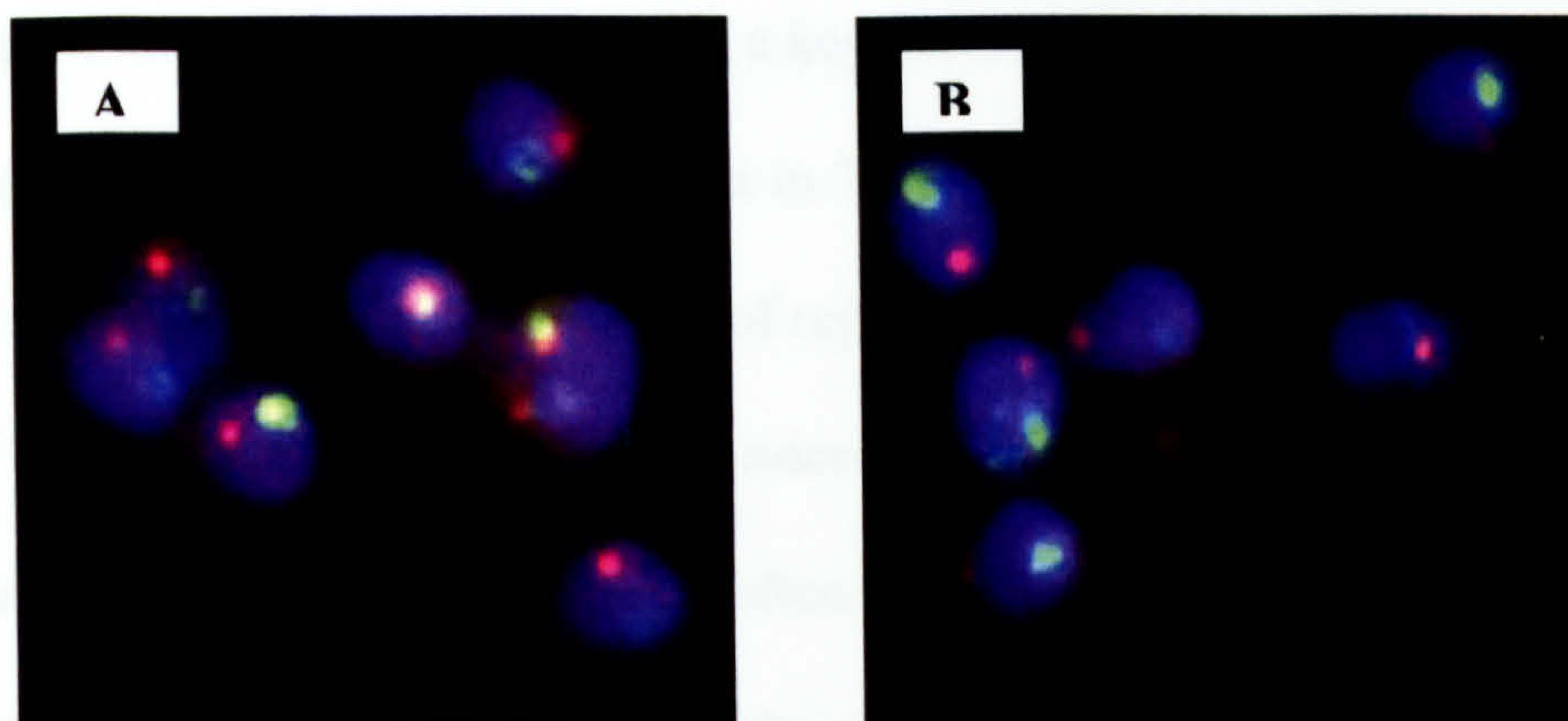
Table 2.2- Concentration and incubation times tested in conjunction with DTT

	1	2	3	4	5	6
C	10mM	25mM	10mM	25mM	10mM	25mM
IT	1hr	1hr	2hr	2hr	3hr	3hr

When comparing the results of the DTT alone and the use of LIS in addition to DTT, the degree of decondensation remained comparable, with no significant difference in the degree of decondensation of the sperm nuclei. It would appear from these results that the use of LIS does not cause sperm nuclei to undergo further decondensation, however, the role of both of these buffers is to enable the DNA probes to access the tightly packed DNA of the sperm nucleus and therefore it may have a role in facilitating access of the probe to the nucleus. We tested the two protocols DTT and LIS (DTT-10mM, 30 minutes and LIS 25mM, 1 hour) and DTT alone (10mM) in several FISH experiments

to determine which protocol at which concentration and incubation time produced the best results.

Figure 2.6 - image panel A illustrates the effects of DTT swelling buffer alone, image panel B illustrates the effect of DTT and LIS combined



The results from these experiments revealed no significant difference between the two treatments (A- 10mM DTT alone, and B- 10mM DTT and 25mM LIS) in the degree of decondensation of sperm nuclei, or hybridisation efficiencies (both of which produced hybridisation efficiencies of >95%). Each protocol produced reliable and reproducible results, with no obvious differences in the results obtained for each test the DTT swelling buffer alone was utilised for the majority of the sperm FISH studies. On a few occasions however, there were individual samples that were not adequately decondensed with weak/absent hybridisation signals, these particular samples were tested on a number of occasions and with other samples that were successful. After several attempts we utilised a combination of the DTT and LIS swelling buffers to determine if the addition of the LIS buffer could better enable the probe to access the sperm nuclei than DTT alone. In 8 out of 14 samples exhibiting this problem the addition of the LIS swelling buffer made analysis possible (previously not possible with DTT treatment alone), producing clear hybridisation signals with hybridisation

efficiency levels of around >90%. In the remaining six samples tested the degree and quality of the hybridisation signals was not good enough for analysis.

2.3.3 Denaturation

The denaturation of the sperm nuclei is a key factor in the reliability of the screening kit, we tested several denaturation times in 70%formamide/2xSSC, and included 7, 8, 9, 10, 11, 12 and 13 minutes. The results of repeated tests revealed that the denaturation times of 7-9 minutes often resulted in under-denaturation (hence absent or weak signals, denaturation times of over 12 minutes often resulted in over-denatured sperm nuclei. The most reliable and reproducible results were obtained using denaturation times of 10-11 minutes, for all FISH procedures the denaturation times of 10/11 minutes were utilised.

2.3.4 Probe protocol development

2.3.4.1 Development of probe protocol 1

Throughout this study two main chromosome probe protocols were used, both commercially available and directly labelled probes obtained from Vysis (Downers Grove Ill. USA). As mentioned previously the chromosomes chosen for this investigation were chromosomes X, Y and 21. In order to distinguish the individual chromosomes they have to be labelled with different fluorochromes, figures 2.1, 2.2 and 2.3 displays ideograms for these chromosomes and the region for which these chromosome probes are specific for. In order to investigate the sex chromosomes Probe protocol 1 is described in section 8.2.1.5.1 (materials and methods) briefly involves a chromosome 21 probe labelled in Spectrum Orange, a Y chromosome probe labelled in Spectrum Green. Chromosome X was produced by combining two probes specific for

the same chromosome region labelled with two different fluorochromes (Spectrum Orange and Spectrum Green) to produce a yellow colour enabling the distinction of the individual chromosomes. The manufacturers recommendations suggest that when using multiple probes 1μl of each probe should be added to an eppendorf along with 7μl of the hybridisation buffer. This protocol was followed for the first few experiments, the results of these experiments produced hybridisation signals for each of the chromosomes. However, several problems with this protocol were encountered the first of which was the chromosome 21 probe was weak and difficult to visualise down the microscope, and the second of which was the inability to distinguish between the chromosome Y and X probe. Using this protocol the colour of the X was not sufficiently different to that of the Y. To solve these problems we set up an experiment with different probe ratios (table 2.3) to determine if we could make the chromosome 21 probe more intense and if we could differentiate between the X and Y probes.

Table 2.3- Probe volumes tested for each of the four probes used in this approach

Chromosome	*Test A*	*Test B*	*Test C*
X (Orange)	1.25μl	1.0μl	1.0μl
X (Green)	0.5μl	0.5μl	0.75μl
Y (Green)	0.5μl	0.5μl	0.75μl
21 (Orange)	1.25μl	1.5μl	1.25μl
Hybridisation buffer	7μl	7μl	7μl
Total	10.5μl	10.5μl	10.5μl

Figure 2.7- Examples of sperm nuclei after FISH for chromosomes 21, X and Y, each panel (A, B and C) corresponds to test A, B and C (table 3.3) to determine the appropriate probe volumes to use

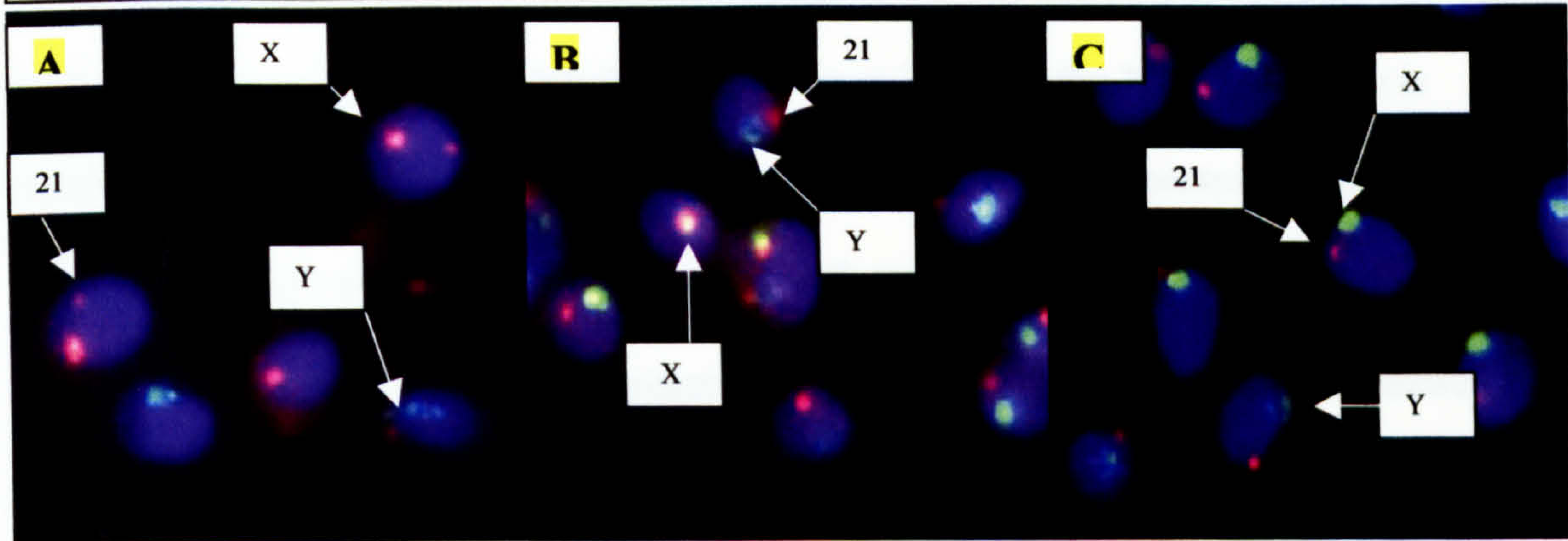


Figure 2.7- Panel A- (test A table 3.3), each chromosome (X, Y and 21) are indicated, the results show chromosome X to be orange in colour similar to that of chromosome 21.
Panel B- (test B table 3.3), chromosomes X, Y and 21 are indicated, in this case chromosome X has the desired yellow colour , hence it is distinguishable from that of chromosomes 21 and Y.
Panel C- (test C table 3.3), indicated are chromosomes X, Y and 21,in this probe protocol chromosome X was a green colour making it difficult to distinguish from that of chromosome Y.

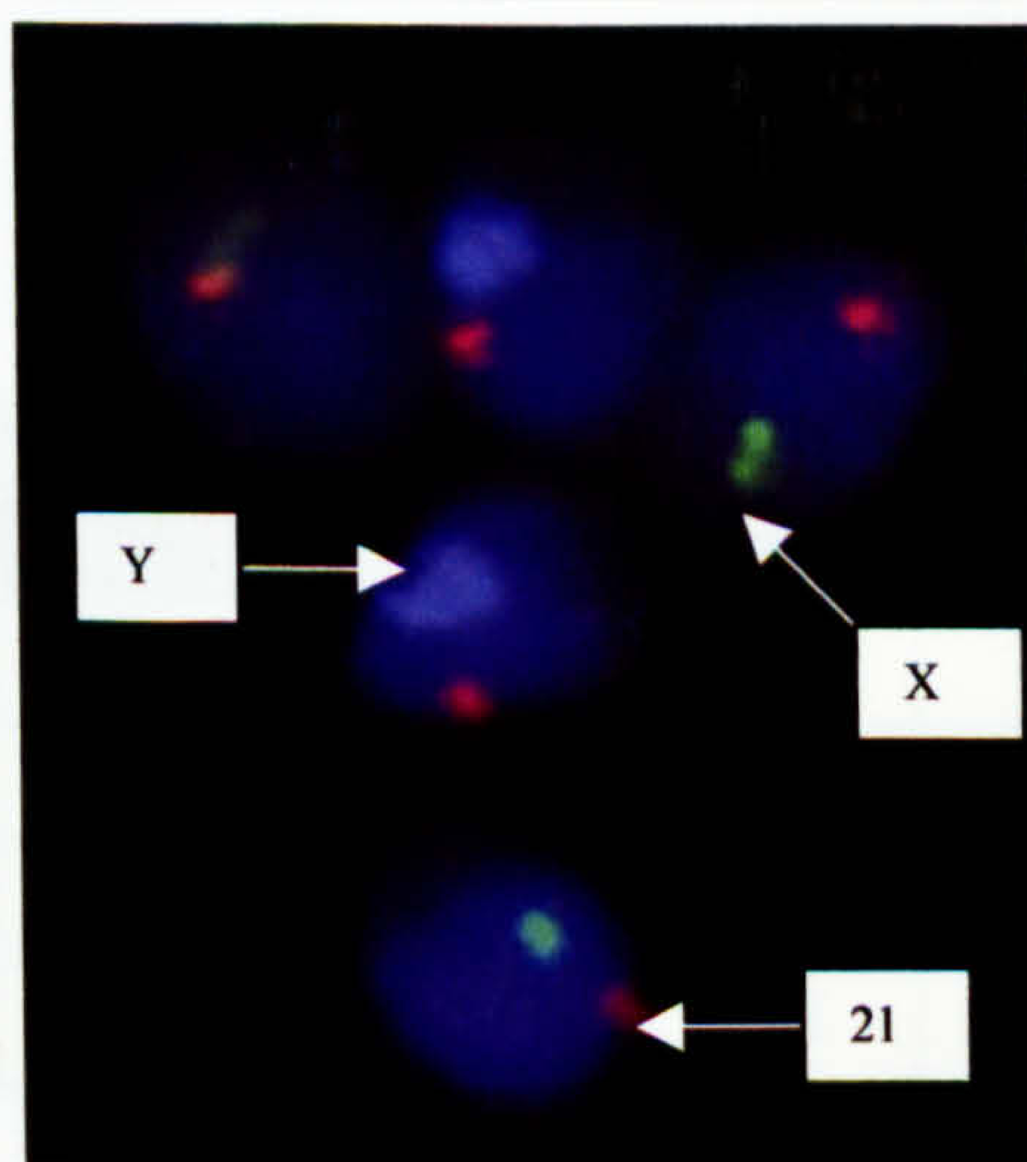
Analysis of each of the slides (figure 2.7) revealed that for test A and C the chromosomes X and Y were not easily distinguished, and the signal for chromosome 21 was also still weak. However test B had a clearly visible chromosome 21 and the X chromosome was distinguishable from that of the Y chromosome as it was yellow-orange in colour. All subsequent FISH studies utilised this probe protocol, however there were problems with the reproducibility of this protocol. The chromosome X was not always yellow-orange in colour, with occasions where it would be greener in colour (hence indistinguishable from chromosome Y), or more orange in colour (hence indistinguishable from chromosome 21).

2.3.4.2 Development of probe protocol 2

As a result we tried a second probe protocol, to try to resolve the reproducibility issues, for this protocol we utilised chromosome probes labelled with three different fluorochromes. The probes were obtained from the same commercial company (Vysis,

Downers Grove, Ill, USA). Ideograms for each chromosomes along with the regions for which the chromosome probe are specific for are found in figure 2.4. In the probe protocol 2 chromosome 21 is labelled in Spectrum Orange; the X chromosome is labelled in Spectrum Green and the Y chromosome labelled in Spectrum Aqua. Initial experiments were carried out in accordance with manufacturers recommendations (utilising 1μl of each chromosome paint and 7μl of hybridisation buffer). As before there were technical difficulties with the ability to visualise the weak chromosome 21 signal, this was rectified by using 1.5μl of the chromosome 21 probe and 1μl of the X and Y probe. The new probe protocol and the adjusted probe ratios resulted in a reliable reproducible assay for which the intensity of chromosome 21 was clearly visible down the microscope. This protocol also resulted in the elimination of analysis problems arising due to the inability of distinguishing chromosome X from that of chromosome Y or 21 (due to the fact that different fluorochromes were utilised for all chromosomes figure 2.8).

Figure 2.8- illustrating the three colour approach, chromosome 21- orange, X- green and Y- aqua



2.3.5 Aneuploidy screening kit results

The aneuploidy screening kit was tested in 165 samples, of which 128 samples successfully yielded results (defined as able to accurately score the frequency of aneuploidy in at least 5,000 spermatozoa). In some of these cases the probes were easily distinguished from that of the other chromosome probes and DAPI counterstain, in this situation scoring of sperm cells was rapid and easily undertaken, ideal for use within a clinical setting. There were however, cases in which the FISH was successful, but that the probes were difficult to distinguish from each other. For example with probe protocol 1 chromosomes X was sometimes difficult to distinguish from chromosomes 21 or Y, or with probe protocol 2 chromosome Y was sometimes difficult to distinguish from the DAPI counterstain. In these cases slides could still be scored however, it required significantly more time. This is a result of each area having to be checked with individual filter sets rather than dual or triple filter sets that enable all probes to be visualised simultaneously. These slides are thus, amenable for analysis however, this situation is not ideal for a clinical setting, as slides can take between 1.5-5 times longer to analyse. The results generated for these 128 individuals, including the probe protocol and swelling treatments used are presented in table 2.4.

Table 2.4- Results produced by the screening kit including that of the type of swelling buffer, probe protocol used along with details on the ability to distinguish the three probes and the DAPI counterstain.

	DTT	LIS	Probe protocol 1	Probe protocol 2	A	B	C	D
No of patients	X		88		48	22	18	
No of patients	X	X	10		7	1	2	
No of patients	X			26	8			18
No of patients	X	X		4	2			2

Table 2.4 X- indicates the type of swelling buffer utilised, **A-** probes easily distinguished from each other and the DAPI counterstain, **B-** chromosome X not easily distinguished from chromosome Y, **C-** chromosome X not easily distinguished from chromosome 21, **D-** chromosome Y not easily distinguishable from the DAPI counterstain.

In addition to this karyotype analysis was carried out in 57 patients, in all cases at least 10 metaphases were karyotyped and the number of chromosomes in over 30 metaphases were counted to rule out chromosome mosaicism (Hook, 1977). All individuals were found to have no karyotype abnormalities (46,XY). Out of these 57 individuals sperm FISH analysis was only possible in 10 individuals, we believe that this is due to the fact that the blood and semen samples were sent through the post; as a result we received them up to three days later. In 8 cases however, the addition of the LIS decondensation buffer to the protocol enabled FISH analysis, with the remaining 2 patients recruited from another study (for which samples were received same day).

2.5 Discussion

We have undertaken a series of experiments to determine the appropriate concentrations and incubation times for various key stages in the sperm FISH assay in order to ensure that the technique and probe protocols are reliable, robust and reproducible, suitable for use in a clinical setting. Our experiments have shown that the concentrations of DTT and LIS found to be the most effective were 10mM and 25mM respectively at incubation times of 30 minutes and 1-2 hours respectively. The use of LIS as a swelling buffer was also found not to be essential (with the exception of a few circumstances). The most effective denaturation of sperm nuclei occurred between 10 and 11 minutes. Two probe protocols were utilised the first of which utilised a combination of two fluorochromes to produce a third colour, however their were reproducibility problems arising with the inability to distinguish between the other investigated chromosomes. As a result this was subsequently changed to a three-colour approach resolving this problem. This approach has also proved problematic as in many situations the aqua Y was not distinguishable from the DAPI counterstain.

Within the literature the concentration of DTT and length of incubation is one of the principal technical differences between studies, for example within FISH studies DTT concentrations ranged from between 0.01mM –25mM (Acar et al., 1999; Calogero et al., 2001), with incubation times of 30 minutes to overnight (Acar et al., 1999; Shultz et al., 2000). In the vast majority of samples the 30 minute DTT (10mM) swelling buffer treatment alone was more than adequate producing reliable and reproducible rates of decondensation with clear distinct punctate signals required for scoring analysis in over 120 patient samples, however, in 14 cases DTT swelling alone did not appear to be adequate enough to produce results amenable for analysis. Upon further investigation it was discovered that these 14 samples included ones processed the following day (refer to section 8.2.3 in the materials and methods) or processed several hours later than in the normal situation. For these samples the addition of the LIS swelling buffer step enabled analysis to be performed in 57% (8/14) of cases. It is apparent that samples need to be processed as soon as possible (preferably within 1-3 hours after production). If this does not occur it may be possible that DTT swelling alone is not capable of producing adequate demembranation or decondensation of the nucleus, thus not enabling the probes to enter the nucleus. The addition of the LIS step in the protocol however, (shown not to have any effect on the swelling of the nucleus) may in fact have an additional role in causing further demembranation of the sperm nucleus, enabling the DNA probes to access the sperm nucleus. We suggest therefore that the addition of the additional swelling buffer step of LIS (concentration for 1 hour) is optional however should be recommended as it has no negative effects and has proved to aid in situations where for whatever reason samples have not been processed in the sperm buffer within several hours of production.

Within this study denaturation times of 10-11 minutes produced the most reliable results with times below this often resulting in under-denaturation and times above this resulting in over-denaturation.

Initial studies used a combinatorial approach for chromosome X, however this produced a great deal of difficulty in the collection of data, due to difficulties in distinguishing individual chromosomes. This approach rarely produced reproducible results and hence was not suitable for the inclusion in a clinical screening kit. Therefore a different three-colour approach (red 21, green X and blue Y) was put in place eliminating the reproducibility problem. This protocol also required only three fluorescent-labelled probes rather than four (as in the previous protocol) and will be less costly and more suitable for inclusion in a screening kit.

In conclusion we have developed a protocol for an aneuploidy screening kit, this protocol has been successfully used by researchers and undergraduate students to screen over 150 control and patient samples (with varying semen quality) with few problems. The results of our initial testing of the various parameters required have ensured that this FISH technique is reliable, robust, reproducible, easy to follow and given the appropriate microscope filter sets will enable the probes to be easily distinguished from each other. The data generated from this kit has resulted in the interest of a company (Stretton Scientific Ltd). Stretton Scientific Ltd are interested in commercialising this kit for sale and distribution to infertility clinics for the use of screening sperm aneuploidy levels prior to ICSI and are currently working with us to achieve that goal.

Chapter 3

The association between male infertility and sperm disomy: Evidence for variation in disomy levels among individuals and a correlation between particular semen parameters and disomy of specific chromosome pairs

Work from this chapter was submitted for publication in Human Reproduction, a resubmission has been invited

Chapter 3: The association between male infertility and sperm disomy: Evidence for variation in disomy levels among individuals and a correlation between particular semen parameters and disomy of specific chromosome pairs

3.1 Introduction

The relationship between male infertility and elevated proportions of aneuploid sperm in any given ejaculate is now extensively documented (refer to section 1.5). Over 30 studies have investigated this effect with the majority suggesting a highly significant relationship between decreased semen quality parameters and increased sperm disomy (refer to section 1.5.4). Differences between the frequencies reported between studies may be due to technical and patient specific differences (refer to section 1.5.6). An alternative explanation however is that, among individuals and individual patient cohorts, some men have elevated levels of sperm disomy associated with infertility whereas others do not. It is possible that perhaps environmental influences could play a role, if this is the case it may account for patient specific differences observed between studies. Indeed, a number of synthetic chemicals have been shown to be able to mimic endogenous hormones and affect the normal pattern of reproductive development in wildlife (Tyler et al., 1998). In humans, levels of sperm disomy can be increased by environmental factors including: alcohol abuse and heavy smoking (refer to section 1.5.9.1). Intrinsic factors such as age, have been well established (refer to section 1.5.3) and DNA polymorphisms have also been implicated. Abruzzo et al. (1996) found no effect of Y chromosome alphoid array size on Y chromosome non-disjunction, however Hobbs et al. (2000) recently identified a genetic polymorphism involved in folate metabolism as a significant risk factor for trisomy 21.

To date a handful of studies have been published investigating the possible existence of a direct relationship between individual semen parameters and aneuploidy (e.g. are specific semen parameters associated with aneuploidy for particular chromosome pairs). Several studies have reported a correlation between teratozoospermia and sperm disomy (Calogero et al., 2001b; Templado et al., 2002; Viville et al., 2000). However Viville et al. (2000) were the only study to investigate teratozoospermia alone, analysing four males presenting with different types of total teratozoospermia (including shortened flagella syndrome, globozoospermia, irregular acrosomes and macrocephalic sperm). In that study no significant difference was reported for three patients however one patient with 100% macrocephalic spermatozoa had a total aneuploidy rate (including nullisomy, disomy and diploidy) of around 90% demonstrating a significant correlation with morphology for patients with macrocephalic spermatozoa. Further studies, demonstrated a negative correlation between sperm disomy and sperm concentration (Rives et al., 1999; Vegetti et al., 2000; Calogero et al., 2001b). Correlations were also found between disomy and progressive motility (asthenozoospermia) (Vegetti et al., 2000; Hristova et al., 2002). In the majority of the above studies either semen parameters and or aneuploidies for individual chromosome pairs were grouped together and thus not considered individually with the exception of the study carried out by Viville et al. (2000).

3.2 Objectives

Given the above information, the purpose of this study was to determine the following:

- To investigate the sperm aneuploidy levels for chromosome 21, X and Y in repeat samples from the same individual to investigate potential variations within the rates of disomy.
- To compare the individual specific semen parameters on a sample-by-sample basis.
- To establish if particular infertility phenotypes were associated with specific chromosome aneuploidy for the investigated chromosomes. To determine chromosome-specific and parameter-specific correlations between male infertility and percentage of aneuploid sperm in an ejaculate as a preliminary step towards understanding the mechanisms of the association between male infertility and chromosome segregation

3.3 Patient cohort and experimental design

A series of males undergoing infertility treatment with a range of andrological phenotypes were assessed for conventional semen parameters and for sperm disomy. All patients were attending IVF clinics in the central London area. Semen samples were taken, with patients' informed consent, from 19 different men on 43 occasions from infertility clinics in central London. None had known constitutional karyotypic abnormalities or Y chromosome deletions (however the majority declined permission to have blood samples taken). We received 1 sample each from 12 men, 2 samples from one man, 3 samples from 2 men, 4 samples from one man, 6 samples from 2 men and 7 samples from one man. In some men one, two or three of the semen parameters measured (concentration, motility and morphology) were within the normal range; these were hence placed in a control group. In other cases (test group) individual parameters were in the abnormal range (refer to

subsequent section for andrological criteria). Given that some samples were taken from individual patients on several occasions, these males sometimes appeared within the control group for some samples and in the test group for others. We restricted our molecular cytogenetic studies to chromosome 21 and the sex chromosomes for three reasons. Firstly, according to previous studies (Blanco et al., 1996; Hassold et al., 2001; Scarpato et al., 1996; Spriggs et al., 1996) these are the most prone to non-disjunction in sperm and hence the most likely to give significant results. Secondly, the large number of sperm that needed to be scored per individual to establish statistically significant results precluded the study of large numbers of chromosome pairs. Finally these pairs are the most clinically significant as they lead to common mutant phenotypes among liveborns. That is, unlike most trisomies that abort in the first trimester, trisomies of the sex chromosomes and chromosome 21 frequently go to term and can lead to Klinefelter syndrome and Down syndrome respectively.

3.3.1 Semen analysis

Patients were assessed for sperm quality, the same operator performed all analyses. Individual samples were then placed in three occasions (for concentration, motility and morphology) into a "test" or "control" group based on the results of the semen assessments. For the studies investigating sperm concentration individuals were placed in the control group if there semen assessment revealed a sperm concentration of ≥20 million/ml, those individuals with <20 million/ml were placed in the test group. When investigating motility individuals were placed into the control groups if individuals had forward motility of ≥20% and those with <20% forward motility were placed in the test group. Finally when

investigating sperm morphology the control group consisted of individuals who had a normal morphology of ≥4% and those with <4% normal morphology placed in the test group. The cut-off points for considering individual samples as being in the test or control groups were based on WHO guidelines for oligozoospermia, severe asthenozoospermia, and severe teratozoospermia and were comparable to those used in other studies for sperm disomy (Pang et al., 1999). In each case the sample was assessed by fluorescent in-situ hybridisation (FISH) for the proportion of disomic sperm for chromosomes X, Y and 21.

3.3.2 Statistical Analysis

The hypotheses of interest were whether the rate of disomy was significantly different for the test and control groups in terms of sperm concentration, morphology or motility. Data on the rate of disomy were generated for chromosome 21 and the sex chromosomes. Initially linear regression was utilised to determine if a correlation could be found between individual semen parameters and chromosome disomy levels for X, Y and 21. The distribution of the data (refer to table 3.1) however, did not lend itself to this particular analysis, as the data points were not normally distributed. This is illustrated for example when considering abnormal morphology (table 3.1), in total 43 semen samples were obtained with a range of abnormal forms (%) of between 86-100%, of these over 70% of the data points were skewed to one side (i.e. between 96 and 100%). In consultation with statistician Dr. D. Wright it was decided that the data would be more suitable for analysis with logistic regression. To test the hypotheses six logistic regression models were fitted in the statistical software package SAS (Version 8.2). Logistic regression is a statistical model that examines the relationship between one or more independent variable and is used to

determine the probability of whether an event occurs by chance or not. For each model an Odds Ratio, a 95% confidence interval and a p-value were calculated. A confidence interval that does not contain 1 implies that there is evidence that the disomy rates were significantly different for that comparison and hence has a corresponding p-value of < 0.05.

3.4 Results

In total for the 43 samples analysed for chromosomes X, Y and 21 a total of 209,188 spermatozoa were scored (approximately 5,000 per sample). The results of the sperm disomy rates calculated for these chromosomes and the semen assessments for each of the 43 samples analysed are presented in table 3.1. The total rate of disomy for the sex chromosomes and chromosome 21 was found to be 0.3% (633/209,188) and 0.19% (398/209,188) respectively. In agreement with previous studies published (refer to intro) we report a high degree of inter-individual differences with disomy levels ranging from 0.02% - 2.00% for the sex chromosomes and 0.00% - 1.46% for chromosome 21. We also found a surprisingly large variation in aneuploidy frequency in patients for which multiple samples were received. Including patient 5, who gave 4 samples, had sex chromosome disomy rates between 0.06% and 0.22% and chromosome 21 disomy rates between 0.1% and 0.24%). Patient 9 (6 samples) ranged from 0.1% and 0.73% for the sex chromosomes, 0.12% and 0.25% for chromosome 21. Patient 10 (3 samples) 0.02%-0.24% (sex chromosomes) and 0.04%-0.38% (chromosome 21). Patient 12 (7 samples) 0.12%-0.44% (sex) and 0.11%-1.46% (21). Patient 14 (6 samples) 0.08%-0.65% (sex) and 0.04%-0.37% (21). Patient 17 (3 samples) 0.08%-0.24% (sex) and 0.04%-0.24% (21) (Table 3.1).

Table 3.1 Sperm disomy for the sex chromosomes and chromosome 21 and the semen analysis in 43 men.

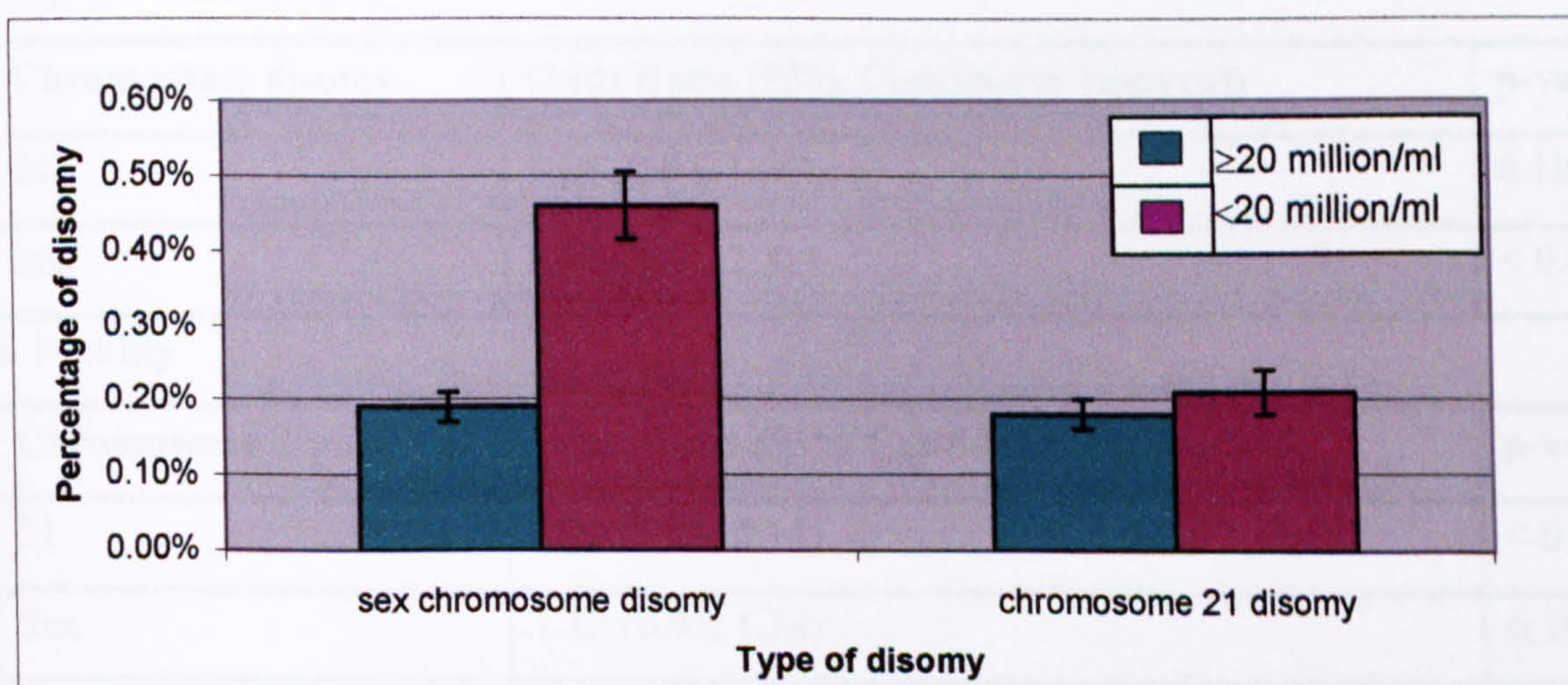
Patient number	*% disomy - sex chromosomes*	*% chromosome 21 disomy*	*Total cells scored*	*count (million/ml)*	*Motility (%)*	*% abnormal forms*	*Semen analysis*
1	0.50%	0.43%	5097	6.9	26	97	OT
2	1.02%	0.10%	5000	6.6	48	98	OT
3	0.29%	0.29%	4864	128	63	86	N
4	0.35%	0.20%	4790	13.5	17	98	OAT
5a	0.17%	0.24%	5400	61	24	95	N
5b	0.22%	0.10%	5092	37.5	7	95	A
5c	0.12%	0.10%	5000	22	45	99	T
5d	0.06%	0.14%	5000	63	13	94	A
6a	0.14%	0.08%	5000	48	44	98	T
6b	0.12%	0.04%	5000	40	50	99	T
7	0.18%	0.20%	5000	4.8	<10	95	OA
8	0.74%	0.00%	544	<0.01	<10	100	OAT
9a	0.73%	0.25%	5066	17	18	96	OAT
9b	0.45%	0.24%	5056	12	11	97	OAT
9c	0.30%	0.14%	5084	21	30	96	T
9d	0.10%	0.22%	5000	80	8	98	AT
9e	0.18%	0.12%	5063	13.3	26	97	OT
9f	0.24%	0.12%	5004	7.9	42	98	OT
10a	0.24%	0.38%	5000	25	48	99	T
10b	0.02%	0.04%	5000	8	38	97	OT
10c	0.16%	0.12%	5000	22	41	98	T
11	0.17%	0.09%	5275	41	46	93	N
12a	0.96%	1.46%	4050	69	10	91	A
12b	0.44%	0.11%	5449	10.3	26	87	O
12c	0.30%	0.12%	5048	9.5	11	97	OAT
12d	0.24%	0.14%	5018	63	17	97	AT
12e	0.18%	0.08%	5031	32	41	96	T
12f	0.12%	0.06%	5061	57	37	96	T
12g	0.14%	0.18%	5000	24	58	87	N
13	0.06%	0.02%	5000	96	60	92	N
14a	0.65%	0.20%	3521	12	36	97	OT
14b	0.24%	0.08%	5009	7.5	53	99	OT
14c	0.14%	0.10%	5001	37	27	97	T
14d	0.08%	0.14%	5037	16	45	98	OT
14e	0.20%	0.04%	5000	30	40	100	N
14f	0.27%	0.37%	4108	16	38	94	O
15	2.00%	0.59%	5293	14	57	96	OT
16	0.31%	0.21%	5120	47	49	89	N
17a	0.24%	0.24%	5000	18	53	92	O
17b	0.08%	0.04%	5000	49	44	96	T
17c	0.08%	0.04%	5000	44	56	92	N
18	0.10%	0.26%	5000	123	10	96	AT
19	0.30%	0.18%	5107	52	46	89	N

Patient number: Letters after patient number indicate consecutive samples from the same patient e.g. 10b is the second sample from patient 10. Semen Analysis: O = oligozoospermia; A = severe asthenozoospermia; T = severe teratozoospermia; N = normozoospermia

3.4.1 The relationship between chromosome aneuploidy and sperm concentration

Each of the 43 samples from 19 different individuals were placed either into a control group or a test group dependent on the results of the sperm concentration of sperm in the ejaculate as determined by the semen assessment (Table 3.1). In total 25 individuals were placed in the control group (those with a sperm concentration of ≥ 20 million/ml) with the remaining 18 samples placed into the test group (those with a sperm concentration of < 20 million/ml). The mean rate of disomy for the control group and test group for the sex chromosomes and chromosome 21 were 0.19% and 0.46%, and 0.18% and 0.21% respectively (Figure 3.1).

Figure 3.1 Graph to show the mean sperm disomy frequencies for the sex chromosomes and chromosome 21 in two groups with sperm concentration of ≥20 million/ml and <20 million/ml, error bars represent SEM.



Statistical analysis using logistic regression analysis clearly demonstrate that men with oligozoospermia (sperm concentration of < 20 million/ml) have significantly elevated levels of sex chromosome disomy (Odds Ratio 2.39, $p < 0.0001$) (table 3.2) in their sperm compared to men with normal sperm count levels (sperm concentration ≥ 20 million/ml).

As the lower limit of the 95% confidence interval for the Odds Ratio is 2.04 these data suggest that the rate of sperm disomy is likely to be at least twice as high in test patients compared to controls. Further analysis revealed that this increase was largely accounted for by an increase in XY disomy (with around 72% of the sex chromosome disomy rate attributed to XY disomy), which is usually associated with non-disjunction errors of meiosis I. Conversely there was no evidence of a significant association between oligozoospermia and sperm disomy for chromosome 21 with disomy rates of 0.18% within the control group and 0.21% in the test group.

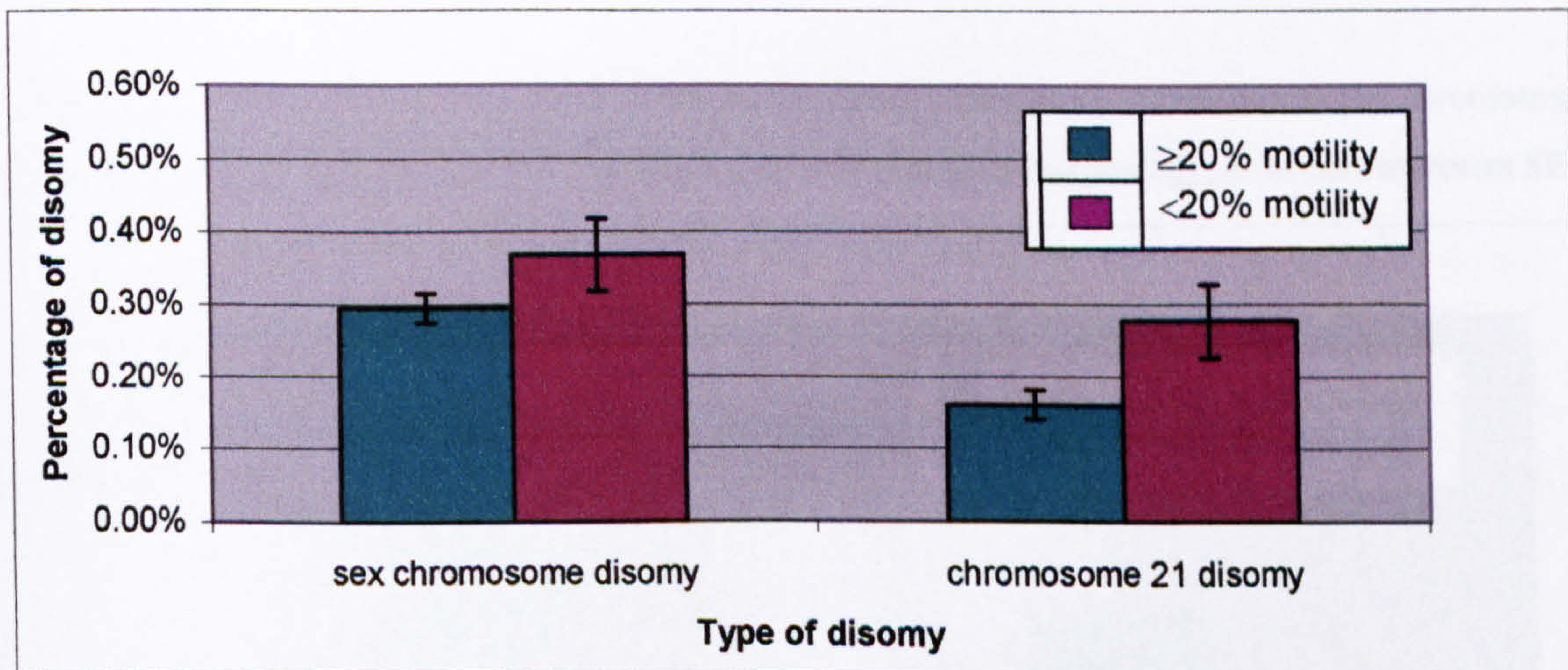
Table 3.2 Logistic regression analysis of individual disomy rates compared to semen parameters. a. Sperm concentration, b. sperm motility, c. sperm morphology.

a. Sperm concentration		
Chromosome disomy	**Odds Ratio (95% Confidence Interval)**	**p-value**
21	1.14 (0.94, 1.40)	0.18
Sex	2.39 (2.04, 2.81)	< 0.0001
b. Motility		
Chromosome disomy	**Odds Ratio (95% Confidence Interval)**	**p-value**
21	1.75 (1.43, 2.14)	< 0.0001
Sex	1.12 (0.95, 1.34)	0.19
c. Morphology		
Chromosome disomy	**Odds Ratio (95% Confidence Interval)**	**p-value**
21	1.54 (1.26, 1.89)	< 0.0001
Sex	1.22 (1.04, 1.43)	0.013

3.4.2 The relationship between chromosome aneuploidy and sperm motility

As for sperm concentration each of the 43 samples from 19 different individuals were placed either into a control or a test group dependent on sperm motility as determined by the semen assessment (Table 3.1). In total 31 individuals were placed in the control group (those with sperm motility of ≥ 20%) with the remaining 12 samples placed into the test group (those with sperm motility of < 20%). The mean rate of disomy for the control group and test group for the sex chromosomes and chromosome 21 were 0.30% and 0.37%, and 0.16% and 0.28% respectively (Figure 3.2).

Figure 3.2 Graph to show the mean sperm disomy frequencies for the sex chromosomes and chromosome 21 in two groups with percentage of motility of ≥20% motility and <20% sperm motility, error bars represent SEM.



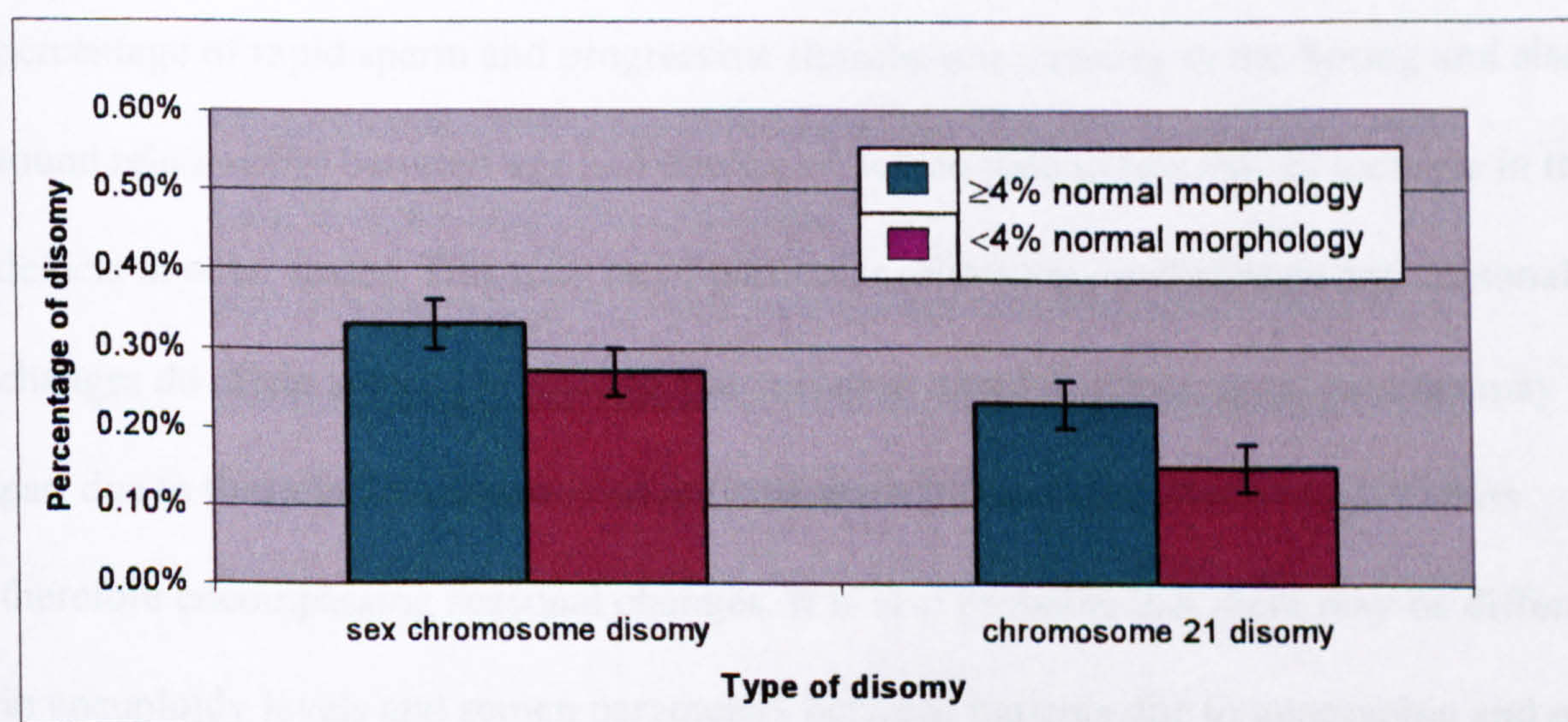
These results provide evidence of a difference between sperm disomy and motility however, the opposite situation to that of sperm concentration pertained. That is, there was no significant difference between sperm disomy levels for the sex chromosomes (XY, XX or XY disomy) whereas men with motility of < 20% (asthenozoospermia) had significantly

elevated levels of chromosome 21 disomy compared to controls (Odds Ratio 1.75, $p < 0.0001$) (Table 3.2).

3.4.3 The relationship between chromosome aneuploidy and sperm morphology

As for sperm concentration, each of the 43 samples from 19 different individuals were placed either into a control or a test group dependent on sperm motility as determined by the semen assessment (Table 3.1). In total 21 individuals were place in the control group (those with normal sperm morphology of ≥ 4%) with the remaining 22 samples placed into the test group (those with normal sperm morphology of < 4%). The mean rate of disomy for the control group and test group for the sex chromosomes and chromosome 21 were 0.33% and 0.27%, and 0.23% and 0.15% respectively (Figure 3.3).

Figure 3.3 Graph to show the mean sperm disomy frequencies for the sex chromosomes and chromosome 21 in two groups with ≥4% abnormal morphology and <4% abnormal morphology, error bars represent SEM.



Finally (and surprisingly) men with severe teratozoospermia (less than 4% abnormal forms) had significantly reduced levels of sperm disomy for both pairs of chromosomes compared

to controls. (Sex chromosome disomy, Odds Ratio 1.22 ($p = 0.013$), Chromosome 21 disomy, Odds Ratio = 1.54 ($p < 0.0001$) (Table 3.2).

3.5 Discussion

The results generated by this study particularly that of the repeat patients were surprising in that there was marked variation between semen samples produced by the same individual on separate occasions. It is clear from previous studies that many factors can influence both that of sperm aneuploidy (refer to section 1.5) and semen parameters including: the number of days abstinence, possible environmental effects for example variations within semen parameters may be due to the climate, seasonal changes and environmental factors for example Centola et al. (1999) reported no changes in volume, count motility or motile count associated with climate changes, however found a significant reduction in the percentage of rapid sperm and progressive straight-line velocity in the Spring and also found relationship between age and decreased semen parameters and an increase in tail defects in older males. This may be of particular relevance, as if climate and seasonal changes do affect semen parameters, the variation noted in these repeat patients may be in part due to these factors as many of patients were treated for 3 months to 1.5 years therefore encompassing seasonal changes. It is also probable that there may be differences in aneuploidy levels and semen parameters between patients due to geographic and ethnic differences. Thirdly it is clear from previous studies (refer to section 1.5) that many factors can influence sperm aneuploidy and semen parameters including: increasing paternal age; alcohol; tobacco; caffeine; stress; chemotherapy; drugs and exposure to chemicals for example whether it is through occupational exposure or through environmental exposure.

To date there have been few studies that have addressed these points and those that have, have been poorly designed, not taking into account other factors that may be involved and not analysing a large enough patient cohort for example. It is clear that these questions are difficult to address (and may even be impossible) to prove any existence of a relationship between reduced semen parameters and aneuploidy with any of these questions, as there are so many compounding factors involved. Hopefully, future studies will be able address these questions without the pitfalls of previous studies.

This study revealed the existence of a possible relationship between individual clinically defined semen parameters and segregation of specific chromosome pairs. The relationship between sex chromosome disomy and the failure of spermatocytes to complete spermatogenesis is manifested by significantly higher sex chromosome disomy levels in oligozoospermic men. These results are similar to those of Rives et al. (1999), Vegetti et al. (2000), Calogero et al. (2001b) who also reported a relationship between sex chromosome disomy and sperm concentration for investigated chromosomes. However the study carried out by Vegetti et al. (2000) investigated total aneuploidy (hence nullisomy and disomy) and considered chromosomes 13, 21 and 18, X and Y as a group. Here however we report on individual chromosomes and also report the absence of such an effect for chromosome 21 which leads us to propose the hypothesis that this effect may be restricted to the sex chromosome bivalent. Further studies on other chromosome pairs will allow us to test this hypothesis further (refer to section 7.3).

Our results also suggest a significant association between asthenozoospermia (poor motility) and non-disjunction of chromosome 21. This is similar to reports by Vegetti et al.

(2000) but in this case, we found no such association with the sex chromosomes. This study however is not in agreement with the study published by Hristova et al. (2002) in which 10 males with asthenoteratozoospermia were investigated, we report that asthenozoospermia is correlated with chromosome 13 and XX disomy but not that of chromosome 21, XY and YY disomy. One possible explanation for the association within our patient cohort is that over expressed genes on chromosome 21 significantly impair the formation of the sperm midpiece through which sperm motility is mediated. This seems unlikely however since chromosome 21 is a gene-poor chromosome and there are thought to be few genes expressed in the spermatocyte itself that impact on spermiogenesis. It is also possible that there are gene products (e.g. micro tubular or motor proteins) common to both normal chromosome segregation of chromosome 21 (or the acrocentric (non-Y) chromosomes, or the autosomes in general) and normal formation of the structures that mediate sperm motility. A final possibility is that our results represent a statistical anomaly. While correlations for individual males who have given multiple (four or more) samples are relatively consistent for sperm concentration, they are less so for motility (see table 4.1). If disomy were related to motility by a genetic cause, then would expect a consistency in chromosomal aneuploidies from individual patients who gave multiple samples. In patient 5 however, his highest motility sample of 45% also had the lowest proportion of autosomal disomy (0.1%) and the highest (0.24%) in a "normal" motility sample. Moreover in patient 9, the lowest motility sample (8%) had a disomy frequency of 0.22% and the 18% motility sample had 0.25% disomy frequency. There was also considerable evidence of varying disomy levels when motility remained relatively constant, for instance, patient 10, had normal motility in all samples, the disomy frequency ranged from 0.12 – 0.38%. In patient

12 the sample with 10% motility had an disomy rate of 1.46%, the sample with 11% motility had a 0.12% rate and the 17% motility sample had 0.14% disomy. A similar situation pertained for patient 14, in which samples of 16% motility had disomy rates of both 0.14 and 0.3% and patient 17 had >40% motility throughout but disomy rates of 0.24, 0.04 and 0.04% respectively. Clearly therefore further studies are necessary before a stronger relationship between autosomal sperm disomy and asthenozoospermia can be established.

The apparent inverse association between sperm morphology and chromosome segregation was surprising and it is, again, possible that this is a statistical anomaly. Indeed although a number of studies have found no significant correlation between morphology and disomy for the investigated chromosomes (In't Veld et al., 1997; Colombero et al., 1999; Vegetti et al., 2000; Ryu et al., 2001), others suggest a positive correlation between disomy and abnormal morphology for disomy 8, 18, XX and YY (Calogero et al., 2001b), disomy 13 and XX and YY (Hristova et al., 2002; Templado et al., 2002), but do not suggest a correlation for disomy 12 or XY (Calogero et al., 2001b), disomy 21 or YY (Hristova et al., 2002; Templado et al., 2002). The high level of statistical significance reported, the fact that the effect is clear in two separate chromosome pairs, and the fact that different effects were seen for concentration and morphology would argue that this is a genuine phenomenon. Moreover this is one of the few studies that has used repeat samples from individual patients and, in some cases, the same individual appeared in different groups depending on his semen parameters at the time of donation. In other words disomy levels appear not to be consistent among individuals, rather they relate more to their semen

parameters on any given day, perhaps as a result of extrinsic factors. Other studies have reported that teratozoospermic males have elevated levels of sperm disomy. Calogero et al. (2001b) found a correlation between increased sperm disomy levels and teratozoospermia as did Viville et al. (2000) but only an association with 100% macrocephalic spermatozoa. For the most part however these individuals were defined as "OAT" i.e. also oligozoospermic and asthenozoospermic and thus it is possible that the association of teratozoospermia alone was not measured fully. Future studies warrant investigating this further, perhaps investigating more chromosome pairs and individuals who display severe teratozoospermia but normal levels of sperm concentration and motility.

Chapter 4

Evidence of an improvement in the genetic quality of sperm coincident with infertility treatment by traditional Chinese medicine (TCM)

Data presented in this chapter were previously published as a poster abstract, Tempest et al., (2001). Looking to the East: Evidence that traditional medicine can significantly reduce the rates of sperm aneuploidy in infertile males. Chromosome Research., Vol 9, pg 146.

Results from this chapter and chapter 5 are in preparation for publication

Chapter 4: Evidence of an improvement in the genetic quality of sperm coincident with infertility treatment by traditional Chinese medicine (TCM)

4.1 Introduction

To date a number of causes of infertility have been identified (reviewed in chapter 1); however, at present our understanding of the causes of infertility is limited, with many cases diagnosed as "unexplained". Currently the only widely used treatment of male factor infertility is that of ICSI. As mentioned in chapter 1, there are a number of concerns over the safety of this technique, the aim of this study was to investigate another possible treatment regime that may, ultimately provide an adjunct or alternative to ICSI. Within the past decade more and more Westerners are turning to alternative therapies also known as complementary and alternative medicine (CAM) for the treatment of illnesses and general well being. CAM includes a wide range of treatments including: herbalism, aromatherapy, osteopathy, chiropractic, massage, meditation, reflexology, reiki, hypnotherapy, homeopathy, acupuncture and traditional Chinese medicine (TCM) amongst others (New Scientist., 2001). Figures released by the British Medical Association reveal that specialists in alternative medicine outnumber GPs in the UK with around 50,000 practioners of CAM compared to 36,600 GPs in 1999 (New Scientist., 2001).

4.2 Why are Westerners turning to CAM therapies

Individuals often turn to CAM when Western medicine fails to provide a diagnosis, they have exhausted all that it has to offer and as CAM treatment is often less radical and invasive. This is of relevance when considering infertility, as in many cases Western medicine fails to provide a diagnosis. CAM therapies offer alternative models of disease

and hence may offer diagnosis and possible cures for these individuals (New Scientist., 2001). Many individuals are turning to CAM therapies for chronic and degenerative conditions that Western medicine has had little success in treating such as asthma, eczema, cancer and infertility. In these cases individuals are often treated indefinitely with potentially serious side effects. CAM is often undertaken to alleviate the serious side effects or in some cases for sole treatment escaping the invasiveness of Western treatment (Bensoussan, 1999).

4.3 Traditional Chinese medicine (TCM)

Studies in this chapter were carried out to investigate the effectiveness of traditional Chinese herbal medicine (TCM), in the treatment of male factor infertility. TCM originated from Taoism over 4,000 years ago in China and has been developed and practised over thousands of years. Treatment includes both acupuncture and individualised prescriptions (the components of which are mainly plant based). In recent years the practice of TCM within Western countries has increased significantly and Chinese herbalists are now commonly found UK high streets. Western medicine as a whole has been very sceptical towards the use of TCM, largely in part due to views that it is an unscientific approach. Traditionally Western drugs contain a single active compound, whereas TCM uses on average 10-20 different herbs within prescriptions, therefore it is likely that in many cases the activity of these plants are based on the interactions of different compounds (Yuan and Lin, 2000), TCM uses an estimated 8,000 plants; 400 of which are commonly used (Pach et al., 2002).

4.3.1 Principles of TCM

The philosophy of TCM is different to that of Western medicine, in that Western medicine often targets specific biochemical pathways. Western medicine has methodically looked at the relationship between structure and function and designed specific drugs to target pathogens and/ or diseased cells and tissues (Yuan and Lin, 2000). TCM differs in diagnostic techniques and therapeutic principles. The theoretical system of TCM is based around the doctrines of Yin-Yang, the Five Elements, 'Zang' and 'Fu' organs (viscera) and 'Meridians' (energy channels that flow through the body, Channels and Collaterals). TCM suggests that the human body is an organic entity in which tissues and sense organs are connected through a network of channels and collaterals (blood vessels), and that the body is unified with nature. TCM defines health in terms of balance and the principles suggest that the body and the environment should be in a balanced state in order to perpetuate good health, and any alteration results in disease.

4.3.2 Zang-Fu theory of TCM

Zang and Fu theory comprises of five zang organs including: the heart, lung, spleen, liver and kidney, and six fu organs including: the gall bladder, stomach, small and large intestine, bladder and sanjiao (the trunk of the body). The theory proposes that these Zang-Fu organs are not just organs but represent the physiology and pathology of a number of systems within humans. The zang organs produce and store essence including: qi, blood and bodily fluids, whereas the fu organs primarily receive and digest food absorbing any nutrients, transmit and excrete any waste products.

4.3.2.1 Zang organs in TCM

Table 4.1 reports the main functions and indicators (leading to diagnosis) of the five Zang organs according to TCM principles.

According to TCM theories the kidney in particular plays a vital role in growth, development and reproduction, its main function is involved in the storing of essence (termed kidney qi) and is of importance in reproduction terms, as it is believed that kidney qi becomes abundant when a male reaches the age of sixteen causing him to have sexual energy and semen. However, kidney qi is said to decrease with increasing age causing a decline in sexual energy and semen quality resulting in infertility, abnormalities can also cause maldevelopment and infantile underdevelopment.

Table 4.1: List of the Zang organs and their function and indicator

Zang organ	*Function and indicator*
Heart	- Domination of blood, vessels and facial complexion - Control of the mind - Opening into the tongue
Lung	- Dominating qi and controlling respiration - Dispersion and descent of qi - Regulating water passage - Connecting externally with skin, hair and internally with the large intestine
Spleen	- Governing transportation and transformation - Controlling blood - Dominating muscles and limbs - Opening into the mouth and the complexion of the lips
Liver	- Storing blood - Creating unrestrained conditions for qi - Controlling tendons and luster reflected in the nails - Opening into the eye
Kidneys	- Controlling human reproduction, storing essence, growth and development - Controlling water metabolism - Receiving qi - Producing marrow, filling up the brain, controlling bones, manufacturing blood and hair luster - Opening into ear, and the bladder which is connected both internally and externally

4.3.2.2 Fu organs in TCM

Table 4.2 reports the main functions and potential disease types that may result due to abnormalities within the six Fu organs according to TCM principles.

Table 4.2: List of the Fu organs, function and associated diseases

Fu organ	Function and diseases associated with abnormalities within these
Gall bladder	(attached to the liver, stores bile), mental disorders, insomnia and emotional symptoms such as fear
Stomach	(receives and digests food, associated with the spleen), anorexia, fullness, nausea and distension of the upper abdomen
Small intestine	(connects with the stomach, receives partially digested food and continues with digestion), affect digestion and can lead to urinary problems
Large intestine	(receives waste material and absorbs fluid coverts its to faeces), diarrhoea and constipation
Bladder	(associated with the kidney, stores and discharges urine), dysfunction in qi will result in urine retention or incontinence
Sanjiao	(split into upper jiao: contains heart and lungs; middle jiao: spleen and stomach, lower jiao: kidney and bladder)

In summary it is proposed that the heart and lung distribute qi and bodily fluid, the spleen and stomach are responsible for the digestion, absorption and transference of the qi, blood and body fluids transformed form essential substances and the kidney and bladder transport fluids and water. Hence if any of the sanjiao areas are affected by pathological problems the organs located within these will be affected.

4.3.4 Yin-Yang theory of TCM

Yin-Yang theory in TCM is important for both diagnosis and treatment, Yin and Yang represent two polar pairs/opposites (e.g. night and day, hot and cold etc). Yin and Yang are said to be found in all things, and are not independent of each other (e.g. day is Yang and night is Yin, however morning is believed to be Yang within Yin and afternoon is Yin within Yang). In summary they are two fundamental principles that oppose and complement each other. In TCM it is postulated that the body's functional activities are perceived to be Yang and the consumption of nutrient substances is Yin, if Yin is decreased Yang increases and when nutrients are metabolised (Yin) functional energy is decreased (hence Yang). In the normal situation the body is thought to undergo mutual increases and decreases in Yin and Yang, however, a relative balance is maintained. In contrast disease is said to result if there is either an excess or deficiency in either Yin or Yang, therefore normal vital activities of the human body are the result

of a balance between Yin and Yang. The Yin-Yang theory is also used in TCM treatment, for example properties, flavour and action of the Chinese herbal medicine (e.g. herbs that are bitter are Yin and those that are sweet are Yang). The treatment aims are supposed to restore a balance between Yin and Yang in individuals.

4.3.5 The Five Elements theory

This theory is based upon the fact that the "main elements" of the material world are fire, water, wood, earth and metal. The characteristics of each element is assigned to humans and anything within the world, for example characteristics of wood are germination, extension, softness and harmony, and hence anything that falls into this category is identified as a wood element. This theory is utilised to express the mutual relationship between humans and the environment including that of seasons, climates and flavours amongst others. Wood is associated with such things as the spring and wind but also with the liver and the eyes of humans. The diagnosis and treatment of disease according to this theory is made possible, as any changes in the internal organs function are hypothesised to be detected in the external appearances of individuals; including complexion, voice sense of taste and pulse. Any changes therefore within these parameters are measured by TCM practioners through the careful monitoring and examining of individuals by four diagnostic methods: inspection ausculation and olfaction, inquiring and palpitation.

4.3.6 Diagnostic methods of TCM

Normally an individual consulting a TCM specialist will undergo a number of diagnostic methods, these include: inspection, olfaction, palpation and interrogation.

4.3.6.1 Inspection

This technique is used to observe vitality (thought to enable prosperity, decline of vital essence and energy of the body, enabling the seriousness of the disease to be established); complexion (changes in colour and lustre of the face, believed to indicate the type and severity of the disease), general appearance and observation of changes in secretions and excretions for example analysis of the seminal fluid including colour and volume.

4.3.6.1.1 Inspection of the tongue

Inspection of the tongue is of key importance in TCM theory, tongue colour and tongue coating being analysed as this is thought to establish the location of the disease. The thinness/thickness and colour of the coating is thought to indicate the prosperity and decline of Qi factors, the colour indicates cold and heat of pathologic changes, if the coating is moist or dry allegedly denotes an insufficiency of body fluids.

4.3.6.2 Ausculation and olfaction

Ausculation (listening) and olfaction (smelling) are also two key methods used in diagnosis. Listening to the voice, breathing and coughing can help indicate the type of syndrome and deficiency of Qi. Olfaction, (smelling) of individual's breath, secretions and excretions such as faeces, urine is thought to indicate the location, type of syndrome and disease.

4.3.6.3 Meridians

Analysing meridians, is based on a complex pulse-taking method (over twenty pulse characteristics have been described in TCM) which analyses the energy channels that

flow through the body. These meridians are believed to identify vitality and health and when these are balanced the individual is healthy. However if one or more of these meridians is unbalanced, it will present itself as illness.

4.3.7 Differentiation of syndromes according to the Eight Principles

In TCM syndromes can be differentiated according to the eight principles, which include: yin and yang, cold and heat, exterior (biao) and interior (li) and finally deficiency (xu) and excess (shi). Yin and yang are the general principles that are used to categorise the other six principles (as listed previously). Exterior and interior are used to indicate the depth and development of disease, exterior syndromes relate to pathological changes in the body surface resulting from pathogenic factors.

The syndromes of each of the mentioned principles are varied but are postulated to be linked with each other, in many cases for example when differentiating syndromes of deficiency (xu) and excess (shi), other syndromes are often also involved including exterior (biao), interior (li) and cold and heat.

4.3.8 Conclusion on the principles of TCM

TCM is based on the theories and relationships of the Zang-Fu organs and tissues of the human body, but also that of the relationship between the environment and the human body. One key factor in TCM theory is that all these elements remain in a relatively balanced state for the body to function normally; any alteration in this balance will result in disease.

4.4 Studies investigating the efficacy of TCM treatments

The practice of TCM has met with much scepticism particularly within the Western world. However, some argue that it would be unlikely that the practice and theory of TCM would have continued over these thousands of years if no positive effects were noted. There are currently a number of journals published based around TCM, however the majority are published in Chinese and hence are not easily accessible. It is clear that definitive literature is scant and as a result has not been integrated within the mainstream medical community (Cohen et al., 2002). To the best of our knowledge no reports have been published within Western literature investigating the role of TCM in the treatment of male infertility. There are however, reports of both open tube experiments and randomised clinical trials within the literature proving the effectiveness of TCM in the treatment of a wide range of diseases including: asthma; atopic dermatitis; atopic eczema; cancer; diabetes mellitus; heart disease; irritable bowel syndrome (IBS); pneumonia; rheumatoid arthritis and viral hepatitis. Several of these studies demonstrating the effectiveness of TCM in the treatment of some of the diseases mentioned previously will now be considered. Sheehan and Atherton (1992) investigated TCM treatment of atopic dermatitis in 40 adults, split into two groups, each group was administered either a standardised TCM mixture (consisting of a mixture of ten herbs) or a placebo for a period of two months. Both groups were then given a four week wash-out period and subsequently switched to the opposite treatment. A clear decrease in the severity of the dermatitis was observed after TCM treatment, identified by both patient and specialist evaluation. Sheehan et al. (1992) carried out a similar trial investigating TCM treatment of atopic eczema in 47 children. As before specialists analysing erythema and surface damage and parental observations monitored the progress of patients. As observed in the atopic dermatitis, patient's conditions

significantly improved with TCM treatment and in both studies the patient's conditions returned to its original state when the placebo was administered. In a follow-up study Sheehan and Atherton. (1994) further investigated 37 of the 47 children enrolled in the previous study for the following year. A total of 18 children demonstrated a 90% reduction in eczema symptoms and 5 had lesser degrees of improvement. Within this study 14 individuals dropped out, 4 due to difficulties in preparation and or ingestion of the TCM treatment and 10 due to no response. However, 7 patients were able to stop treatment as their symptoms returned to normal and 16 were able to control their eczema through treatment.

At least two studies have investigated the efficacy of TCM treatment in bronchial asthma, one study evaluated the effects on childhood bronchial asthma (Hsieh, 1996) and will be considered first, with the second study investigating the effects in adults (Egashira and Nagano, 1993).

A multi-centre, double-blind, placebo controlled trial was carried out in 310 children over a period of 6 months (Hsieh, 1996). This study split patients into three groups, dependent on the diagnosis by TCM specialists. Each of these three groups were divided further into 2 groups, with one receiving a TCM treatment specific to the type of deficiency found by the TCM specialists, with the other group receiving a placebo. The authors reported an improvement in all groups, therefore suggesting some evidence of a placebo effect, however, those individuals treated by TCM demonstrated better results. Egashira and Nagano, (1993) investigated 90 adults with steroid-dependant bronchial asthma for a 12 week period, patients were administered either a TCM treatment or a placebo. All groups also received steroid (prednisolone) treatment, this

study reported an improvement in the bronchial asthma of individuals treated with TCM compared to that of the placebo group. Two patients were reported to have stopped their steroid usage with eleven patients reducing their steroid levels by over 50%, compared to only three patients reducing their steroid levels by 50% in the placebo group. Egashira and Nagano, (1993), suggest that TCM is effective in the treatment of bronchial asthma and that when used in conjunction with steroid treatment may result in the stoppage and reduction of steroid usage, and as a result may lead to a reduction in the side effects of steroid use.

Bensoussan et al. (1998) investigated the use of TCM in the treatment of irritable bowel syndrome (which at present, no Western drug treatment has been identified). 116 patients participated, these were split into three groups (group 1- received individualised TCM treatments, group 2- received a standardised TCM treatment with group 3- receiving a placebo for 16 weeks). This study interestingly reported significant improvements in both groups 1 and 2 (individualised and standardised treatment) compared to group 3. The results of this study suggest that standardised treatments are as effective as tailored treatments, however a follow-up study was carried out 14 weeks after treatment completion. The authors suggest from these results that the continued improvement of the condition was only found in those individuals administered the tailored treatment.

4.5 Objectives

To the best of our knowledge no empirical studies have been performed to assess sperm aneuploidy levels as a result of, or coincident with, any medical intervention. Moreover we are not aware of studies in the Western literature that have investigated the role of

TCM in the treatment of infertility. Finally we are not aware of any study that has reported the efficacy of any alternative treatment of a disease with a genetic basis. With this in mind we investigated the role of TCM as a possible means of improving aneuploidy frequencies and semen parameters within the sperm of infertile males.

- To do this we established a patient cohort of males undergoing TCM treatment in conjunction with the Zhai Clinic (112 Harley Street), for each patient repeat semen samples were obtained and semen assessments performed in each case.
- We carried out investigations into the rates of aneuploidy for chromosomes X, Y and 21 within this patient cohort to determine if there is any difference/ improvement in aneuploidy frequencies over repeat semen samples.

4.6 Materials and Methods

4.6.1 Patient cohort

In total 10 individuals were enrolled in this study and comprised of four control males (defined as males with proven fertility and semen parameters within the normal range) and six patients (with a history of fertility problems and reduced semen parameters) undergoing TCM treatment at the Zhai clinic for male infertility. In each of these individuals the aneuploidy frequencies for chromosomes X, Y and 21 were established, (in each case an average of 5,000 sperm nuclei were scored). Due to difficulties in recruiting control individuals repeat semen samples were only received from one individual on five occasions, with semen samples obtained on one occasion for the remaining three control individuals. Semen samples from all six patients undergoing TCM treatment were received on a minimum of three; up to seven occasions, over a period of eighteen months, with repeat samples being received on average at 6 weekly intervals. In all cases semen assessments were carried out as standard according to

World Health Organization and Kruger's strict scoring criteria, all assessments were also carried out by the same operator (Dr. S. Homa), ensuring any observed differences in semen assessments between patients was not accountable to different operators. The sperm FISH method used can be found in section 8.2, all slides were coded and scored blind, details of the scoring criteria can be found in section 8.3.

4.6.2 Statistical analysis

Within this section of work the statistical test Chi squared was utilised to determine if there was any evidence of a statistically significant difference between the control males and the six infertile male patients undergoing TCM treatment. Firstly Chi squared analysis was performed to establish whether any of the six patients enrolled in the study exhibited statistically significant increases in disomy rates for the investigated chromosomes compared to controls. The mean value obtained for the chromosome disomy investigated (e.g. X, Y or 21) within the control group was used in Chi squared calculations to establish whether sperm aneuploidy levels in each of the six patients was significantly higher than those obtained for the controls. The results of this analysis are presented in table 4.4, which show significant increases in the rate of disomy for at least one of chromosome investigated in the six patients compared to controls. Chi squared analysis of this type, has previously been published sperm aneuploidy studies investigating the levels of aneuploidy in control and infertile males (refer to table 1.3). Secondly Chi squared analysis was performed for each individual patient to establish if there was evidence of a significant difference in sperm aneuploidy in the initial sample from each patient and subsequent samples received during the TCM treatment for male infertility. The analysis of this data revealed a significant difference (reduction) in

sperm disomy coincident with treatment and these aneuploidy rates were found to decline to levels comparable to controls.

Ideally Chi squared analysis is not the most appropriate test to use, in particular with reference to the second analysis performed, however due to the small sample size and the wide variation in repeat samples obtained in both the control and infertile group, makes applying a more powerful statistical test difficult. Such a test for example is the student's t-test, however this analysis revealed no significant difference between the groups (this however, was borderline) and is most likely due to factors mentioned previously. These factors suggest that in this case Chi squared is the most appropriate test to use in this situation and describes the interesting trends shown within the data.

4.7 Results

Detailed semen assessments from each of the patient samples enrolled in the study can be found in the appendix section C. In six patients analysed either prior to treatment or early on in the treatment regime, all had disomy levels significantly higher than that of the control group for at least one of the investigated chromosomes (X, Y or 21). Semen samples from the patient cohort were received at intervals of approximately 8 weeks.

4.7.1 Control Group

Four males with semen parameters all within the normal range were recruited to this study. Semen samples were obtained for C1 on five separate occasions, with one sample received for each of the other control males C2-4.

4.7.1.1 Aneuploidy frequencies for chromosomes X, Y and 21 in the spermatozoa of control subjects as determined by three colour FISH

In this study the aneuploidy frequencies for chromosomes 21, X and Y were established in four control subjects with 5,000 spermatozoa scored per sample. The number of normal spermatozoa monosomic for the tested chromosomes scored (and the percentage), along with the disomy frequencies for each of the sex chromosomes XX (figure 4.3), XY (figure 4.4) and YY (figure 4.5), the sex chromosomes as a whole (figure 4.2) and chromosome 21 (figure 4.1) are displayed in table 4.3.

4.7.2 Patient Group

In this study a total of six patients (NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW) were recruited, from each of these patients repeat semen samples on different occasions were obtained, (approximately at 8 weekly intervals). We received between 3-7 repeat semen samples from each patient, for each sample the semen parameters were assessed (semen assessment results for each patient can be found in appendix C). Each of these six patients were undergoing TCM treatment for infertility, with the first sample analysed either being prior to any TCM treatment or early on within the treatment regime (within 1-4 weeks). The six patients enrolled within this study were aged between 33 and 39 years with a mean age of 35.5 years at the time of their first semen assessment.

4.7.2.1 Aneuploidy frequencies for chromosomes X, Y and 21 in the spermatozoa of patient group as determined by three colour FISH

In this study aneuploidy frequencies for chromosomes X, Y and 21 were established in twenty-eight semen samples from six patients undergoing TCM treatment for male

infertility. The average number of cells scored per sample per patient was as follows: NLCL- 4,945; IDSS- 5,046; PHEH- 4,714; ALTAL- 5,123; JFEF- 5,000 and SGSW- 5,000. The rates of disomy for all twenty-eight samples from the six patients investigated was established. The number of normal spermatozoa monosomic for the tested chromosomes scored (and the percentage), along with the disomy frequencies for each of the sex chromosomes XX (figure 4.3), XY (figure 4.4) and YY (figure 4.5), the sex chromosomes as a whole (figure 4.2) and chromosome 21 (figure 4.1) are displayed in table 4.4.

Table 4.3- showing number of normal X and Y bearing spermatozoa scored (%), and the rates of disomy for the sex chromosomes individually and totalled as well as disomy rates for chromosome 21 in all four control subjects (each with three repeat samples).

Patient	*Normal X (%)*	*Normal Y (%)*	*X, X + 21 (%)*	*X, Y + 21 (%)*	*Y, Y + 21 (%)*	*Sex chr disomy (%)*	*Chr 21 disomy (%)*	*Total*
C1-1	2656 (53.12)	2332 (46.64)	1 (0.02)	8 (0.16)	1 (0.02)	10 (0.2)	2 (0.04)	5000
C1-2	2532 (50.64)	2458 (49.16)	1 (0.02)	4 (0.08)	1 (0.02)	6 (0.12)	4 (0.08)	5000
C1-3	2603 (52.06)	2380 (47.6)	1 (0.02)	7 (0.14)	0 (0)	8 (0.16)	9 (0.18)	5000
C1-4	2553 (51.06)	2443 (48.86)	0 (0)	3 (0.06)	0 (0)	3 (0.06)	1 (0.02)	5000
C1-5	2497 (49.94)	2498 (49.96)	0 (0)	2 (0.02)	0 (0)	2 (0.04)	3 (0.06)	5000
C2	2539 (50.78)	2454 (49.08)	1 (0.02)	3 (0.06)	0 (0)	4 (0.08)	3 (0.06)	5000
C3	2593 (51.86)	2401 (48.02)	0 (0)	2 (0.04)	0 (0)	2 (0.04)	4 (0.08)	5000
C4	2603 (52.06)	2392 (47.84)	1 (0.02)	2 (0.04)	0 (0)	3 (0.06)	2 (0.04)	5000

Table 4.4- showing number of normal X and Y bearing spermatozoa scored (%), and the rates of disomy for the sex chromosomes individually and totalled as well as disomy rates for chromosome 21 in the six patients enrolled in the TCM study.

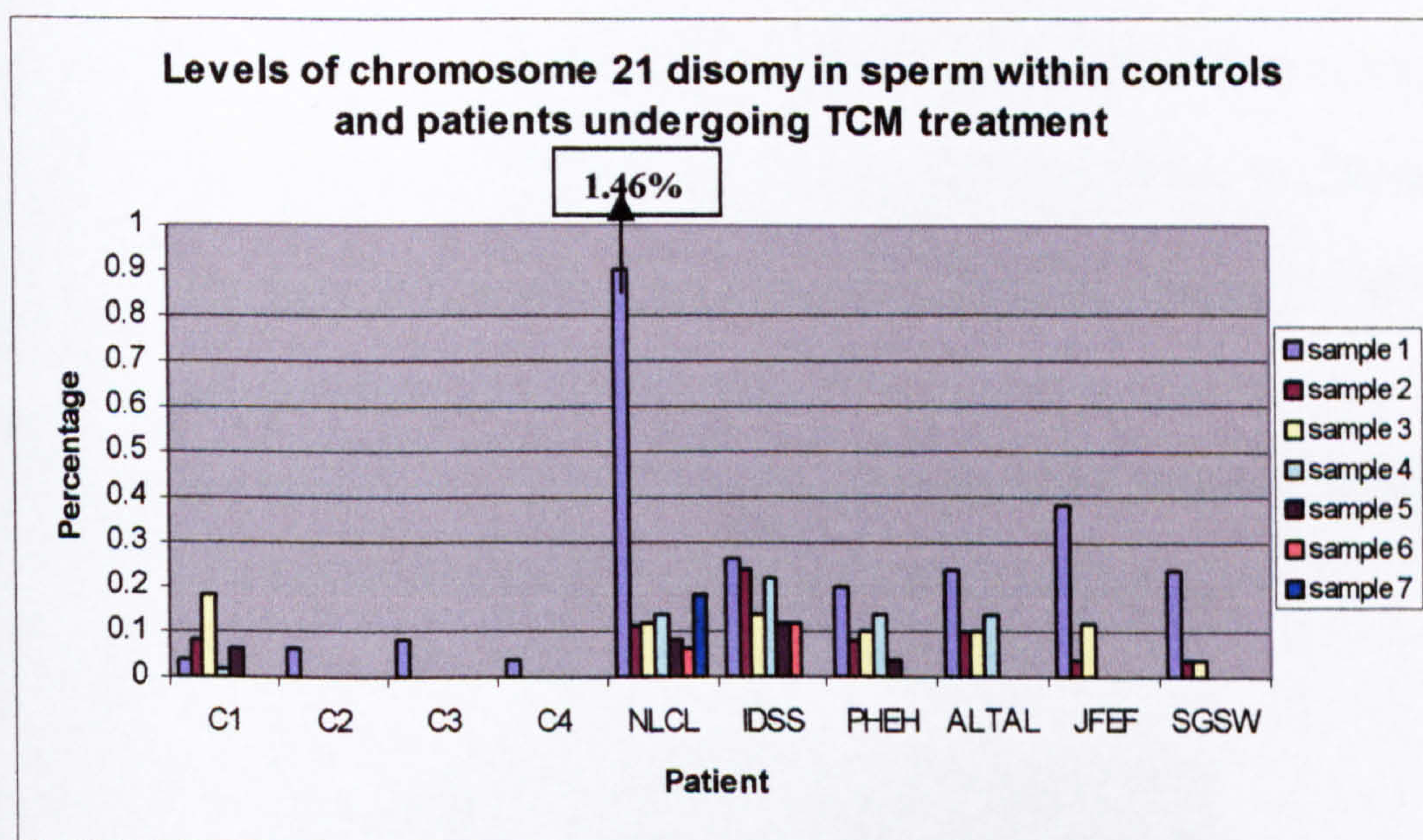
Patient (Date)	*Normal X (%)*	*Normal Y (%)*	*X, X + 21 (%)*	*X, Y + 21 (%)*	*Y, Y + 21 (%)*	*Sex chr disomy (%)*	*Chr 21 disomy (%)*	*Total*
NLCL 1 (8/12/99)	1957 (48.3)	1976 (48.8)	**6 * (0.15)**	**29 * (0.72)**	**4 * (0.10)**	**39 * (0.96)**	**59 * (1.46)**	4050
NLCL 2 (19/1/00)	2730 (50.1%)	2658 (49.3%)	2 (0.04)	**16 * (0.30)**	**6 * (0.11)**	**24 * (0.44)**	6 (0.11)	5449
NLCL 3 (8/3/00)	2337 (46.3)	2670 (52.8)	**4 * (0.08)**	**10 ~ (0.20)**	1 (0.02)	**15 * (0.30)**	6 (0.12)	5048
NLCL 4 (16/5/00)	2408 (47.9)	2578 (51.4)	2 (0.04)	**10 ~ (0.20)**	0 (0.00)	**12 ~ (0.24)**	7 (0.14)	5018
NLCL 5 (8/8/00)	2414 (47.9)	2599 (51.6)	**3 ~ (0.06)**	6 (0.12)	0 (0.00)	9 (0.18)	4 (0.08)	5031
NLCL 6 (7/11/00)	2340 (46.2)	2699 (53.3)	0 (0.00)	6 (0.12)	0 (0.00)	6 (0.12)	3 (0.06)	5016
NLCL 7 (3/7/01)	2538 (50.8)	2436 (48.7)	2 (0.04)	5 (0.10)	0 (0.00)	7 (0.14)	**9 ~ (0.18)**	5000
IDSS 1 (8/12/99)	2511 (49.6)	2457 (48.5)	**3 ~ (0.06)**	**28 * (0.55)**	**6 * (0.12)**	**37 * (0.73)**	**13 * (0.26)**	5066
IDSS 2 (19/1/00)	2500 (49.4)	2556 (50.5)	0 (0.00)	**16 * (0.32)**	**7 * (0.14)**	**23 * (0.46)**	**12 * (0.24)**	5056
IDSS 3 (23/2/00)	2478 (48.7)	2517 (49.5)	1 (0.02)	**14 * (0.28)**	0 (0.00)	**15 * (0.30)**	7 (0.14)	5084
IDSS 4 (29/3/00)	2373 (47.5)	2577 (51.5)	1 (0.02)	3 (0.06)	1 (0.02)	5 (0.10)	11 (0.22)	5000
IDSS 5 (2/5/00)	2121 (41.9)	2858 (56.4)	2 (0.04)	6 (0.12)	1 (0.02)	9 (0.18)	3 (0.06)	5063
IDSS 6 (13/6/00)	2295 (45.8)	2641 (52.7)	1 (0.02)	**10 ~ (0.20)**	1 (0.02)	**12 ~ (0.24)**	6 (0.12)	5004
PHEH 1 (12/1/00)	1658 (47.1)	1779 (50.5)	**9 * (0.26)**	**13 * (0.37)**	1 (0.03)	**23 * (0.65)**	7 (0.2)	3521
PHEH 2 (16/2/00)	2218 (44.3)	2753 (55.0)	1 (0.02)	**11 * (0.22)**	0 (0.00)	**12 ~ (0.24)**	4 (0.08)	5009
PHEH 3 (22/3/00)	2389 (47.8)	2574 (51.5)	0 (0.00)	7 (0.14)	0 (0.00)	7 (0.14)	5 (0.10)	5001
PHEH 4 (16/5/00)	2415 (47.9)	2594 (51.5)	0 (0.00)	3 (0.06)	1 (0.02)	4 (0.08)	7 (0.14)	5037
PHEH 5 (30/6/00)	2398 (47.9)	2564 (51.3)	0 (0.00)	**10 ~ (0.20)**	0 (0.00)	10 (0.20)	2 (0.04)	5000
ALTAL 1 (15/12/99)	2686 (49.8)	2672 (49.5)	0 (0.00)	8 (0.15)	1 (0.02)	9 (0.17)	**13 * (0.24)**	5400
ALTAL 2 (19/1/00)	2565 (50.4)	2503 (49.2)	2 (0.04)	7 (0.14)	**2 * (0.04)**	**11 ~ (0.22)**	5 (0.10)	5092
ALTAL 3 (16/2/00)	2495 (49.9)	2482 (49.6)	2 (0.04)	4 (0.08)	0 (0.00)	6 (0.12)	5 (0.10)	5000
ALTAL 4 (22/3/00)	2550 (51.0)	2421 (48.4)	1 (0.02)	2 (0.04)	0 (0.00)	3 (0.06)	7 (0.14)	5000
JFEF 1 (5/4/00)	2511 (50.2)	2422 (48.5)	2 (0.04)	8 (0.16)	**2 * (0.04)**	**12 ~ (0.24)**	**19 * (0.38)**	5000
JFEF 2 (1/8/00)	2537 (50.7)	2458 (49.1)	0 (0.00)	1 (0.02)	0 (0.00)	1 (0.02)	2 (0.04)	5000
JFEF 3 (26/9/00)	2379 (47.6)	2592 (51.8)	**4 * (0.08)**	4 (0.08)	0 (0.00)	8 (0.16)	6 (0.12)	5000
SGSW 1 (16/2/00)	2427 (48.6)	2515 (50.3)	1 (0.02)	8 (0.16)	**3 * (0.06)**	**12 ~ (0.24)**	**12 * (0.24)**	5000
SGSW 2 (22/3/00)	2502 (50.0)	2457 (49.5)	2 (0.04)	2 (0.04)	0 (0.00)	4 (0.08)	2 (0.04)	5000
SGSW 3 (18/7/00)	2367 (52.7)	2328 (46.6)	0 (0.00)	2 (0.04)	**2 * (0.04)**	4 (0.08)	2 (0.04)	5000

Chi squared analysis was performed to determine if the levels of aneuploidy in the patients were significantly higher than that of the controls (refer to section 4.5.3). Aneuploidy frequencies significantly higher than controls are highlighted in bold, * denotes a significance level of <0.0005 and ~ denotes a significance level of <0.005

4.7.3 Chromosome 21 disomy frequencies in the spermatozoa of both control and patient group.

The chromosome 21 disomy rates within the control group ranged from 0.02- 0.18% (table 4.3) (with an average of 0.07%), the rate of disomy 21 within the infertile group ranged from 0.04-1.46% (table 4.4) (with a mean rate of 0.19%). The disomy 21 rates for individual patients ranged from 0.06-1.46% (NLCL), 0.06-0.26% (IDSS), 0.04-0.2% (PHEH), 0.1-0.24% (ALTAL), 0.04-0.38% (JFEF) and 0.04-0.24% (SGSW). The chromosome 21 disomy frequencies for all semen samples investigated for both control and infertile males are presented in figure 4.1.

Figure 4.1 chromosome 21 disomy levels in repeat samples for controls and patients undergoing TCM treatment



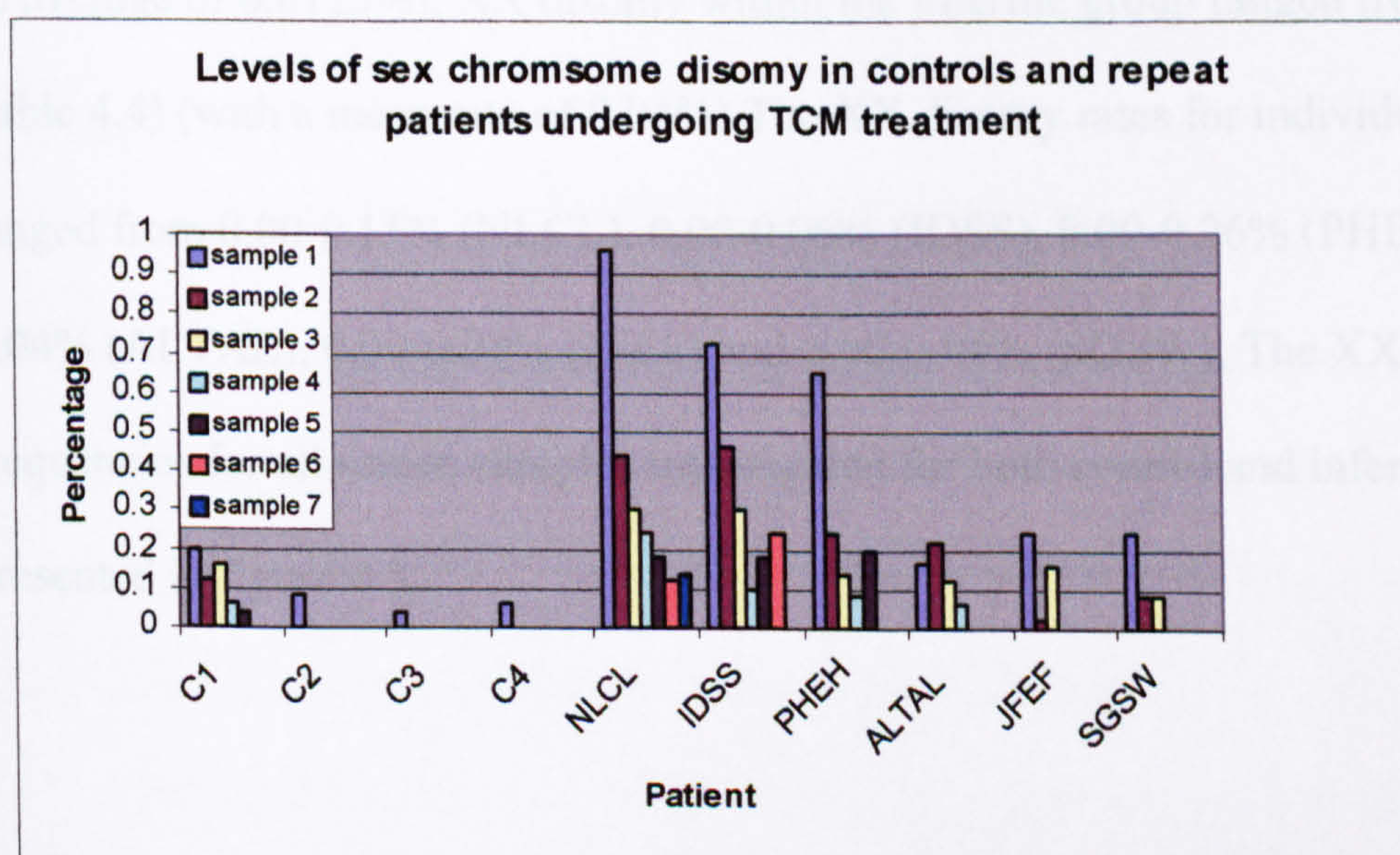
The Chi squared statistical analysis (table 4.4) revealed that significantly higher frequencies of chromosome 21 disomy compared to control levels (p=<0.0005) were found in the initial samples received for all patients with the exception of PHEH. Of significance is the fact that in all cases the levels of chromosome 21 disomy decreased to levels not significantly different to that of the controls, by the second sample (NLCL,

ALTAL, JFEF and SGSW) or the third sample (IDSS). When analysing figure 4.1 and table 4.4 we can say that in all patients disomy 21 levels have declined to rates comparable to controls coincident with TCM treatment, with all patients found to have a significant reduction from the first to last sample.

4.7.4 Sex chromosome disomy frequencies (including XX, XY and YY) in the spermatozoa of both control and patient group.

The sex chromosome disomy rates within the control group ranged from 0.04- 0.20% (table 4.3) (with an average of 0.095%), the rate of sex chromosome disomy within the infertile group ranged from 0.02-0.96% (table 4.4) (with a mean rate of 0.27%). The sex chromosome disomy rates for individual patients ranged from 0.12-0.96% (NLCL), 0.10-0.73% (IDSS), 0.08-0.65% (PHEH), 0.06-0.22% (ALTAL), 0.02-0.24% (JFEF) and 0.08-0.24% (SGSW). The sex chromosome disomy frequencies for all semen samples investigated for both control and infertile males are presented in figure 4.2.

Figure 4.2 sex chromosome disomy levels in repeat samples for controls and patients undergoing TCM treatment



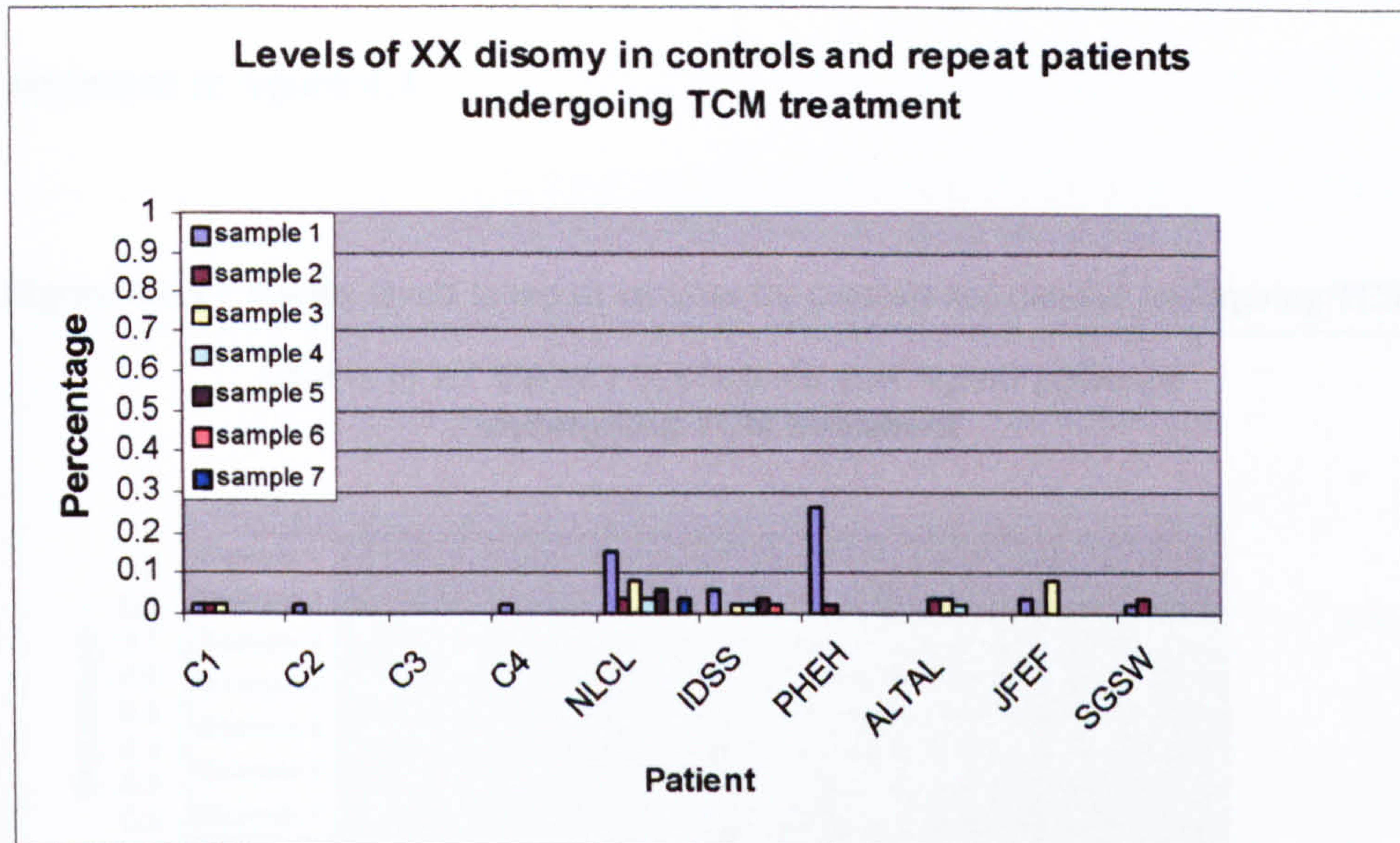
The Chi squared statistical analysis (table 4.4) revealed that significant increases in sex chromosome disomies were found in the initial samples of each of the patients compared to controls ($p=<0.0005$ or $p=<0.005$ refer to table 4.4 for individual patient p-values). As reported for chromosome 21 in all cases these frequencies were found to drop to levels not significantly different to the control group, after the first sample (in patients ALTAL, JFEF and SGSW), second sample (PHEH), third sample (IDSS) and fourth sample (NLCL). Figure 4.2 and table 4.4 demonstrates that coincident with TCM treatment, levels of disomy were all found to decline to rates reported within the control group.

4.7.5 Sex chromosome disomy rates broken down into the individual types of disomy (XX, XY and YY)

4.7.5.1 XX disomy frequencies in the spermatozoa of both control and patient group

The XX disomy rates within the control group ranged from 0- 0.02% (table 4.3) (with an average of 0.0125%), XX disomy within the infertile group ranged from 0.00-0.26% (table 4.4) (with a mean rate of 0.04%) The XX disomy rates for individual patients ranged from 0.00-0.15% (NLCL), 0.00-0.06% (IDSS), 0.00-0.26% (PHEH), 0.00-0.04% (ALTAL), 0.00-0.08% (JFEF) and 0.00-0.04% (SGSW). The XX disomy frequencies for all semen samples investigated for both control and infertile males are presented in figure 4.3.

Figure 4.3 XX disomy levels in repeat samples for controls and patients undergoing TCM treatment



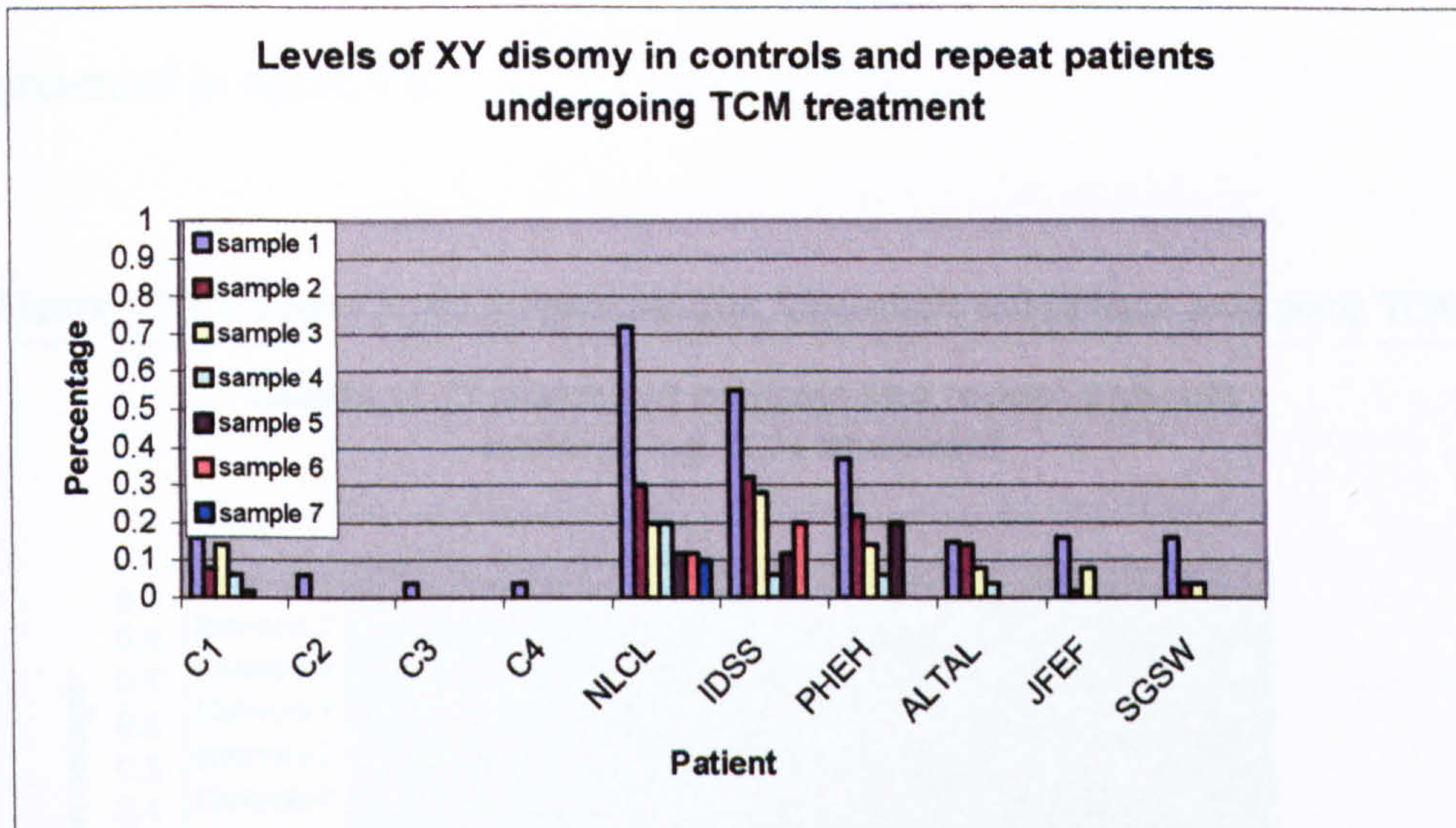
Chi squared analysis revealed significant differences in the rates of XX disomy in the first samples for patients NLCL (p=<0.0005), IDSS (p=<0.0005), and PHEH (p=0.005) compared to the mean rate of aneuploidy for chromosome X. Patients IDSS and PHEH, both dropped to levels not significantly different to that of the controls. Whereas NLCL was found to have significantly higher levels in 3 of the 7 samples analysed (table 4.4). It is clear that XX disomy frequencies in the majority of cases are within control levels with the vast majority of samples displaying rates ranging between 0.00-0.04% (except in 6 samples).

4.7.5.2 XY disomy frequencies in the spermatozoa of both control and patient group.

The XY disomy rates within the control group ranged from 0.04- 0.16% (table 4.3) (with an average of 0.0775%), XY disomy within the infertile group ranged from 0.02-0.72% (table 4.4) (with a mean rate of 0.20%) The XY disomy rates for individual patients ranged from 0.10-0.72% (NLCL), 0.06-0.55% (IDSS), 0.06-0.37% (PHEH), 0.04-0.15% (ALTAL), 0.02-0.16% (JFEF) and 0.04-0.16% (SGSW). The XY disomy

frequencies for all semen samples investigated for both control and infertile males are presented in figure 4.4.

Figure 4.4 XY disomy levels in repeat samples for controls and patients undergoing TCM treatment



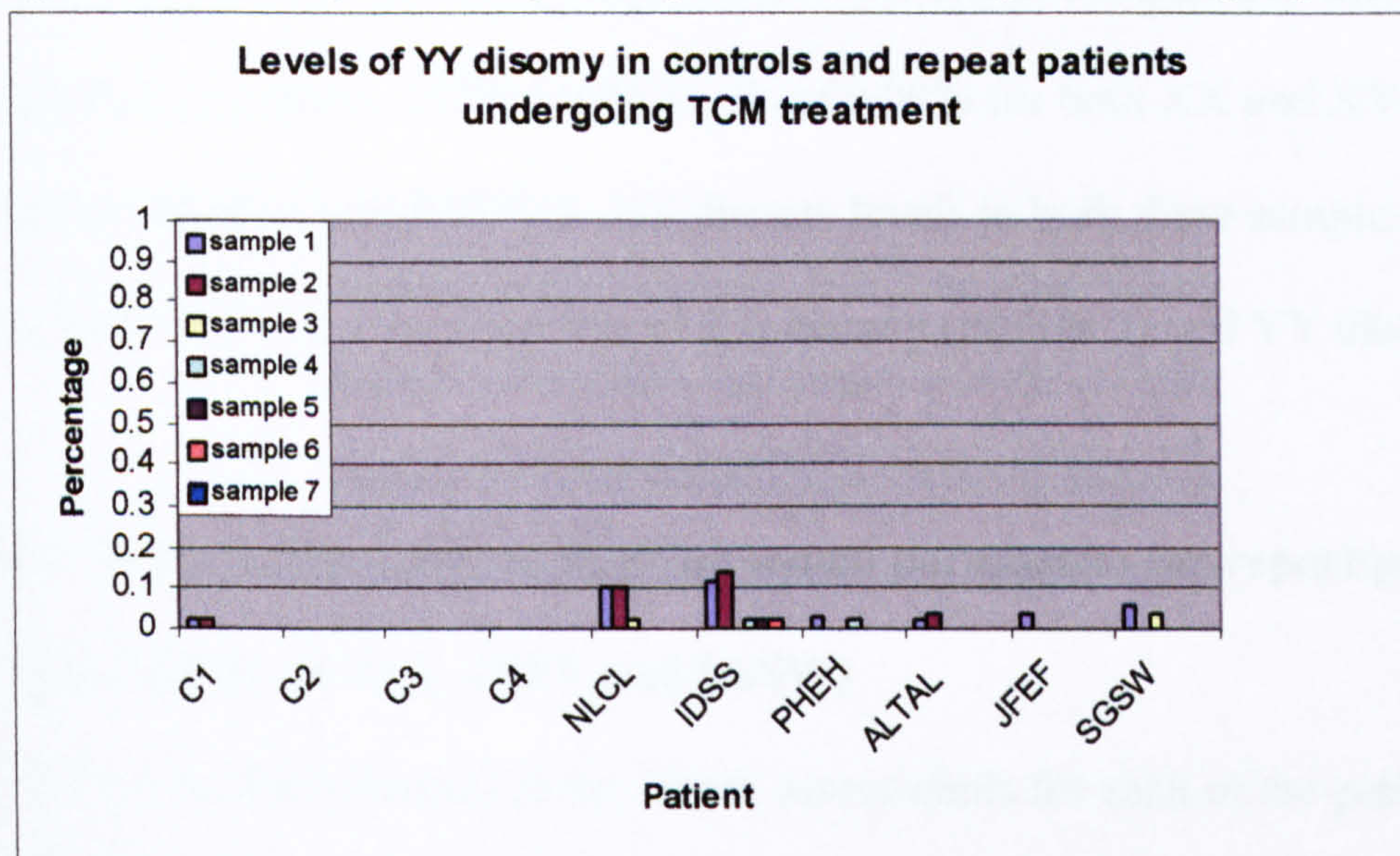
Chi squared statistical analysis (table 4.4) has established that three out of the six patients (NLCL p=<0.0005, IDSS p=<0.0005 and PHEH p=<0.005) were found to have significantly higher levels of XY disomy in their initial samples compared to controls. As reported for the other investigated chromosomes these levels declined to those within the control group after the second (PHEH), third (IDSS) and fourth (NLCL) sample (table 4.4) Figure 4.4 and table 4.4 demonstrates that coincident with TCM treatment, levels of disomy decline to levels found within the controls in the 3 patients with significantly higher levels of XY disomy.

4.7.5.3 YY disomy frequencies in the spermatozoa of both control and patient group.

The YY disomy rates within the control group ranged from 0- 0.02% (table 4.3) (with an average of 0.0005%), YY disomy within the infertile group ranged from 0.00-0.14%

(table 4.4) (with a mean rate of 0.03%) The XY disomy rates for individual patients ranged from 0.00-0.11% (NLCL), 0.00-0.14% (IDSS), 0.00-0.03% (PHEH), 0.00-0.04% (ALTAL), 0.00-0.04% (JFEF) and 0.00-0.06% (SGSW). The YY disomy frequencies for all semen samples investigated for both control and infertile males are presented in figure 4.5.

Figure 4.5 YY disomy levels in repeat samples for controls and patients undergoing TCM treatment



Chi squared analysis (table 4.4) revealed statistical differences between the control group and initial samples in all patients with the exception of PHEH refer to table 4.4 for levels of significance in each case. As reported for the other investigated chromosomes these levels were found to decline in the majority of cases to those reported in the controls.

As previously mentioned (refer to section 1.5) analysis of aneuploidy for the sex chromosomes can enable the stage of origin of the segregation error to be determined. Errors within the segregation at the MI stage results in a XY bearing spermatozoa and an error at the MII stage results in either an XX or YY bearing spermatozoa. As can

been seen when comparing the individual sex chromosome disomies (figures: 4.3, 4.4 and 4.5) it is clear that in the majority of cases MI errors (figure 4.4) form a significant proportion of the total sex chromosome disomy rates with MII errors (figures: 4.3 and 4.5) contributing to a lesser extent. For example in the first sample analysed from patient NLCL the levels of XY disomy are 0.72% compared to 0.15% and 0.1% for XX and YY disomy respectively. This holds true for all patients investigated, all of which were found to have increased frequencies of MI errors compared to MII errors with the exception of patients: JFEF 3 with levels of 0.08% for both XX and XY disomy and in patients SGSW 2 and SGSW 3, XY disomy levels in both these samples were recorded as 0.04% which was equal to that of XX disomy (SGSW 2) and YY disomy (SGSW 3).

4.8 Analysis of the effect of TCM on semen parameters for repeat patients (NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW)

Utilising the data obtained in the semen assessments for each of the patients (NLCL, IDSS, PHEH, ALTAL JFEF and SGSW) presented in appendix C it was possible to determine if there was any improvement within the three main semen parameter categories. These include sperm concentration, percentage of motility and finally the percentage of abnormal forms. In order to investigate the effect of TCM on semen parameters the appropriate values for each category was plotted onto a graph to enable any relationship to be established using Chi-squared statistical analysis.

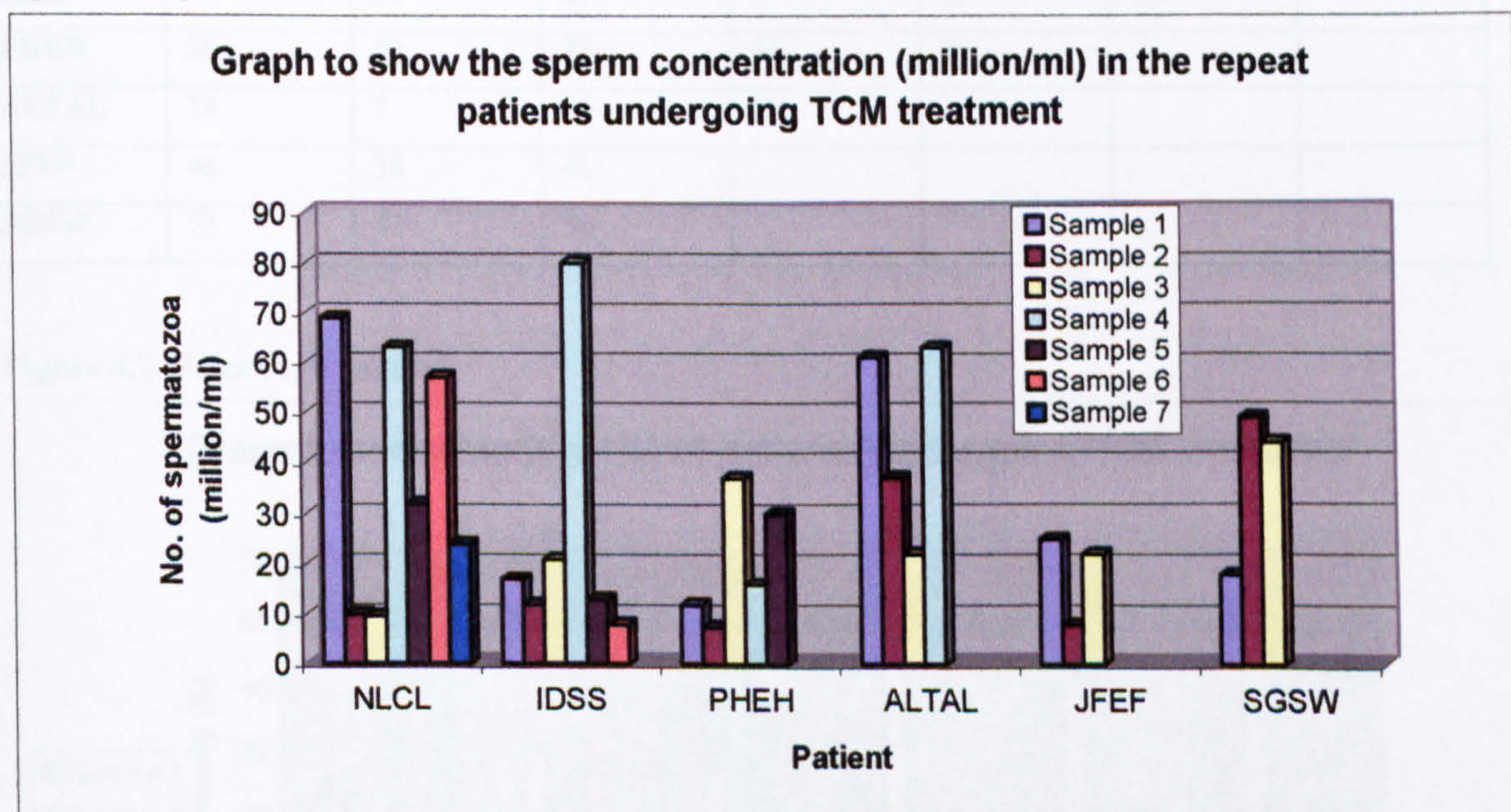
4.8.1 Investigation into the effect of TCM on sperm concentration in patients NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW

The sperm concentration for each of the samples received from the patients enrolled in this study as determined by their semen assessments (appendix C), this information can also be found in table 4.5 and figure 4.6.

Table 4.5:- sperm concentration in patients NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW and their corresponding sample number as determined by semen assessments

	Sperm concentration per million/ml						
Patient	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Sample 7**
NLCL	69	10	9.5	63	32	57	24
IDSS	17	12	21	80	13	7.9	
PHEH	12	7.5	37	16	30		
ALTAL	61	37	22	63			
JFEF	25	8	22				
SGSW	18	49	44				

Figure 4.6- sperm concentration



Analysis of the data in figure 4.6 shows no clear improvement in sperm concentration in the patients undergoing TCM treatment, however there is marked variation between samples derived form the same patients at different time points. This is most notable in

patients NLCL, IDSS and ALTAL, in which ranges of 9.5-69 million spermatozoa/ml, 7.9-80 million spermatozoa/ml and 22-63 million spermatozoa/ml recorded respectively.

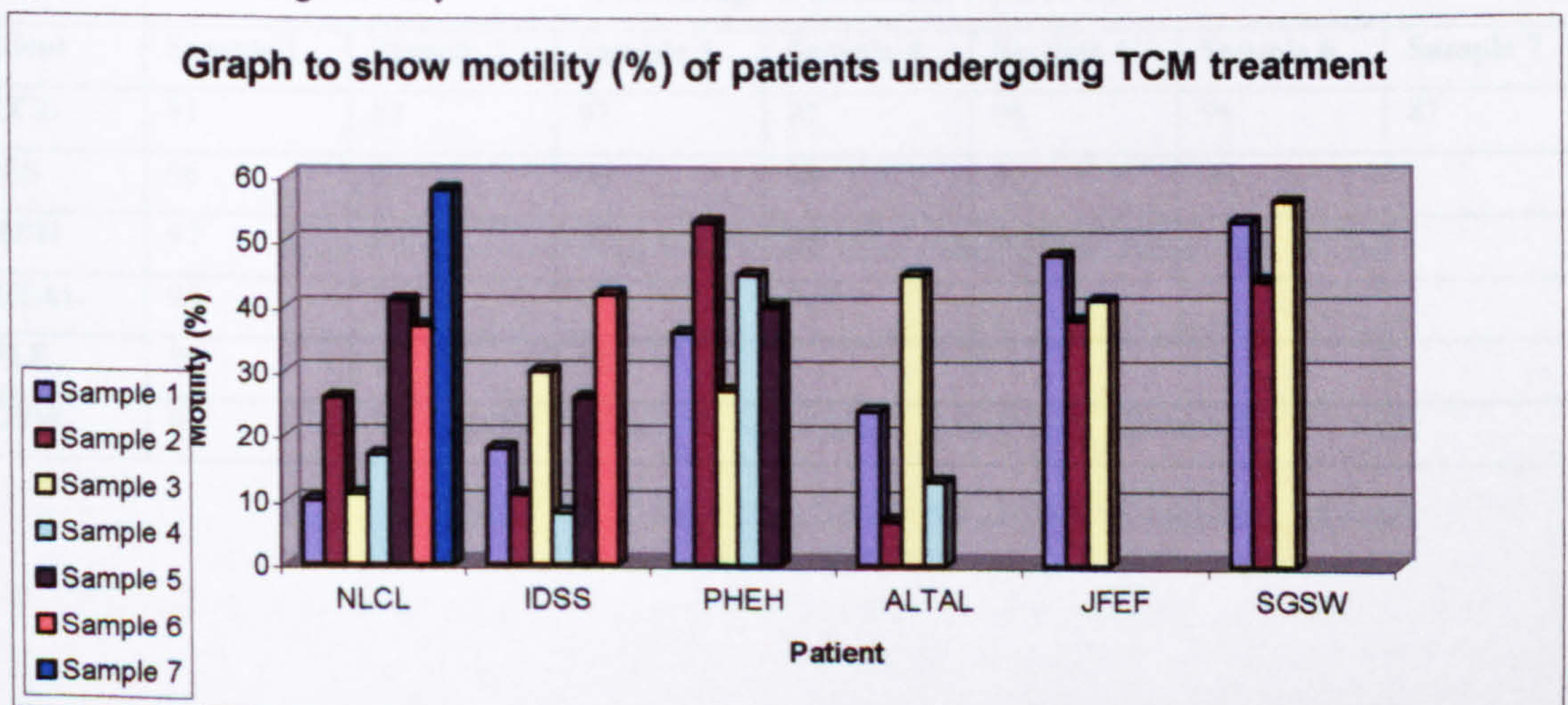
4.8.2 Investigation into the effect of TCM on sperm motility in patients NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW

The sperm motility for each of the samples received from the patients enrolled in this study as determined by their semen assessment (appendix C), table 4.6 and figure 4.7 displays the results obtained for sperm motility in these patients.

Table 4.6:- percentage of sperm motility in patients NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW and their corresponding sample number as determined by semen assessments

	Percentage of motility (%)						
Patient	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Sample 7**
NLCL	10	26	11	17	41	37	58
IDSS	18	11	30	8	26	42	
PHEH	36	53	27	45	40		
ALTAL	24	7	45	13			
JFEF	48	38	41				
SGSW	53	44	56				

Figure 4.7- Percentage motility



It is clear that as in figure 4.7 (sperm concentration) there is a marked variation in percentage of motility within samples at different time points from the same patient. The percentage of motility within patient JFEF and PHEH appears less varied with percentages ranging from 38-48% and 27-53%. However, despite the fluctuation in rate of motility there is a general significant trend in the improvement of motility observed in patient NLCL with values of 10%, 26%, 11%, 17%, 41%, 37% and 58% ($p=<0.0005$).

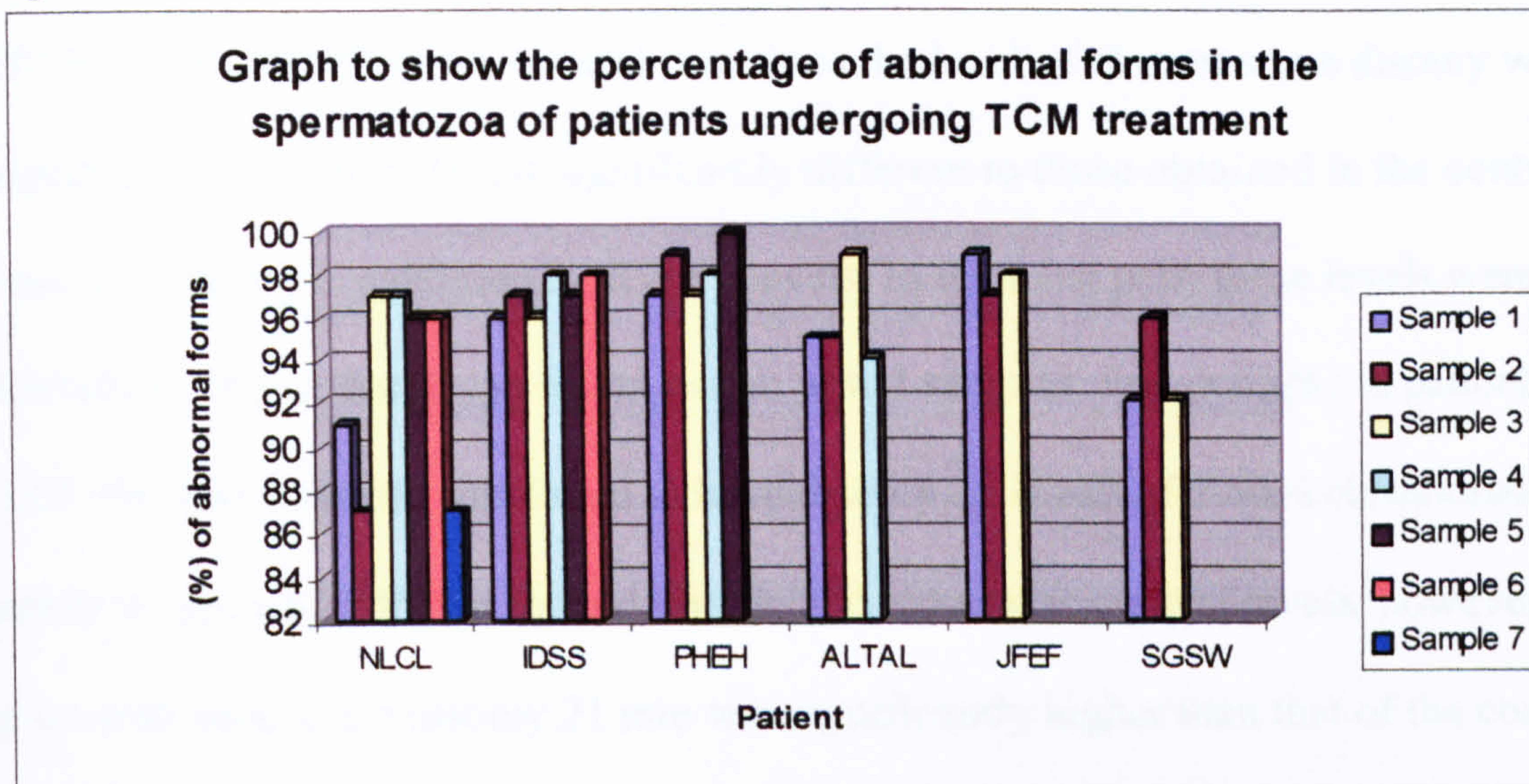
4.8.3 Investigation into the effect of TCM on percentage of abnormal forms within the spermatozoa of patients NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW

The percentage of abnormal forms for each of the samples received from the patients enrolled in this study as determined by their semen assessment can be found in appendix C. Table 4.7 and figure 4.8 present the results obtained from these semen assessments.

Table 4.7:- percentage of abnormal forms in patients NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW and their corresponding sample number as determined by semen assessments

	Percentage of abnormal forms (%)						
Patient	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Sample 7**
NLCL	91	87	97	97	96	96	87
IDSS	96	97	96	98	97	98	
PHEH	97	99	97	98	100		
ALTAL	95	95	99	94			
JFEF	99	97	98				
SGSW	92	96	92				

Figure 4.8- Abnormal forms



Analysis of the semen assessment data (can be found in appendix C) within these patients has enabled the investigation into the effect of TCM on the percentage of abnormal forms as individuals undergo treatment. Unlike in figure 4.6 and 4.7 analysing sperm concentration and motility respectively there is less variation within individuals with levels remaining at similar levels with the exception of NLCL in which there was a wide variation in the levels ranging from 87-97%. There is no significant relationship with TCM treatment and any improvement in the percentage of abnormal forms.

4.9 Discussion

Our results show a clear association between a reduction in disomy levels for the sex chromosomes (in all patients), chromosome 21 (in 5 patients) and XY disomy (in 3 patients) coincident with TCM treatment compared to our control group. The levels of XX and YY disomy were also found to be significantly higher than that of the controls in a number of patients (table 4.4), however the reported frequencies are much lower than that of the other investigated chromosome disomies. In the majority of cases it is likely that the significant difference between the control group and these patients may,

in part, be due to the low levels found in the control group for XX and YY disomy (0.0125% and 0.0005%). On several occasions the level of chromosome disomy within the patient group rose to levels significantly different to those obtained in the control group after having a reduction in disomy levels. In the most part, these levels were substantially lower than those found within initial samples. For example in patient NLCL the initial sample was found to have disomy 21 levels of 1.46% chromosome 21 disomy levels, which by the second sample had reduced to control levels, however in the seventh sample the disomy 21 rate was significantly higher than that of the controls with a rate of 0.18%, clearly however this is still significantly lower than that found in the initial sample ($p=<0.0005$). In contrast no such effect was observed for conventional semen parameters except for a weak correlation with an improvement in sperm motility in patient NLCL. In some ways this is surprising given the results of chapter 3, however the existence of any strong correlations may be due to the small sample size analysed.

Although there are potentially many factors involved, the results presented in this chapter, demonstrate the significant decrease in aneuploidy frequencies for chromosomes 21, X and Y in six infertile males with compromised semen parameters undergoing TCM treatment. Evidence of an effect on the semen parameters of these individuals is less clear, there may be the existence of a possible weak correlation between improved semen parameters including motility for specific patients. We have nevertheless provided some strong preliminary evidence for the effectiveness of TCM treatment and improving sperm quality. It remains difficult to unequivocally identify any strong positive correlations with TCM treatment, aneuploidy frequencies and semen parameters due to the vast number of compounding factors including lifestyle and environmental factors mentioned in chapter 1. The outcome of this open tube

experiment forms a good basis of evidence in the effectiveness of TCM in the treatment of male infertility. This study however is not comparable to a double-blind placebo controlled trial, nevertheless studies such as these are crucial in providing the necessary preliminary evidence of the efficacy of treatment but can also play important roles in designing comprehensive clinical trials (Yuan and Lin, 2000). To the best of our knowledge this is the first report of a study demonstrating an association between an improvement in a disorder with a clear genetic basis, specifically that of a decline in aneuploidy in the spermatozoa of infertile males in association with TCM treatment. As mentioned previously we are not the first to report evidence of the efficacy of TCM. Within the literature there have been reports of at least 450 clinical trials (reviewed in Pach et al., 2002) undertaken investigating the efficacy of TCM in a wide range of diseases including: asthma; atopic dermatitis; atopic eczema; cancer; diabetes mellitus; heart disease; irritable bowel syndrome (IBS); pneumonia; rheumatoid arthritis and viral hepatitis (Pach et al., 2002; Tang et al., 1999; Yuan and Lin. 2000). The majority of these studies however, are Chinese, Japanese and Taiwanese in origin and hence are not easily accessible, it is also important to note that in the large majority of cases these trials were not randomised or double-blind placebo controlled trials (Tang et al., 1999; Yuan and Lin. 2000). There have been, however several well designed randomised, placebo controlled clinical trials investigating several diseases including: atopic dermatitis; atopic eczema; bronchial asthma and irritable bowel syndrome within the literature (reviewed in section 4.2.8).

The conventional method of male infertility treatment within Western medicine is ICSI (refer to section 1.6). As mentioned in section 1.6.3 there have been concerns raised over the potential risks of this technique, in particular the increased risk of generating

aneuploid offspring especially for the sex chromosomes attributed in part to the increased proportion of aneuploid sperm in infertile males (refer to section 1.5). As reported in chapter 1 one partial solution to this issue would be the implementation of an aneuploidy screening kit prior to ICSI, (chapter 2 reports on the development of such a kit). Clearly this can only inform a couple of their specific risks, but will not result in a reduction of those risks. What is required in the case of individuals who have been identified as having an increased risk is a treatment that has been shown to reduce the levels of aneuploid sperm. If such a treatment was identified it could be used instead of or in conjunction with ICSI to reduce such risks. Whilst we are not suggesting that we have found such a treatment, we have shown that high aneuploidy levels in sperm can be reduced. Clearly, this is a subject that warrants further comprehensive investigations in order to identify the biological activities of the treatments used.

Chapter 5

Investigations into the biological activities of TCM herbs used in the treatment of male infertility

Chapter 5: Investigations into the biological activities of the TCM herbs used in the treatment of male infertility

5.1 Introduction

In light of the data presented in chapter 4, a question that remains to be addressed is whether the improvements in aneuploidy levels, coincident with TCM treatment in the six patients investigated were the result of a placebo effect or a result of the treatment itself. While this would take some years to address in full, evidence of any relevant measurable biological activity of the herbs used in the treatment of these individuals might provide some evidence supporting the latter hypothesis. It would stand to reason that if these herbs are in fact having a positive effect on sperm aneuploidy they would be likely to exert an effect on the process of spermatogenesis. Spermatogenesis is a process that is under strict hormonal control (refer to section 5.3), and operates through a delicate balance and negative feedback mechanisms. Any disruption to the hormonal balance can result in a number of disease phenotypes. Thus we might expect any effects of therapeutic treatment to target the endocrine control processes.

5.2 Spermatogenesis

The adult male testis has two main functions, firstly the testes is responsible for spermatogenesis (the production of mature male gametes) and secondly steroidogenesis (the production of steroid hormones). Both these functions are reliant on stimulation by pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that are part of a family of peptide hormones (derived from amino acids) that phosphorylate other proteins, which leads to altered cell function. Gonadotrophins are heterodimers that consist of a α- and β-subunit, the α-subunit is common in FSH and

LH and are identical. These are non-covalently bound to the specific β-subunits that confer there activity (Achermann and Jameson., 1999). Steroid hormones on the other hand are synthesised from cholesterol. Both of these types of hormone are intimately involved in the process of spermatogenesis.

Spermatogenesis is a process that is initiated when males reach puberty and continues throughout their life. Spermatogenesis takes place within the seminiferous tubules of the testes. The process of spermatogenesis occurs over 60-70 days, and is of importance to note that at any one time, all stages of spermatogenesis will be found within the seminiferous tubules. The regulation of spermatogenesis is under endocrine control, which involves the hypothalamic-pituitary-gonadal (HPG) axis, and is of importance as any imbalance/disruption occurring within the HPG results in infertility: (Klinefelter syndrome, hypopituitarism, primary testicular failure, hypothroidism, 5α-reductase deficiency, AIS and PAIS amongst others). Briefly this process requires the hypothalamus to secrete gonadotrophin-releasing hormone (GnRH). This then acts on the anterior pituitary (adenohypophysis) and that in turn causes the release of both FSH and LH. LH subsequently acts on the Leydig cells of the testes stimulating the production of testosterone by these cells. FSH acts on the Sertoli cells stimulating spermatogonia division. Figure 5.1 illustrates this pathway along with feedback mechanisms in place, these will be considered later.

5.3 Endocrine control of spermatogenesis

Figure 5.1 illustrating the HPG axis, the hormones involved and the feedback controls that are in place and where these act.

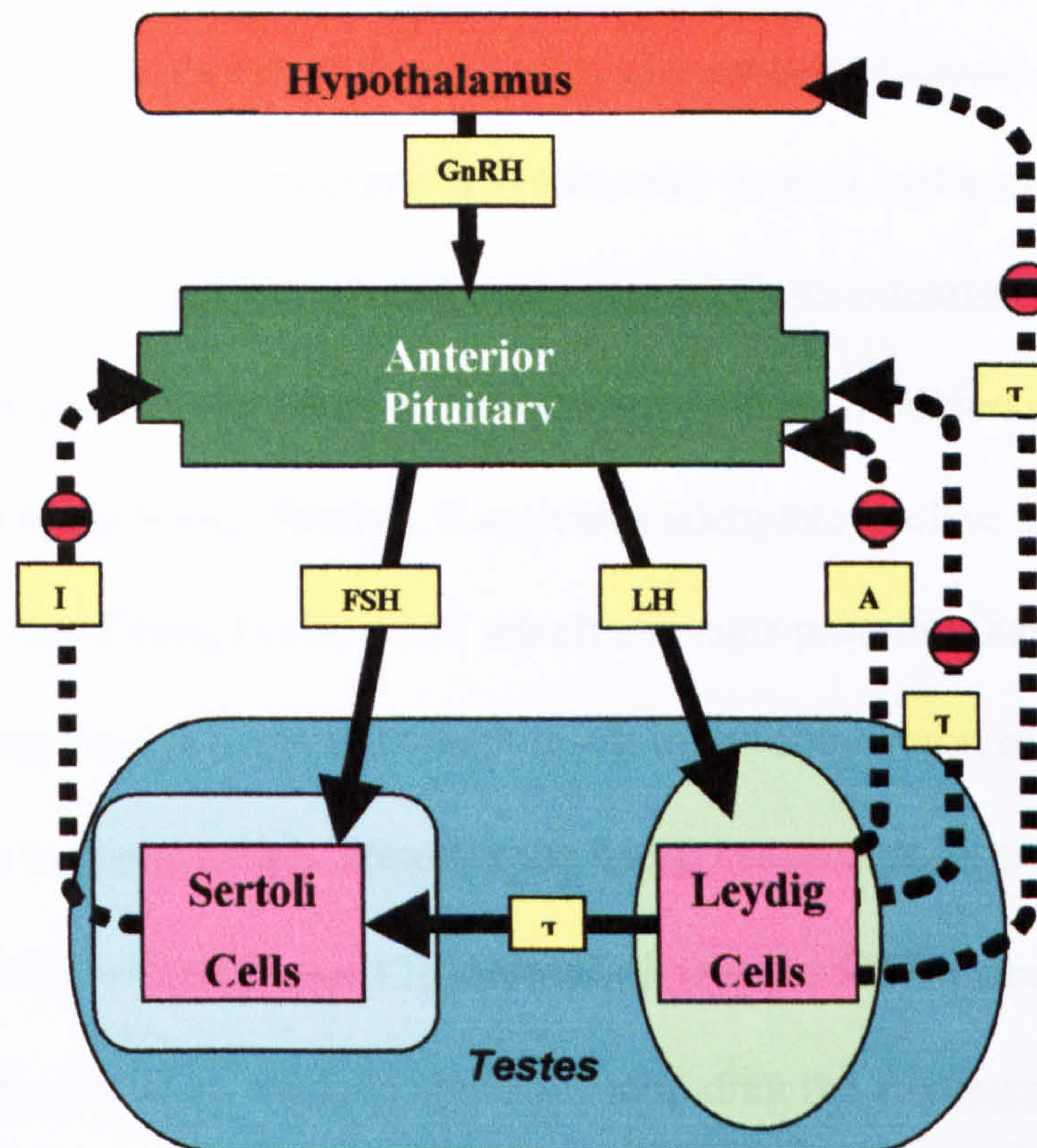


Figure 5.1: GnRH= gonadotrophin-releasing hormone, T= testosterone, FSH= follicle-stimulating hormone, LH= luteinizing hormone, I= inhibin, A= activin.
All hormones are indicated in yellow boxes and feedback controls are indicated by dashed lines. Adapted from Endocrine Reviews.

Figure 5.1 illustrates the endocrine control of spermatogenesis, the hypothalamus releases GnRH in pulses, the frequency that this occurs varies from hourly to several times a day (varying with age and hormonal status). This in turn binds to receptors on the anterior pituitary that stimulate the release of FSH and LH, both of which are only known to exert effects in the gonads. These two hormones will now be considered individually starting with LH.

5.3.1 The role of luteinizing hormone (LH) in spermatogenesis

LH is released in pulses, and the frequency of these varies depending on GnRH release. The primary function of LH is to stimulate steroid production in the testes, this is done so by stimulating the interstitial Leydig cells to synthesise testosterone from cholesterol. Approximately 95% of testosterone is synthesised in the Leydig cells with the remainder being derived from the adrenal cortex, in both situations the precursor to testosterone is cholesterol. There are a number of stages involved in the conversion of cholesterol to testosterone, firstly LH activates adenylate cyclase via a G-protein linked LH receptor, stimulating cyclic AMP which activates protein kinase A. Subsequently a number of enzymes are activated which result in the conversion of cholesterol to pregnenolone to testosterone. Testosterone itself, has two major metabolites including dihydrotestosterone (DHT) and 17β-oestradiol. (Figure 5.2 Illustrates the pathway of testicular androgen and oestrogen synthesis including the enzymes involved).

Figure 5.2- pathways of testicular androgen and oestrogen synthesis

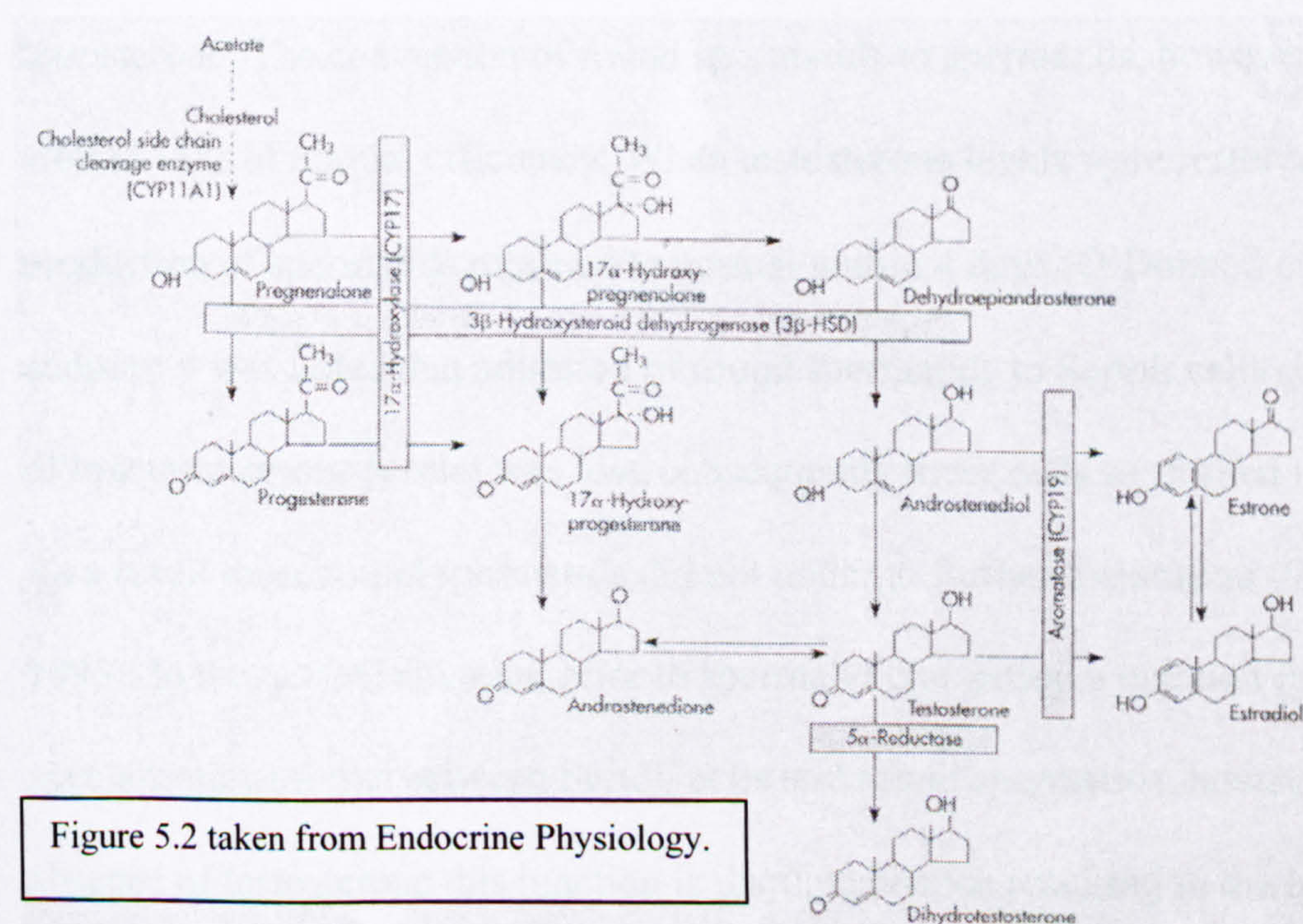


Figure 5.2 taken from Endocrine Physiology.

5.3.2 The role of testosterone in spermatogenesis

The testosterone produced by the Leydig cells is secreted, however information on the mechanism of testosterone action on spermatogenesis is limited. At present it is thought that testosterone exerts its effects via the Sertoli cells. One function identified is that testosterone stimulates the Sertoli cells to produce of androgen-binding protein (ABP), which binds both testosterone and DHT (McLachlan et al., 1996). ABP acts as a transport protein in both the Sertoli cell and following its release into the lumen of the reproductive tract, sequestering testosterone preventing its free diffusion out of the seminiferous tubules (Martinez-Zaguilian, 2002). DHT has double the binding affinity for the androgen receptor (AR) than that of testosterone and as a result is considered to be a more potent androgen than testosterone. Despite this increased affinity to the AR, research suggests that testosterone is likely to be the main ligand as concentrations are approximately five fold greater than that of DHT. Testosterone appears to have an important function in spermatid maturation, studies in testosterone-suppressed rats revealed that steps 1-7 round spermatids were maintained with low levels of testosterone. The conversion of round spermatids to spermatids, however, occurred at around 15% of normal efficiency. When testosterone levels were restored the production of spermatids returned to normal within 4 days (O'Donnell et al., 1994). In addition it was noted that adhesion of round spermatids to Sertoli cells (in the presence of low testosterone levels) was lost, consequently these cells were shed into the lumen. As a result these round spermatids did not undergo further maturation (O'Donnell et al., 1995). In the normal situation prior to spermatid elongation a junction (the ectoplasmic specialisation) forms between Sertoli cells and round spermatids, however in the absence of testosterone this junction is disrupted hence resulting in the loss of the majority of round spermatids (Muffly et al., 1994). As can be seen in figure 5.1 (HPG

axis) the testosterone produced by the Leydig cells also functions as a negative feedback to both the hypothalamus and the anterior pituitary resulting in a decrease in GnRH and LH secretion.

5.3.3 The role of Follicle- stimulating hormone (FSH) in spermatogenesis

The primary target of FSH is the Sertoli cell within the seminiferous tubules of the testes. The biochemical pathway of FSH binding to Sertoli cells is similar to that described in LH, with the activation of cyclic AMP. As in the case of the mechanism of testosterone action on spermatogenesis, knowledge on the roles FSH play in this process is also limited. To date research suggests that FSH is likely to have a role stimulating spermatogonial division and meiosis as well as potentially having an 'anti-apoptotic' function resulting in an increase in germ cell survival. FSH and testosterone are likely to also act in a synergistic manner it is hypothesised that FSH stimulates spermatogonia division and meiosis but it appears that testosterone is required for later maturation steps into spermatozoa (McLachlan et al., 1996). Muffly et al. (1994) also suggest that FSH and testosterone may both play a role in the formation and maintenance of the junction required for round spermatid adhesion to Sertoli cells. FSH has been shown to be responsible for the alignment of actin and vinuclin filaments of the Sertoli cell, which are involved in the specialised junctions leading to round spermatid adhesion for which testosterone is believed to be required for the adhesion (Muffly et al., 1994).

5.3.4 The role of inhibin in spermatogenesis

Yet to be mentioned is the role of the two non-steroidal hormones produced in the gonads, inhibin and activin, within the HPG axis (figure 5.1). FSH stimulates the Sertoli cells to produce the hormone inhibin, which in turn acts as a feed back mechanism on

the anterior pituitary to reduce the rate of FSH release (Plant and Marshall, 2001). At present the only known role of activin in spermatogenesis is that activin is secreted from the Leydig cells and acts as a negative feedback mechanism on the anterior pituitary to reduce the rate of FSH (Plant and Marshall, 2001).

5.3.5 The role of oestrogens in spermatogenesis.

To date the role of oestrogen in spermatogenesis is unclear, for several decades it has been established that oestrogens are involved in the negative feedback mechanisms in place in spermatogenesis (Santen, 1975; Winters et al., 1979). The administration of oestrodiol in males exhibiting idiopathic hypogonadotropic hypogonadism, was found to suppress LH, by reducing the pulse frequency of GnRH from the hypothalamus (Finkelstein et al., 1991). The opposite situation pertained when individuals were administered testolactone (an inhibitor of aromatase), resulting in an increase in LH secretion and a reduction in the inhibitory effects of testosterone (Bagatell et al., 1994). In recent years several discoveries including the finding of two oestrogen receptor (ER) isoforms (ERα and ERβ), that are expressed throughout the male reproductive system. At present there are conflicting reports as to where these receptors are localised within the reproductive system, however the majority of studies find little expression of the ERα receptor (except in the Leydig cells). The ERβ receptor, however has been found to be expressed in a variety of cells including Sertoli cells and various stages of germ cells (spermatogonia, spermatocytes and round spermatids) (Bremner et al., 1994; Turner et al., 2001). This indicates that the role of oestrogen in the testis is not restricted to a feedback mechanism alone. There is evidence that oestrogen is also required for normal fertility, this is apparent in the mice knockout (KO) models ERKO (targeted inactivation of ERα) (Eddy et al., 1996). In the ERKO model male mice were found to

be infertile due to the accumulation of fluid in the seminiferous tubules resulting in the dilution of sperm and testicular atrophy (thought to be attributed to the accumulation of fluid). This provides evidence of a potential role of ERα in the regulation of fluid transport in the reproductive system of males (Zhou et al., 2001). The following mouse KO models have also been generated BERKO (targeted inactivation of ERβ); αβERKO (inactivation of ERα and ERβ) and ArKO (inactivation of aromatase), however the fertility in these individuals were not found to be grossly altered, although there is evidence in some KO that fertility decreases with age (Couse et al., 1999; Krege et al., 1998; Roberton et al., 1999). The role of oestrogen in the maintenance of normal fertility is uncertain, the molecular mechanisms behind the infertility phenotypes within the mouse KO models is unclear, however the problem also lies in the lack of information about genes that are directly regulated by oestrogens within the male reproductive system (Turner et al., 2001).

5.4 Concern over possible decline in sperm counts and possible causes

Within recent years there has been considerable debate over several studies reporting a decline in sperm counts of up to 50% over the last 50 years (Auger et al., 1995; Carlsen et al., 1992; De Mouzon and Thonneau, 1996; Irvine et al., 1996), and the apparent increased occurrence of disorders of the male reproductive tract (reported to have doubled) within the last 30-50 years (Sharpe and Skakkebaek, 1993). These issues are yet to be resolved, however if this is the case these dramatic changes have occurred over a relatively recent period of time and have been reported in a number of countries. It is possible that these reflect adverse effects of environmental or lifestyle factors (Sharpe and Skakkebaek, 1993). There have been a number of reports within the literature on endocrine disruption in wildlife affecting the fertility of animals (reviewed by Tyler et

al., 1998). There is some evidence that environmental factors may be responsible in humans also. During 1945 and 1970 some women were administered a synthetic oestrogen (diethylstilboestrol) during pregnancy. The sons of these women have been found to have decreased sperm counts, semen volume and increased incidence of cryptorchidism and hypospadias. Spermatogenesis in the adult testis is also vulnerable to adverse environmental effects, there are environmental chemicals including chemicals used in the home, workplace and agriculture as well as certain drugs (refer to section 1.5.9) that can interfere the process of spermatogenesis. Occupational exposure to heat or radiation has also been shown to impair spermatogenesis (Sharpe, 1992). There is currently some evidence to suggest that hormonal disruption resulting in decreased fertility is occurring in wildlife, however at present, despite receiving much attention there is little evidence to support this theory in humans. There is growing circumstantial evidence however, that in certain cases particularly those for which no cause has been identified for infertility that these factors may play a role. One study providing circumstantial evidence of this was carried out by Rozati et al. (2002), in this study the levels of polychlorinated biphenyls (PCBs) and phthalate esters (PEs) in seminal plasma of infertile males was significantly higher than that found in controls, the highest concentrations were identified in fish eaters and vegetarians. The infertile males with the higher concentrations of PCB's and PE's were found to have significantly lower sperm: volume, concentration, progressive motility, normal morphology and fertilising capacity.

5.5 The role of oxidative stress in infertility

Oxidative stress has also been implicated in infertility with significant levels of reactive oxygen species (ROS) detected in the semen of 25% of infertile males, however normal

fertile counterparts have no detectable levels of ROS (Iwasaki and Gagnon, 1992). Sperm is susceptible to oxidative stress as a result of the abundance of unsaturated fatty acids in the sperm plasma membrane, required for sperm motility and fusion events (including the acrosome reaction and the sperm-egg interaction) essential for fertilisation. The unsaturated fatty acids are targets for free radical attack and ongoing lipid peroxidation throughout the sperm plasma membrane resulting in the accumulation of lipid peroxides on the sperm surface causing sperm dysfunction and cell death. Oxidative damage to the sperm DNA is also found as a result of the adverse reaction of ROS in sperm. This has relevance to our study in that given the information it would seem prudent also to assess for anti-oxidant activity, in the herbs used. The anti-oxidant potential of the herbs used in the treatment of male infertility was therefore assessed, primarily as medicinal plants have been shown to exhibit high anti-oxidant activity (Rice-Evans et al., 1995). This is of importance as oxidative stress can be reduced by the intake of additional anti-oxidants, and have been related to the prevention of degenerative diseases including that of degenerative diseases such as cancer, oxidative stress dysfunctions, cardiovascular and neurological diseases (Szollosi and Varga, 2002).

5.6 Objectives

The results presented in chapter 4 have raised a number of questions, primarily whether the improvements in disomy levels observed in the six infertile patients coincident with TCM treatment represent a genuine effect of the herbal prescriptions administered or if they are as a result of a placebo effect. If these herbs were exerting a genuine effect on chromosome segregation we might expect them to have a biological activity. Given the fact that the process of spermatogenesis is under strict hormonal control and is subjected

to the effects of oxidative stress, these herbs may possess endocrine or anti-oxidant activity.

- The aim of this study was to test the 37 individual herbs and 7 patient prescriptions (mixtures of the 37 individual herbs) in biological assays to determine if this hypothesis is true.
- Each of the individual herbs and herbal prescriptions were tested for endocrine activity (including that of oestrogenic, androgenic, anti-oestrogenic and anti-androgenic activity) utilising a recombinant yeast based assay (refer to section 8.3 within the materials and methods).
- The individual herbs and herb prescriptions were also tested for anti-oxidant activity using the ferric reducing anti-oxidant assay (FRAP) assay (refer to section 8.6).

5.7 Validation of assays chosen to test for endocrine and anti-oxidant activity

In order to establish if any herbs exhibited endocrine activity a recombinant yeast screen was utilised (refer to section 8.4), this assay was chosen as there are a number of reports within the literature stating that this assay is capable of responding to oestrogens, xeno-oestrogens and androgens (both that of steroidal and non-steroidal oestrogens and androgens). All assays used in this section of work have been fully validated and published (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998).

5.7.1 Principles behind the recombinant yeast screen assay:

The yeast cells used in this assay have been genetically modified with the human oestrogen receptor gene (hER) or a human androgen receptor gene (hAR) inserted within the yeast genome. In the yeast cells there is also an expression plasmid that

contained oestrogen responsive sequences (ERE) (Routledge and Sumpter, 1996), or androgen response elements (Sohoni and Sumpter, 1998), within a strong promoter sequence and also a reporter gene *lac-Z*, (encoding the enzyme β-galactosidase, which enables the activity of the receptor to be measured).

The yeast-based assay was developed to test compounds that can interact with the human oestrogen or androgen receptor, and works through the following reaction/principle. For example in the oestrogen based assay, when an active ligand that has occupied the hER, this in turn causes the hER to bind to ERE (located within the expression plasmid), which in turn results in the initiation of transcriptional factors resulting in gene transcription. In turn this causes the expression of reporter gene *lac-Z* to secrete β-galactosidase into medium. In the normal situation the medium is yellow in colour, however, in the presence of a chromogenic substrate such as chlorophenol red-B-D-galactopyranoside (CPRG), β-galactosidase metabolises CPRG causing a colour change to a red product. This colour change (yellow-red) can then be measured at an absorbance of 540nm (Routledge and Sumpter, 1996). A schematic representation of the modified yeast and the process that is undertaken is represented in figure 5.3.

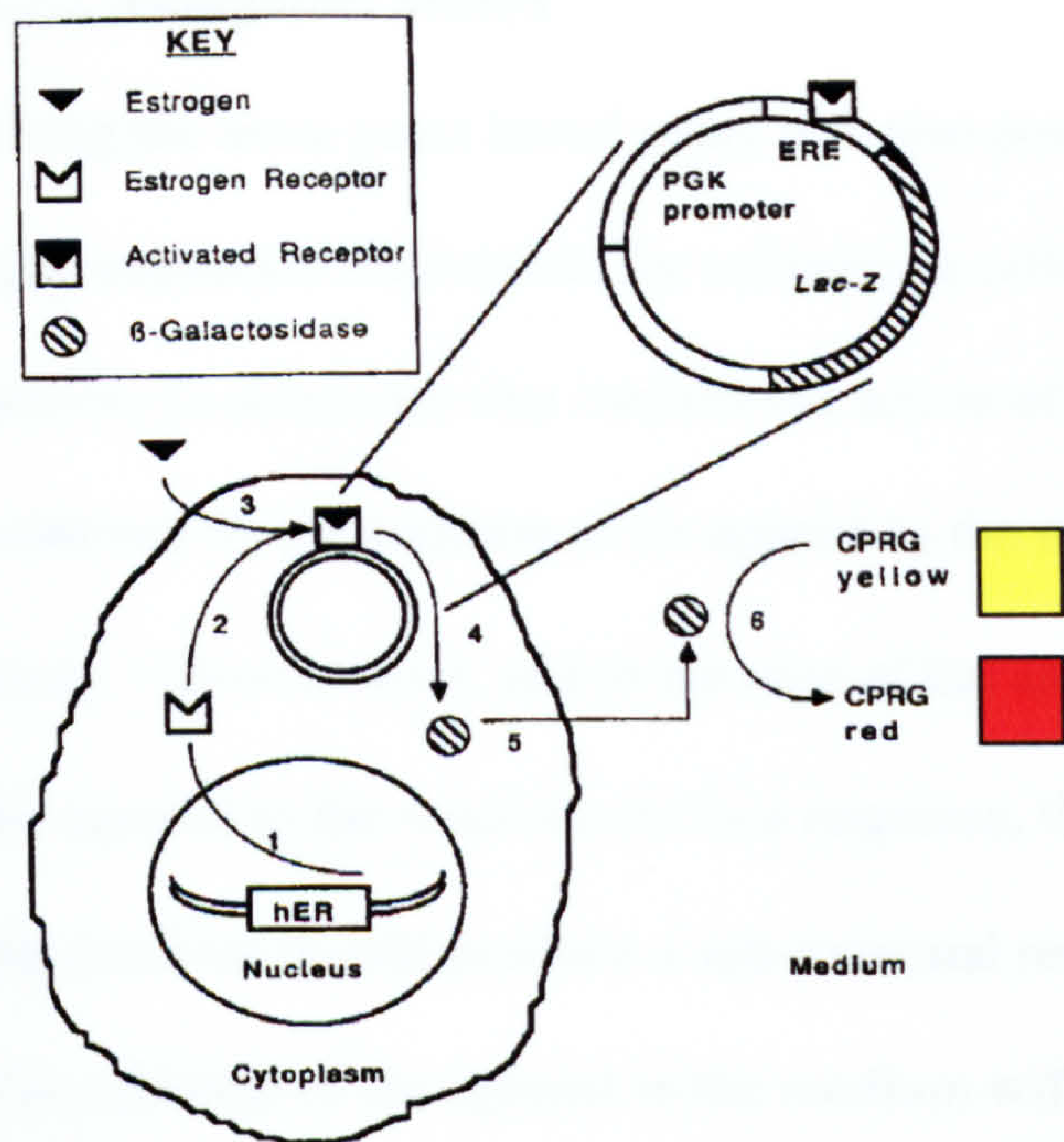


Figure 5.3 Schematic diagram of the oestrogen-inducible expression system in yeast. The human oestrogen receptor gene is integrated into the main genome and is expressed (1) in a form capable of binding oestrogen response elements (ERE) within a hybrid promoter on the expression plasmid (2). Activation of the receptor (3), by binding of ligand, causes expression of the reporter gene *Lac-Z* (4) which produces the enzyme β-galactosidase. This enzyme is secreted into the medium (5) and metabolises the chromogenic substrstrate CPRG (normally yellow) into a red product (6) which can be measured by absorbance (taken from Routledge and Sumpter., 1996).

Figure 5.4 provides an example of an oestrogen yeast-based assay, illustrating the colour change response seen with this assay.

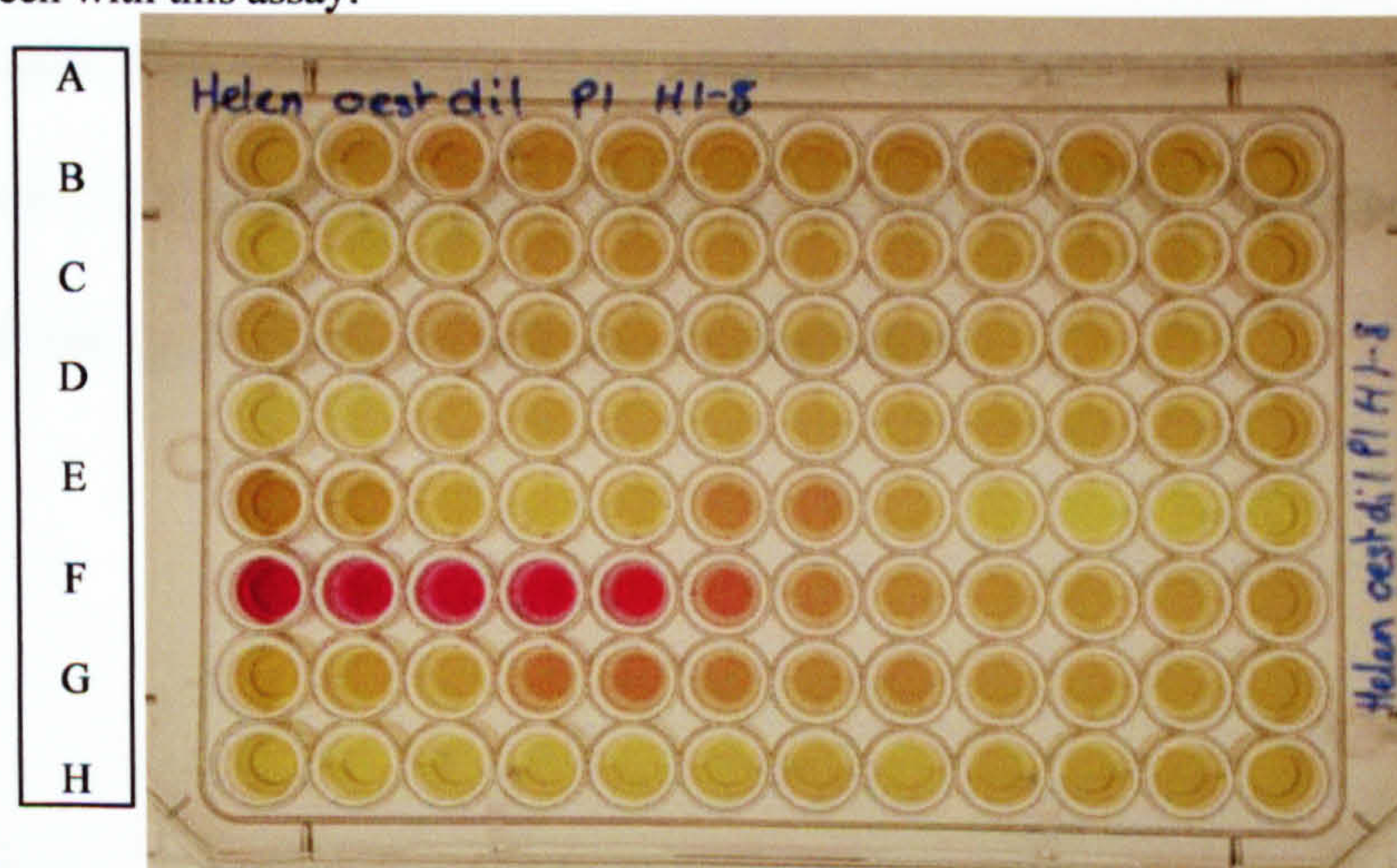


Figure 5.4 is an example of a oestrogen yeast-based assay plate at the end of the assay, as stated above a positive response is obtained when the medium within the wells changes from a yellow to a red colour (this colour change is read on a spectrophotometer at 540nm). This assay plate shows samples serially diluted with the highest concentration found in the first well (on the left-hand side) with the lowest concentration in the final well (on the right-hand side). As can be seen from this plate row F display oestrogenic activity whereas the remaining rows do not display any oestrogenic activity.

5.7.2 Antagonist assays

Using the same yeast based assay it is also possible to detect not only agonist activity (any substance responsible for initiating a cellular response) but also that of antagonist activity (a substance that inhibits the action of one substance on another). This is achieved by the addition of an agonist to the medium (in the case of the anti-oestrogen assay 17β-oestrodiol, and in the case of the anti-androgen assay DHT). The addition of the agonist to the medium elicits a response, the concentration of the agonist added to the medium should produce a sub-maximal response (preferably of around 50-75%). The addition of the agonist to the medium will cause a colour change yellow to red, however in the presence of an antagonist the activity of the agonist will be inhibited in a dose dependant fashion. Hence the antagonist will compete for the receptor and inhibit the medium from changing from yellow to red (therefore in the antagonist assays a positive response is found for samples tested in which the medium remains yellow). Figure 5.5 provides an example of an anti-androgen assay plate. The antagonist assay protocol was carried out as before (refer to section 8.5) with the exception of the addition of the agonists to elevate the background.

Figure 5.5 provides an example of an anti-androgen yeast-based assay, illustrating the colour change response seen with this assay.

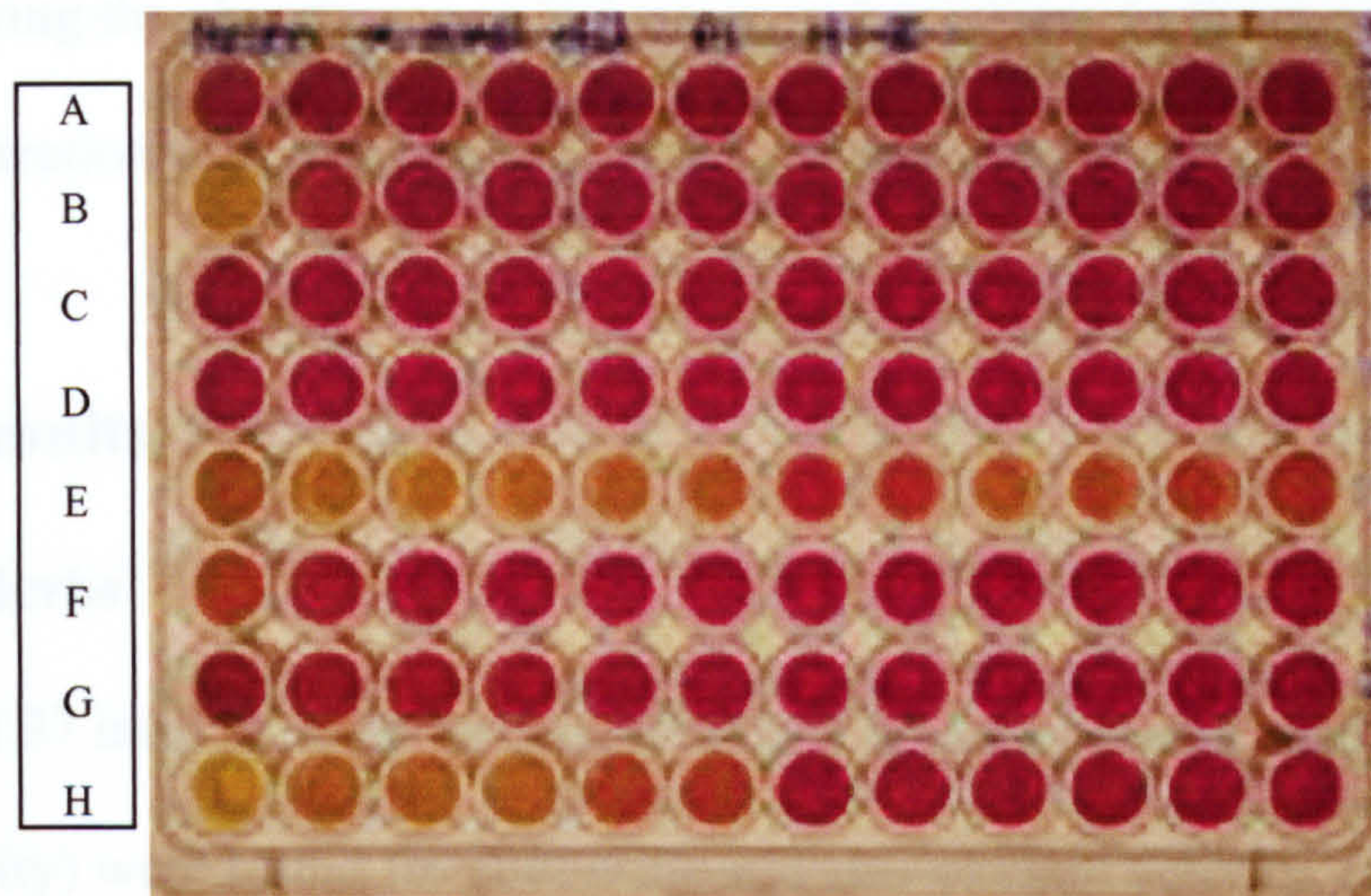


Figure 5.5 is an example of a anti-androgen yeast-based assay plate at the end of the assay, as stated above a positive response is obtained when the medium within the wells remains a yellow colour (this colour change is read on a spectrophotometer at 540nm). This assay plate shows samples serially diluted with the highest concentration found in the first well (on the left-hand side) with the lowest concentration in the final well (on the right-hand side). As can be seen from this plate rows B, E and H display anti-androgenic activity whereas the remaining rows exhibit no anti-androgenic activity at the concentrations investigated.

5.8 Anti-oxidant assays

The ferric reducing antioxidant potential (FRAP) assay was utilised to determine the anti-oxidant potential of the plant-based herbs due to its reproducibility, the inexpensive cost, simplicity and speed (Benzie and Strain, 1996). This technique has also been successfully utilised in a number of studies investigating the anti-oxidant potential of various plants homogenates and pharmacological plant products including a variety of herbal teas (including green and black), medicinal plants (including 6 members of the Labiatae plant family) (Szollosi and Varga, 2002), and common vegetables (Ou et al., 2002).

5.8.1 Principles behind the FRAP assay

The FRAP assay (refer to section 8.6) works on the principle that the presence of an anti-oxidant results in ferric to ferrous ion reduction at a low pH resulting in a coloured

ferrous-tripyridyltriazine complex to form. The FRAP assay values are obtained by comparing the absorbance at 593 nm in test reactions to those with a known concentration of ferrous ions (Benzie and Strain, 1996).

5.9 Results

5.9.1 Herbs

In total 37 individual herbs (used in the TCM preparations used in the treatment of male infertility) were tested for endocrine and anti-oxidant activity, (refer to figure 8.8 and table 8.1 for a list of all the 37 herbs including the Chinese name known as the Pin Yin name and the Latin name and common name of the herb where possible). As well as testing the individual herbs, a total of 7 herb mixtures (4 prescribed to patient NLCL, and three prescribed to patient IDSS), the herb mixtures containing between 10 -20 of the 37 individual herbs were tested using the same assays, the individual herbs within these mixtures cannot be revealed due to confidentiality agreements. These herbs were prepared as described in section 8.3.

5.9.2 Endocrine activity

The assay (refer to section 8.4) was set-up using the two plate formats including the dilution plate (which covered a range of $5x10^{-3}$-10 µl of the herb extract) and the "spot plate" (which covered a range of 1.25-40µl). In each case the assay was repeated for each herb to ensure that the results produced were representative. For each assay a plate containing a dose response curve was set-up: the following standards were used in each assay: 17β oestrodiol (oestrogen assay), DHT (androgen assay), OHT (anti-oestrogen assay) and flutamide (anti-androgen assay) thereby testing the assay system is responding to substances of known activity and enabling the response of any activity

produced by the herbs to be measured (refer to section 5.9.3). Several negative controls were also set-up in each assay to ensure that any response was not seen as a result of contamination. The controls set-up included: a row of the yeast media only (to give a baseline value and ensure that there was no contamination during the preparation of the media); a row of ethanol blanks and boiled ddH_2O (serially diluted in ethanol in the same manner as the herbal extracts) used in the preparation of the herbal extracts (ensuring there was no contamination in the ethanol used to set-up the dilution plates and ddH_2O used to prepare the herbs). In all cases (for each of the 37 individual herbs and the 7 herb mixes tested) experiments were repeated a minimum of two times with appropriate standards each time, similar responses were generated in each case. Figures 5.7, 5.8, 5.9 and 5.10 display representations of the results generated for each standard, individual herb and herb mixes.

5.9.3 Conversion of results to oestrodiol, DHT, OHT and flutamide equivalents.

Using the data generated in the yeast-based endocrine activity assays it is possible to establish estimates of the oestrogenic, androgenic, anti-oestrogenic and anti-androgenic activities of the herbs directly with those of the known standard assays.

In each case the appropriate standards for each agonist and antagonist assays were plotted with the concentration (ng/l) against Absorbance measured at 540nm, whilst for the herb samples the volume of herb extract (in µl) within the yeast was also plotted against the Absorbance (540nm).

From the graphs produced for each of the individual herbs and the herb mixtures an absorbance and its corresponding volume of extract were noted for each herb. The

absorbance used was in the mid response range, along a linear section, in order to make extrapolation of the results more accurate. Using that same absorbance the appropriate standard graph was used and reading from the dose response curve the equivalent concentration of the standard found.

The standard's concentration (ng/l) was then used to calculate the mass of standard in each assay well (containing 200μl). From this we were able to calculate the activity of the four standards used in each assay, in terms of the amount of standard (ng/l) required to cause the response seen in each assay, it is therefore possible to determine the equivalent amount of herb required to elicit the same response. This task however, is not an easy one as we have a set of herbs with of unknown constitution, however we do know the initial amounts of herb added to a known volume of ddH_2O to generate the herbal infusion tested in the assay. The conversion of standard equivalents from the responses exhibited from the herb are difficult as we do not know the herb components and their concentrations, and the fact that a known mass of herb was not added directly to the assay plates but boiled in water to create an aqueous extract that was used in the assay. One method to be able to determine an estimate of herb activity (compared to that of a known standard) is to go back to the initial starting amounts of herb and ddH_2O added to create the aqueous extract, from this we can determine the mass of herb used to produce the aqueous extract. As a result we determined that each 1μl of herb extract $1.5x10^{-4}$ grams of herb was present to create the aqueous extract, thus we can deduce an approximate idea of the amount of herb (in grams) that is boiled in the infusion that elicits a response, enabling a standard equivalent to be calculated. From this it is possible to extrapolate the daily intake (of standard equivalent) that take places when the herbs are taken as prescribed by Dr. P.Zhai. This is clearly not going to produce

accurate equivalent values, however this will provide an indication of the activity of the herbal extracts.

5.9.4 Endocrine activity of individual and prescription herbs

The results generated for each assay (oestrogenic, androgenic, anti-oestrogenic and anti-androgenic) are presented in figures 5.7-5.10 for each of the individual herbs, prescription herbs and dose response curves. The endocrine activity of each herb is determined from these graphs. In the case of the oestrogen screen, for example, the results obtained for the standard (in this case 17β-oestrodiol) and the negative control (the assay readings in the wells that contained only yeast) were plotted onto a graph. The graph plot for the standard illustrates the assay response in the presence of a substance possessing oestrogenic activity (in this case the standard 17β-oestrodiol). The negative control (yeast medium only) illustrates the response of a substance with no oestrogenic activity (see figure 5.6A). Figure 5.6B shows the results of the individual herbs 9-14. Clearly we can see that all the herbs, with the exception of herb 14, exhibited responses similar to the control yeast (with spectrophotometer readings at 540nm of around 1). It would therefore indicate that these herbs do not exhibit any oestrogenic activity. Herb 14 however, exhibits a similar response observed in the standard with spectrophotometer readings (at 540nm) of around 2.5. The results of the androgen screen are determined in the same manner.

Figure 5.6- Illustrates an example of the response given by a standard of known activity and an example of the responses given by 6 individual herbs.

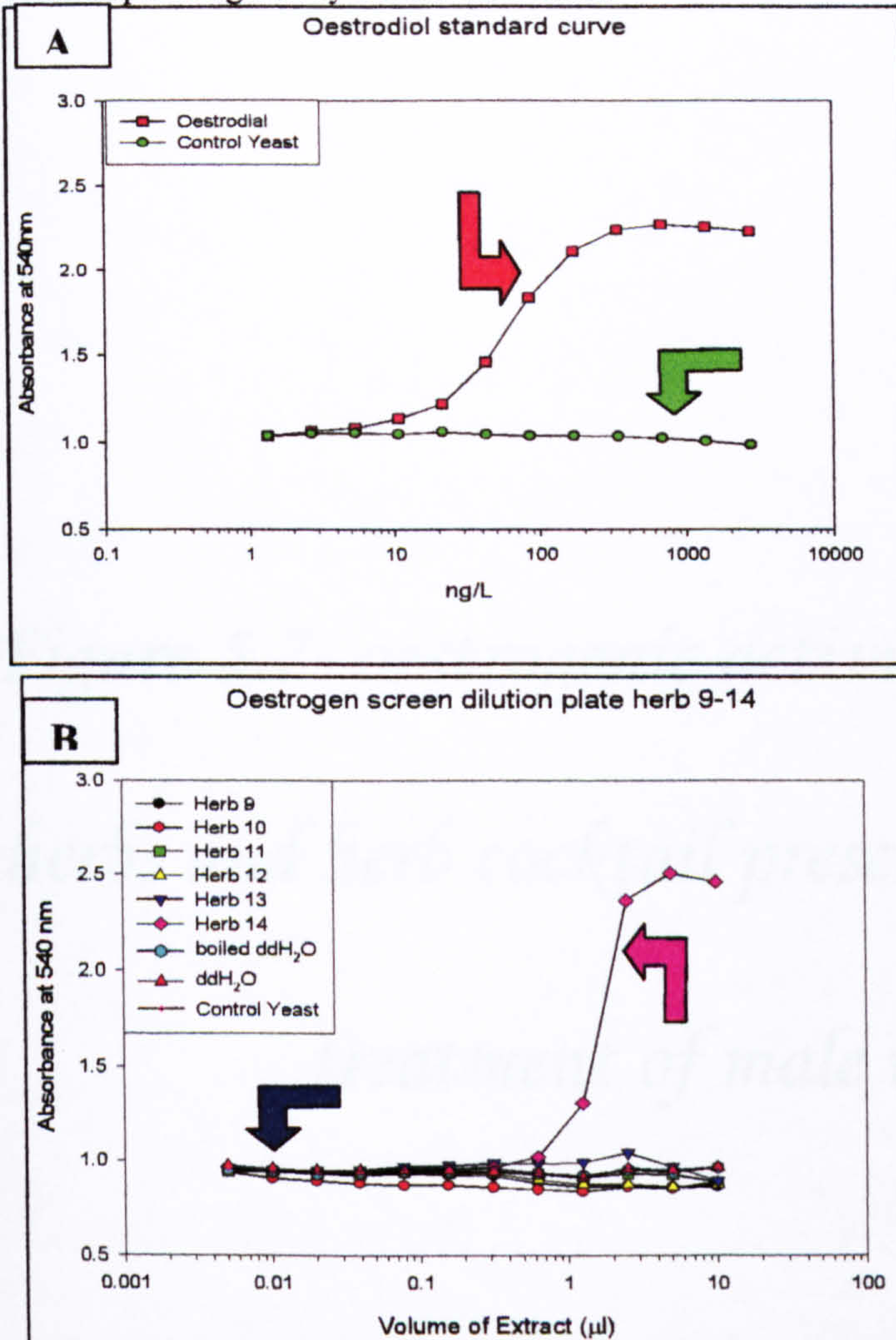
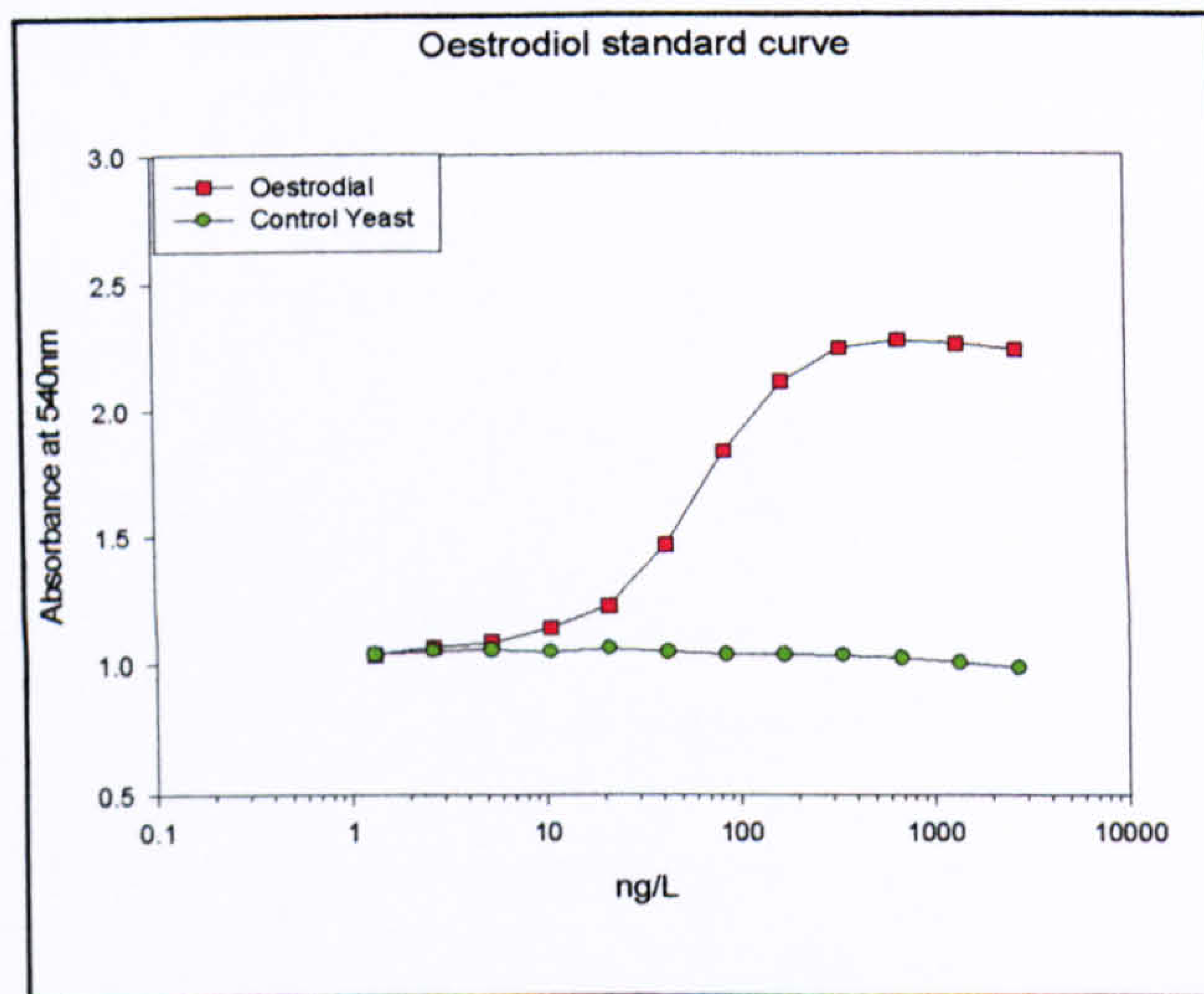
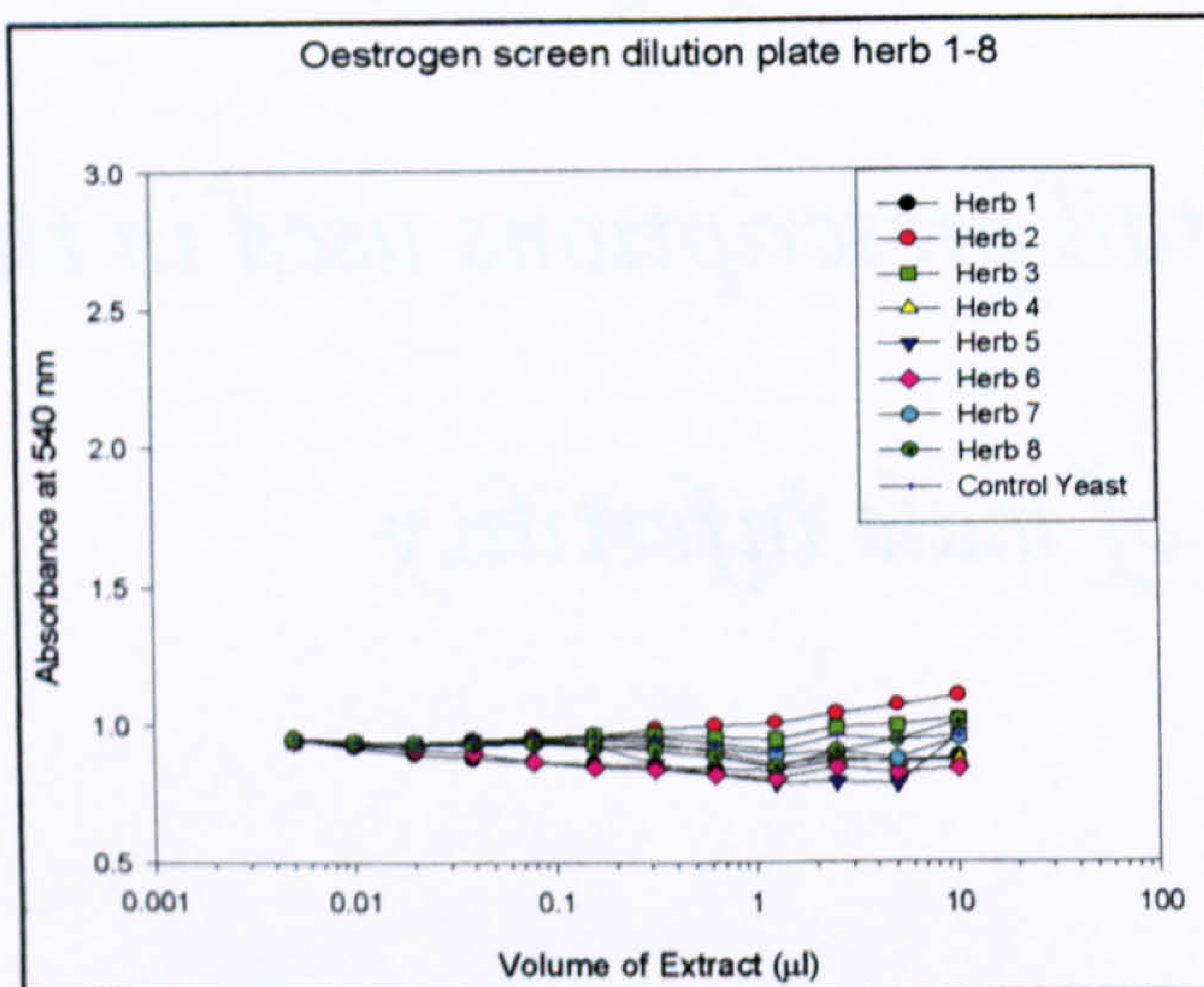


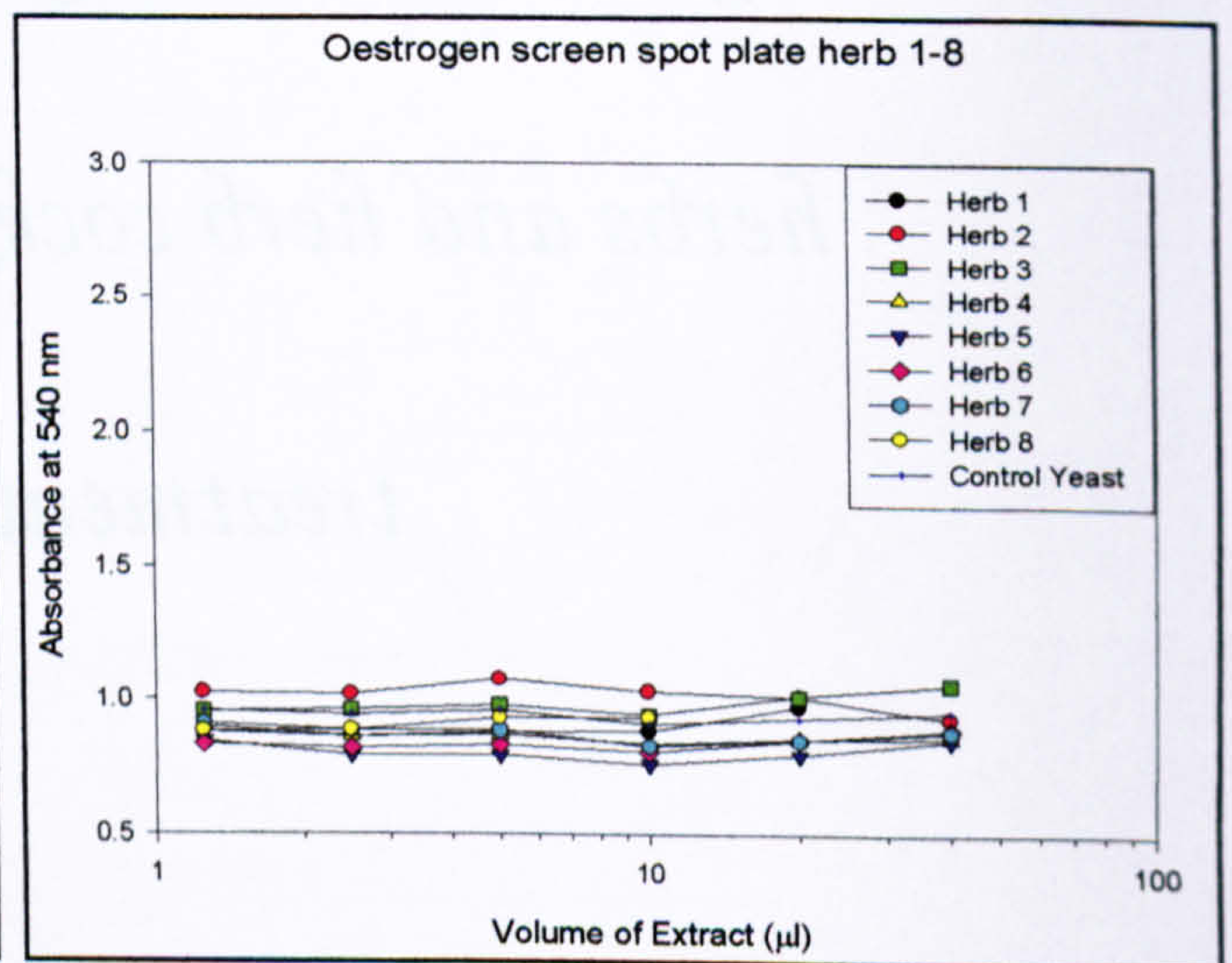
Figure 5.6 A- this provides an example of the response seen on the yeast screen of the control yeast (yeast media with the addition of nothing), which acts as a negative control (indicated by the green arrow). The absorbance measured at 540nm gives a value of approximately 1.0. The red arrow indicates the response of the standard of known activity (in this case oestrodiol), the maximal response gives an absorbance of approximately 2.3 at 540nm. Figure 5.6 B shows the response of six herb extracts on the oestrogen yeast screen. The blue arrow indicates that the majority of the herbs produces no oestrogenic response with an absorbance comparable to the control yeast. The pink arrow however illustrates that herb 14 displays oestrogenic activity displaying a similar result to the standard oestrodiol.

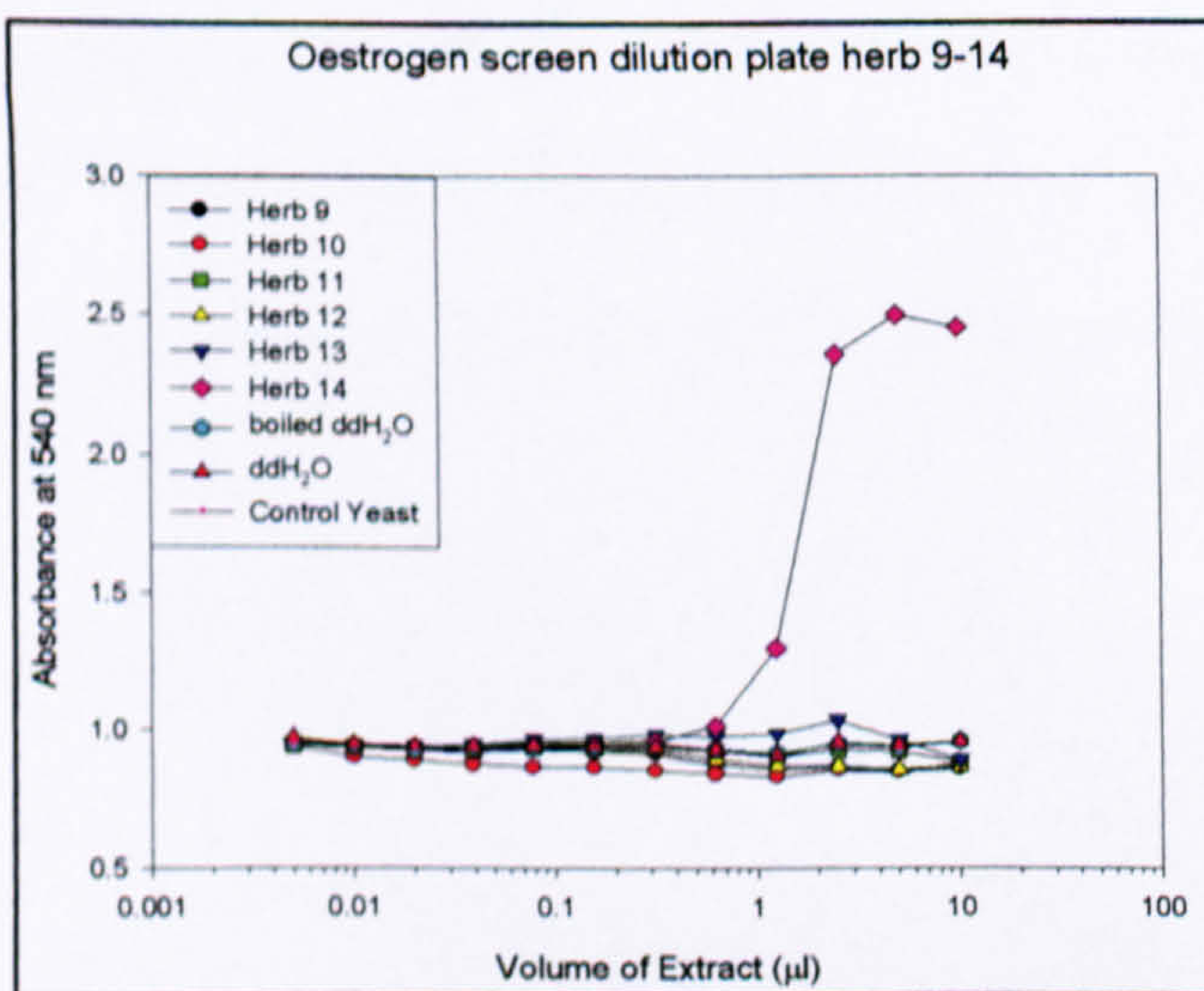
The results of the anti- screens are determined in much the same way with samples that exhibit no activity having levels similar to the control yeast (with absorbance readings of around the 2-3, as the background has been elevated), and samples exhibiting a positive response shown to have activities similar to standards, with absorbance readings dropping below the control yeast.

Figure 5.7- oestrogenic activity of the individual herbs and herb cocktail prescriptions used in the treatment of male infertility









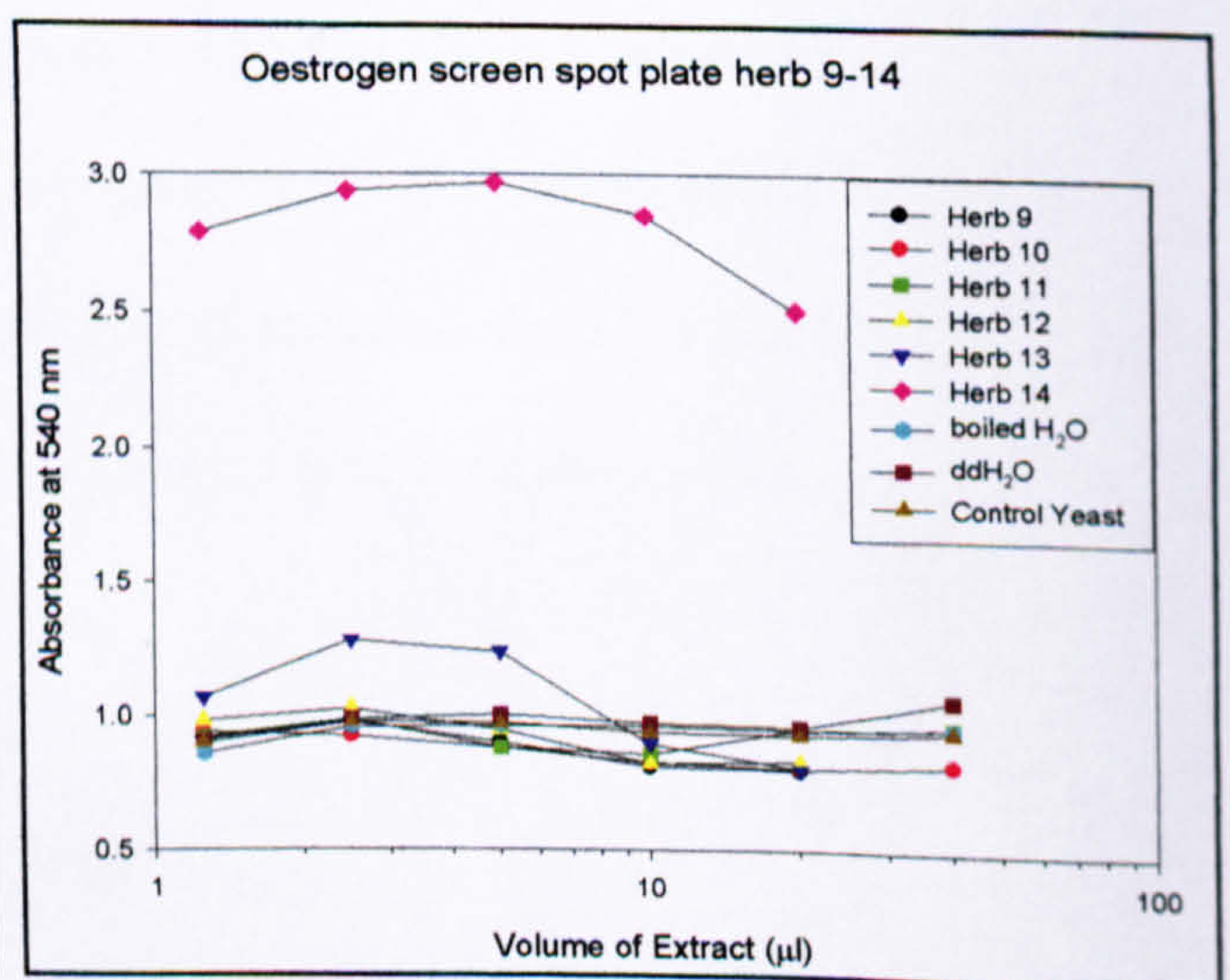
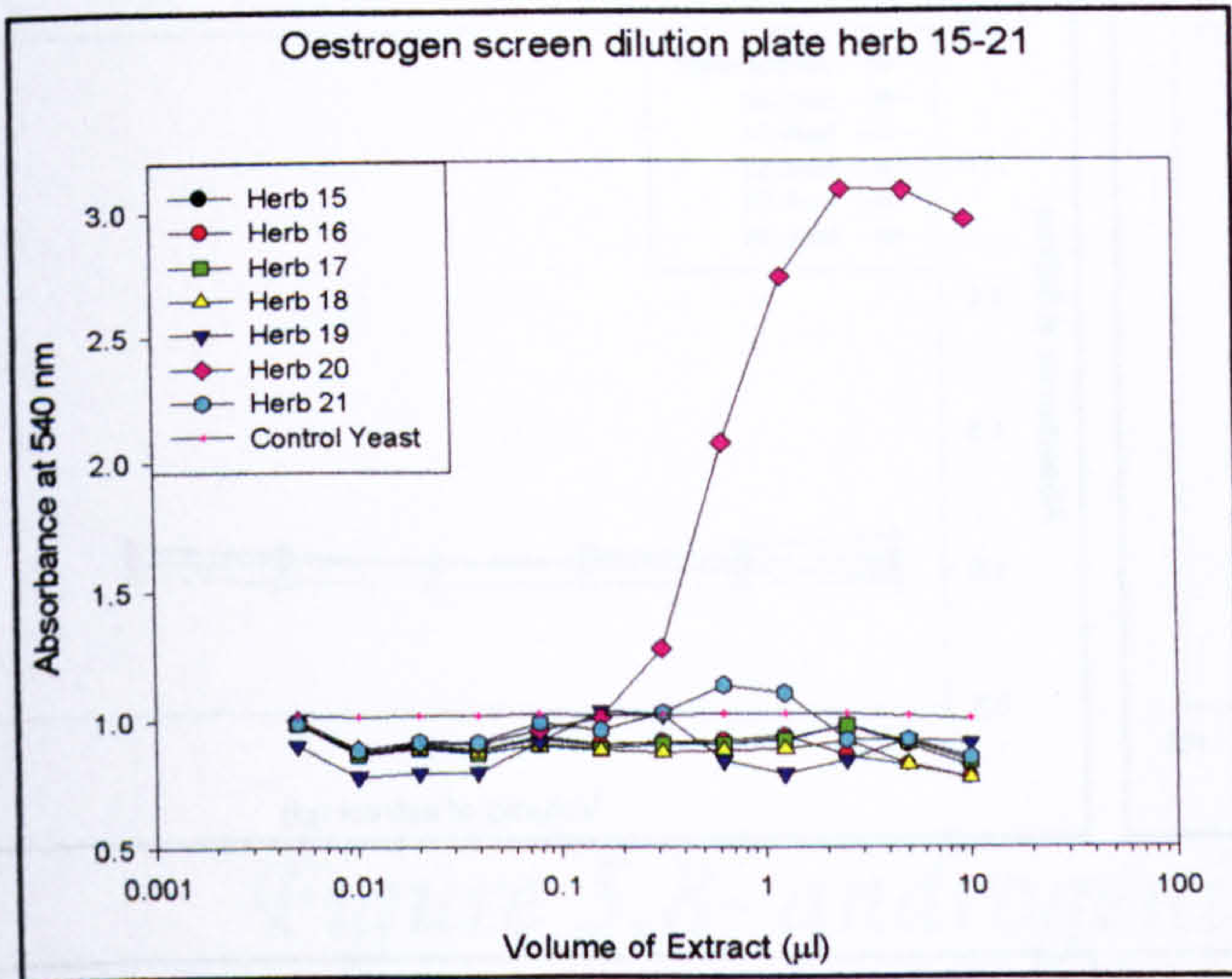
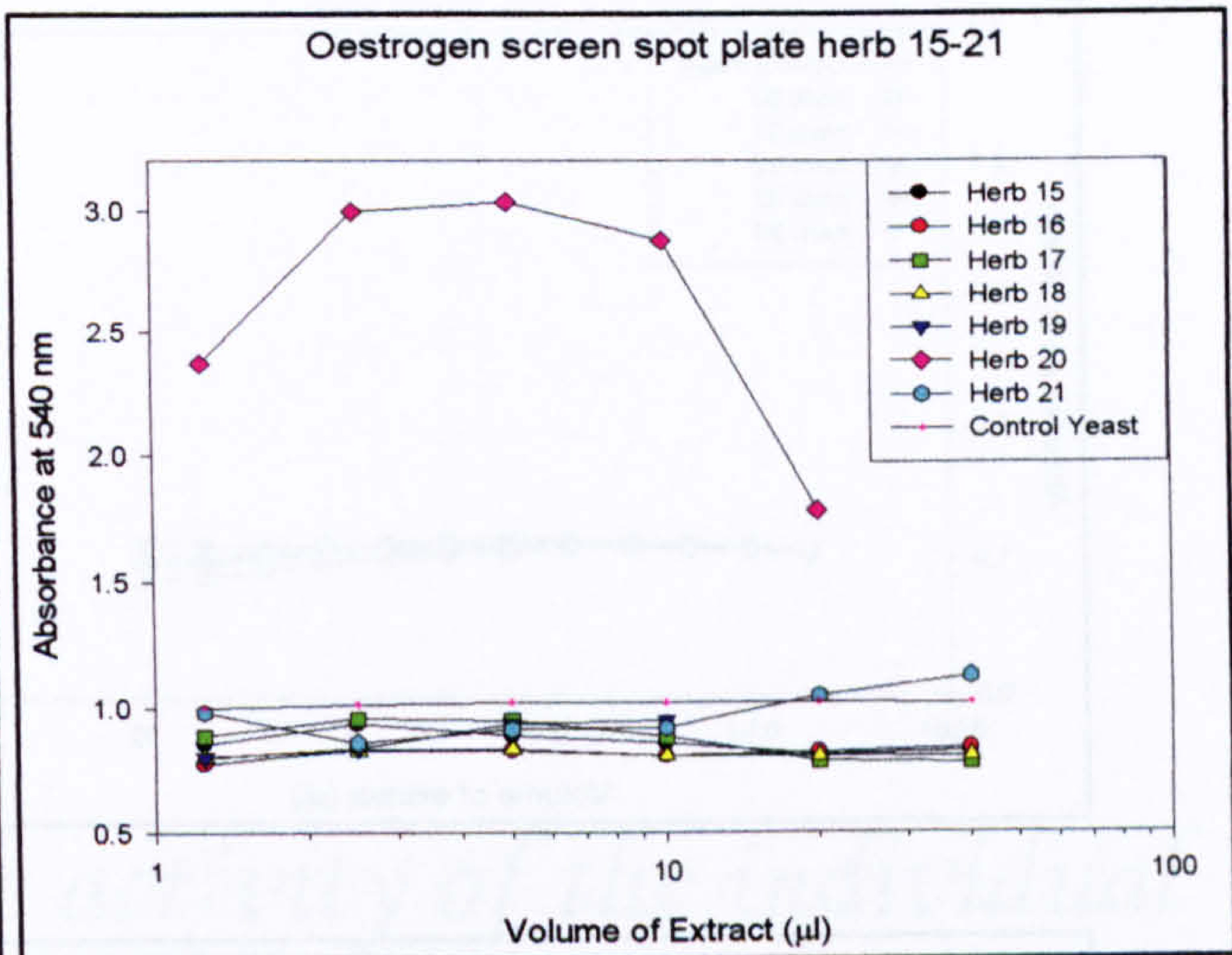
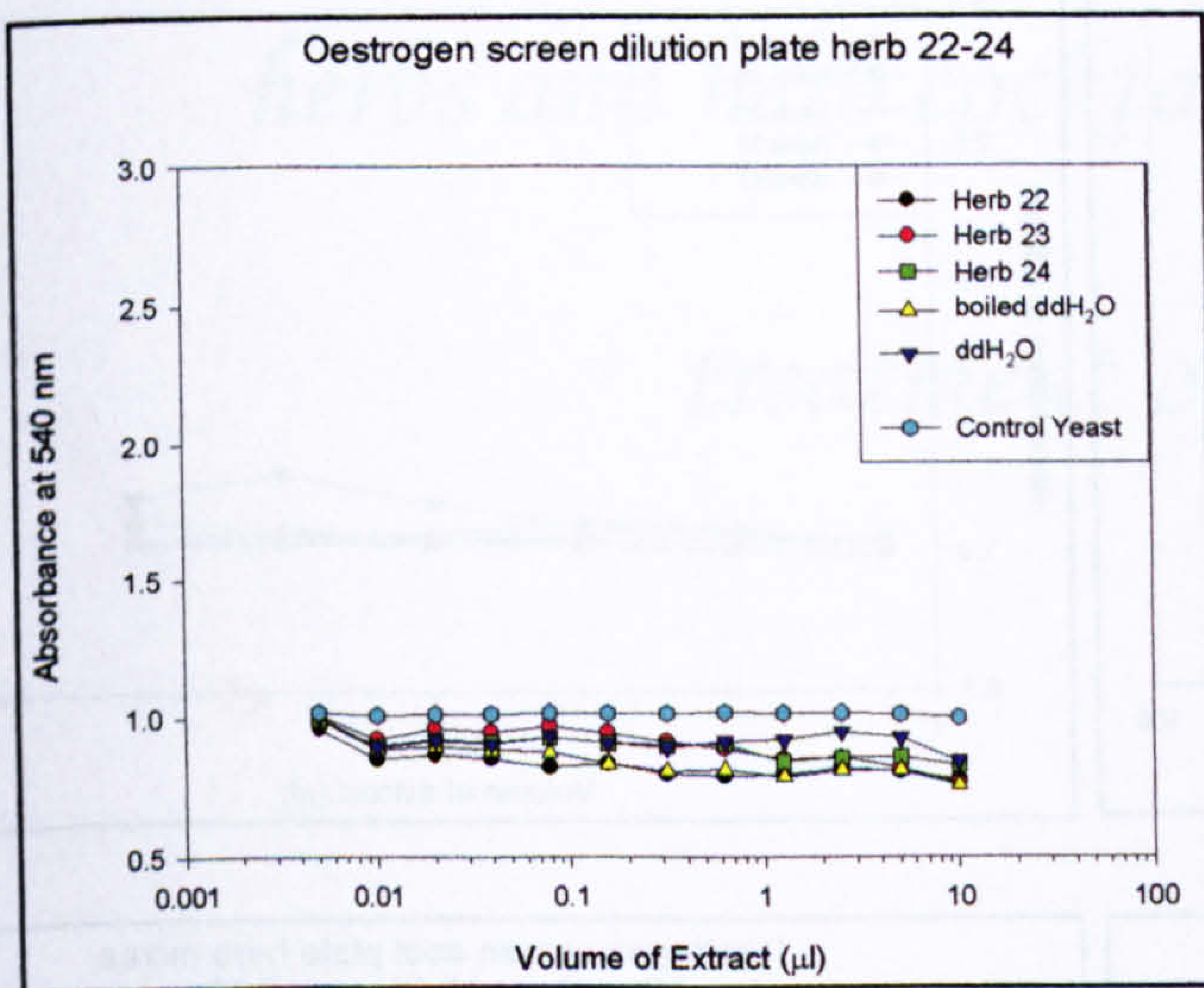
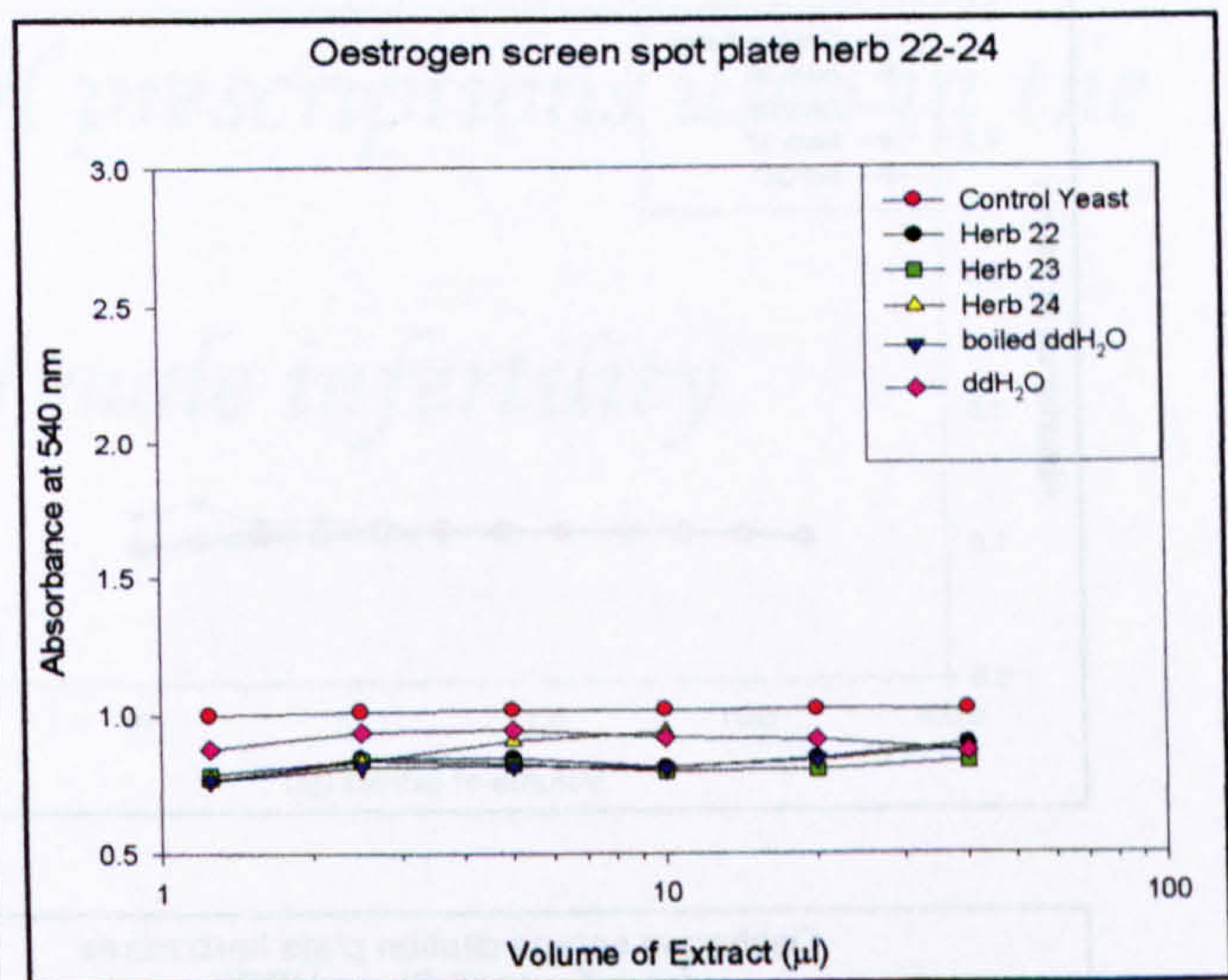


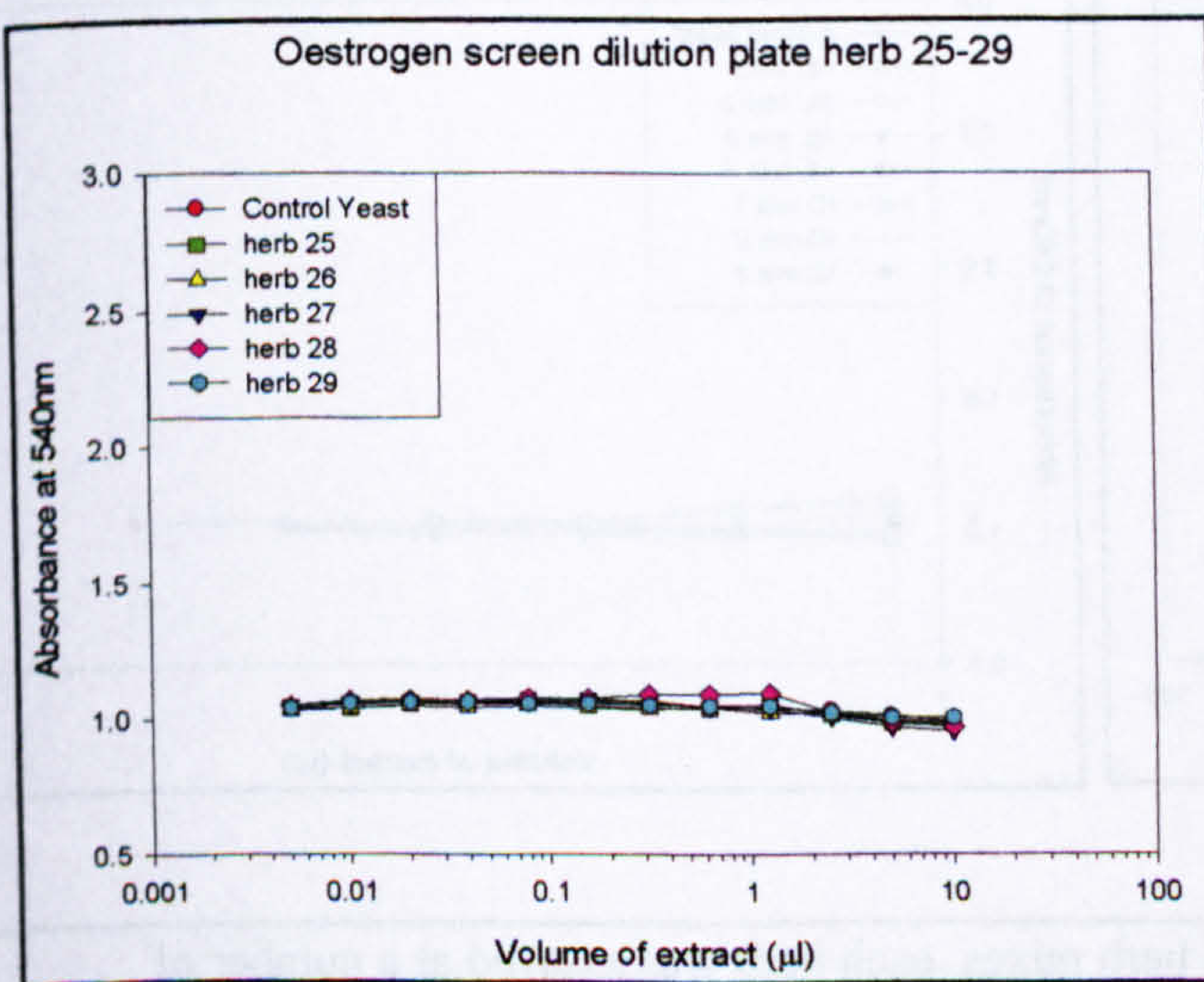
Figure 6.7- oestrogenic activities of herbs 1-14, each herb was assayed at a number of dilutions inluding: $5x10^{-3}$-10ul (dilution plates) and 1.25-40ul (spot plates), responses are compared to those of known concentrations of 17B- oestrodiol











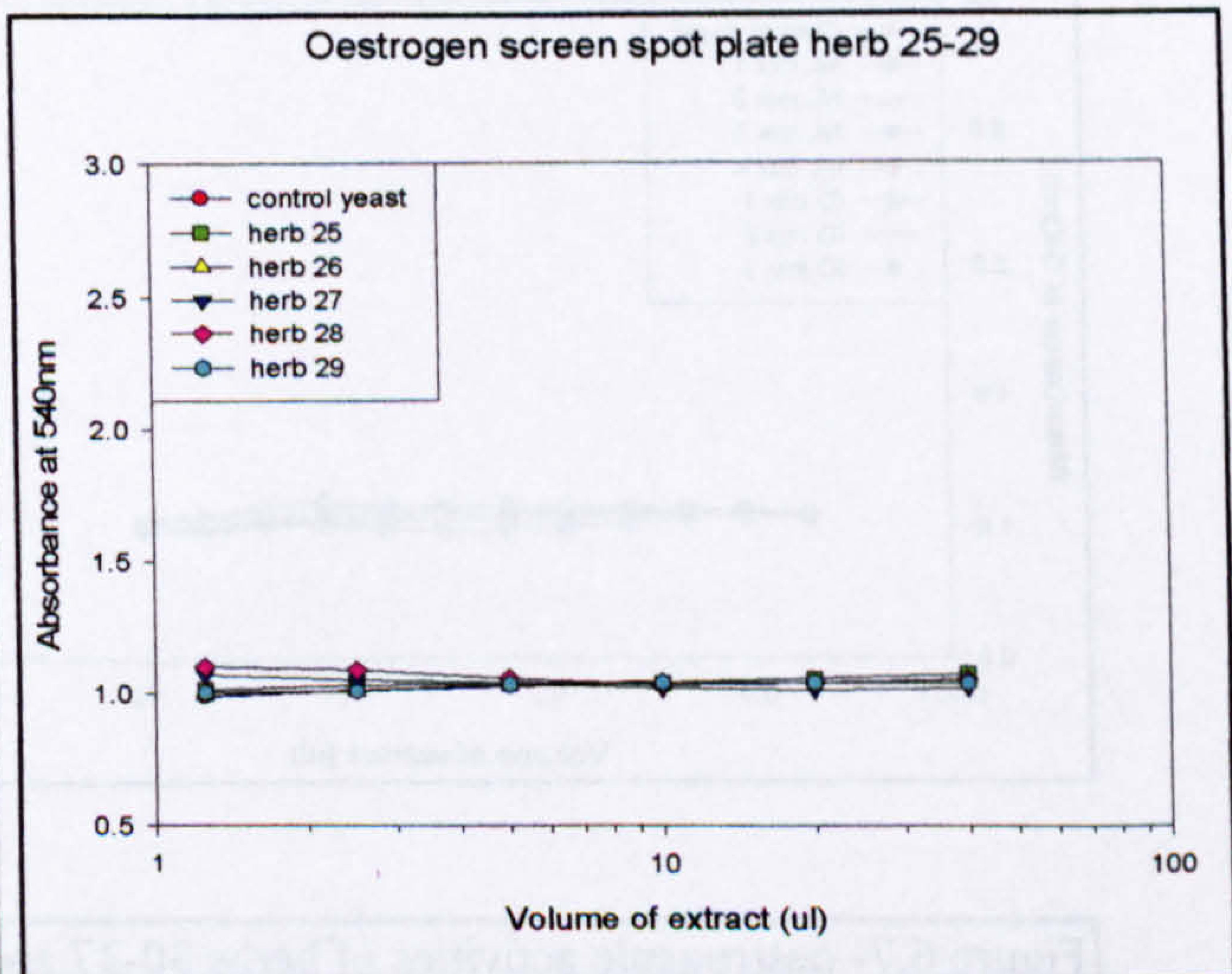
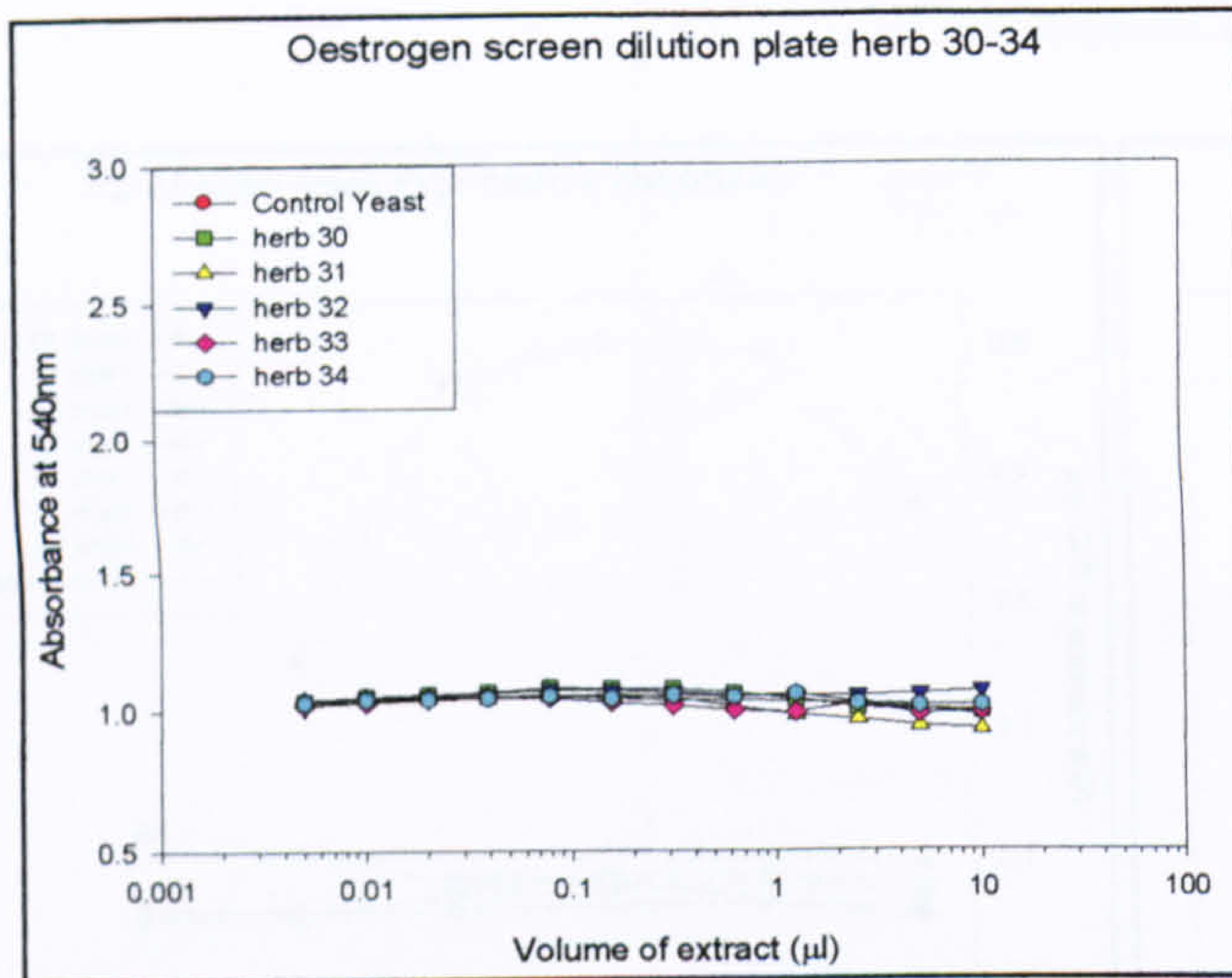
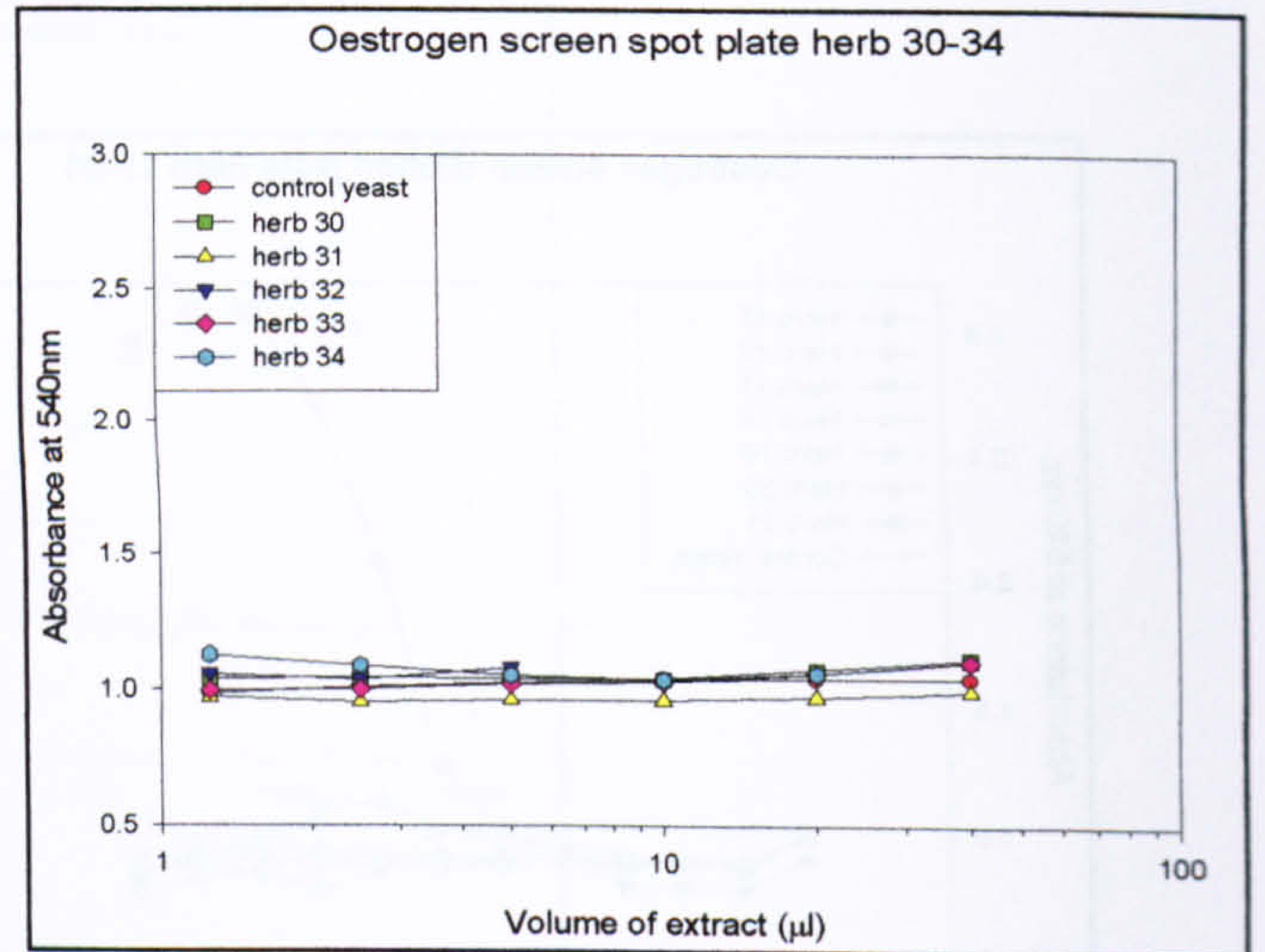
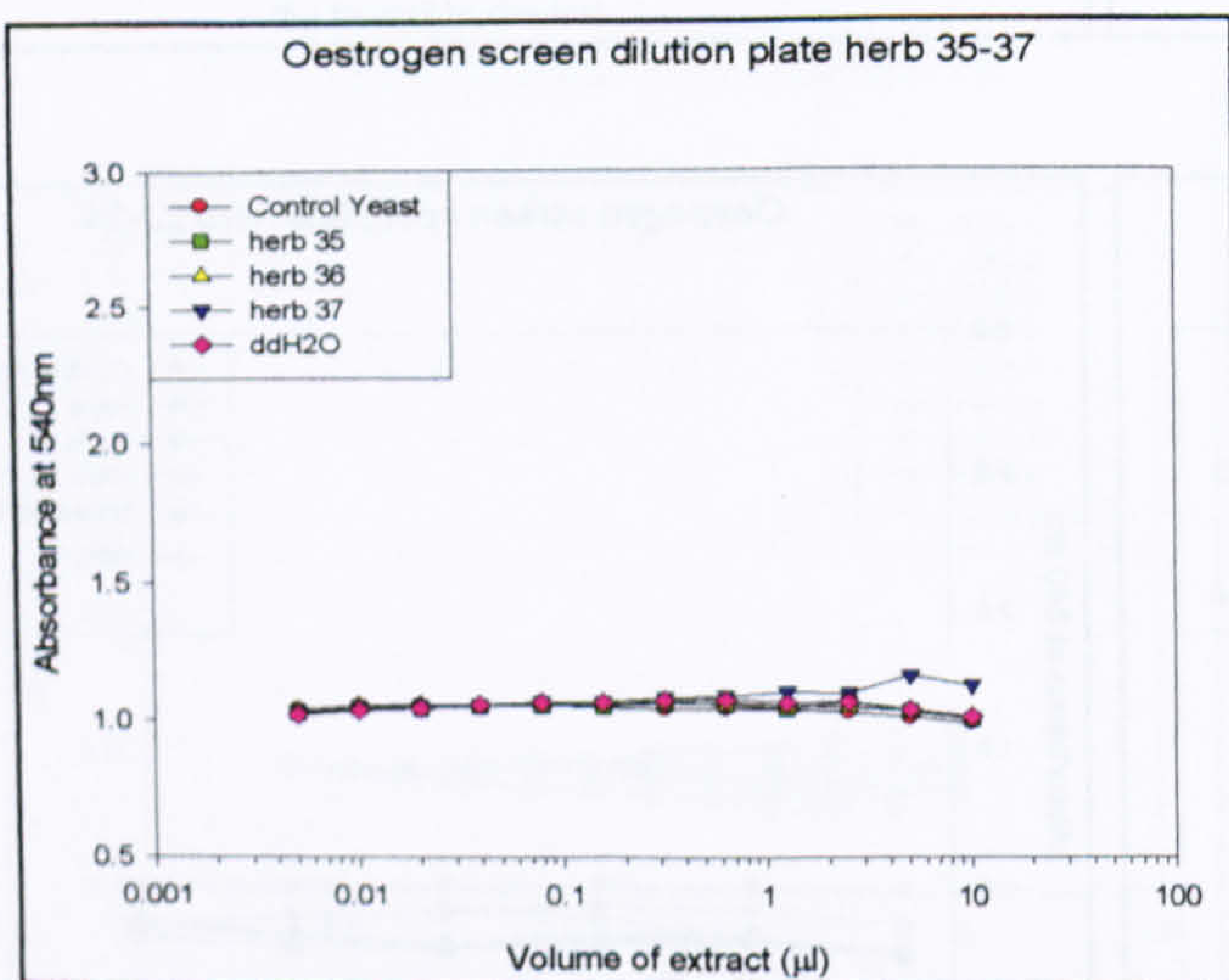
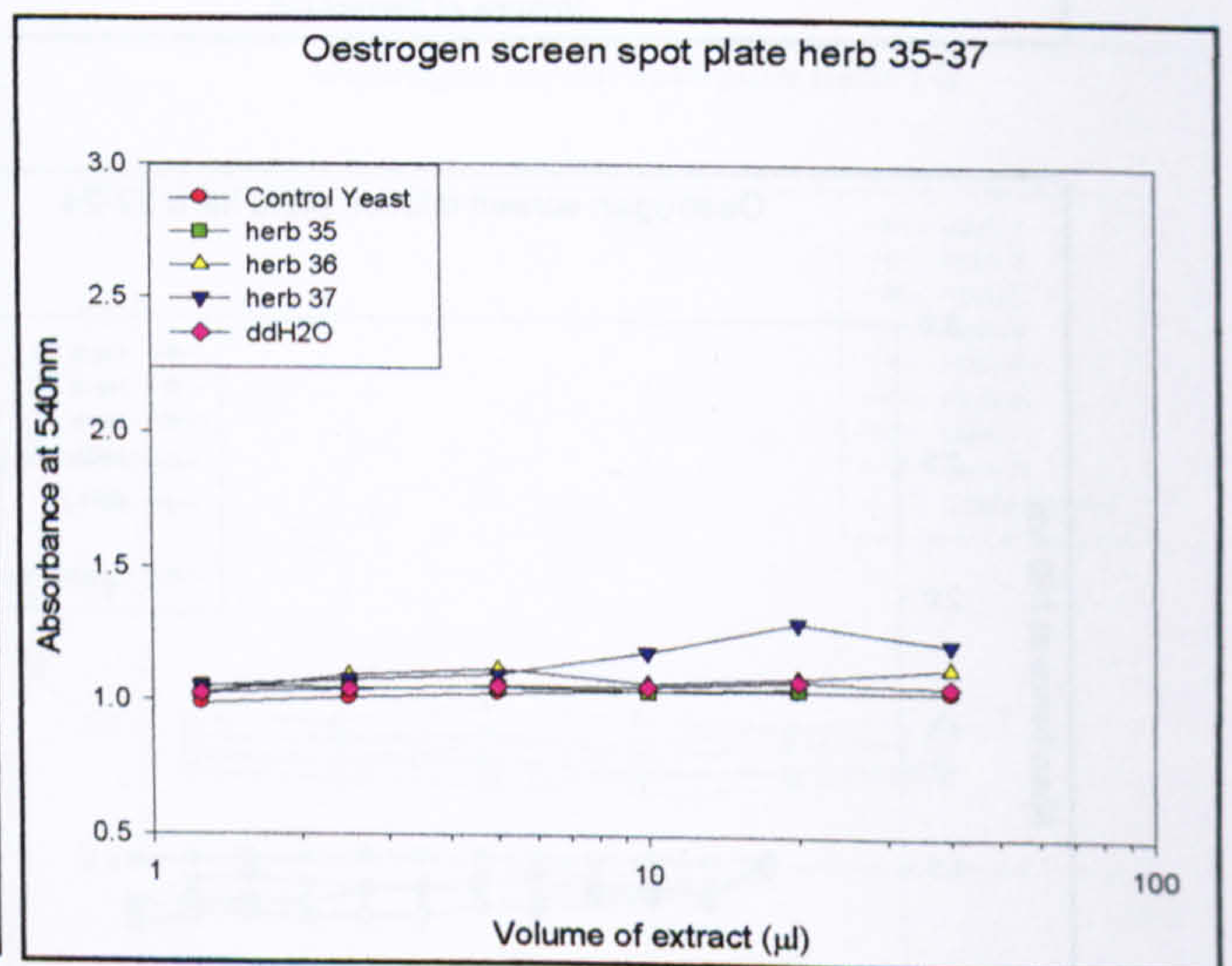


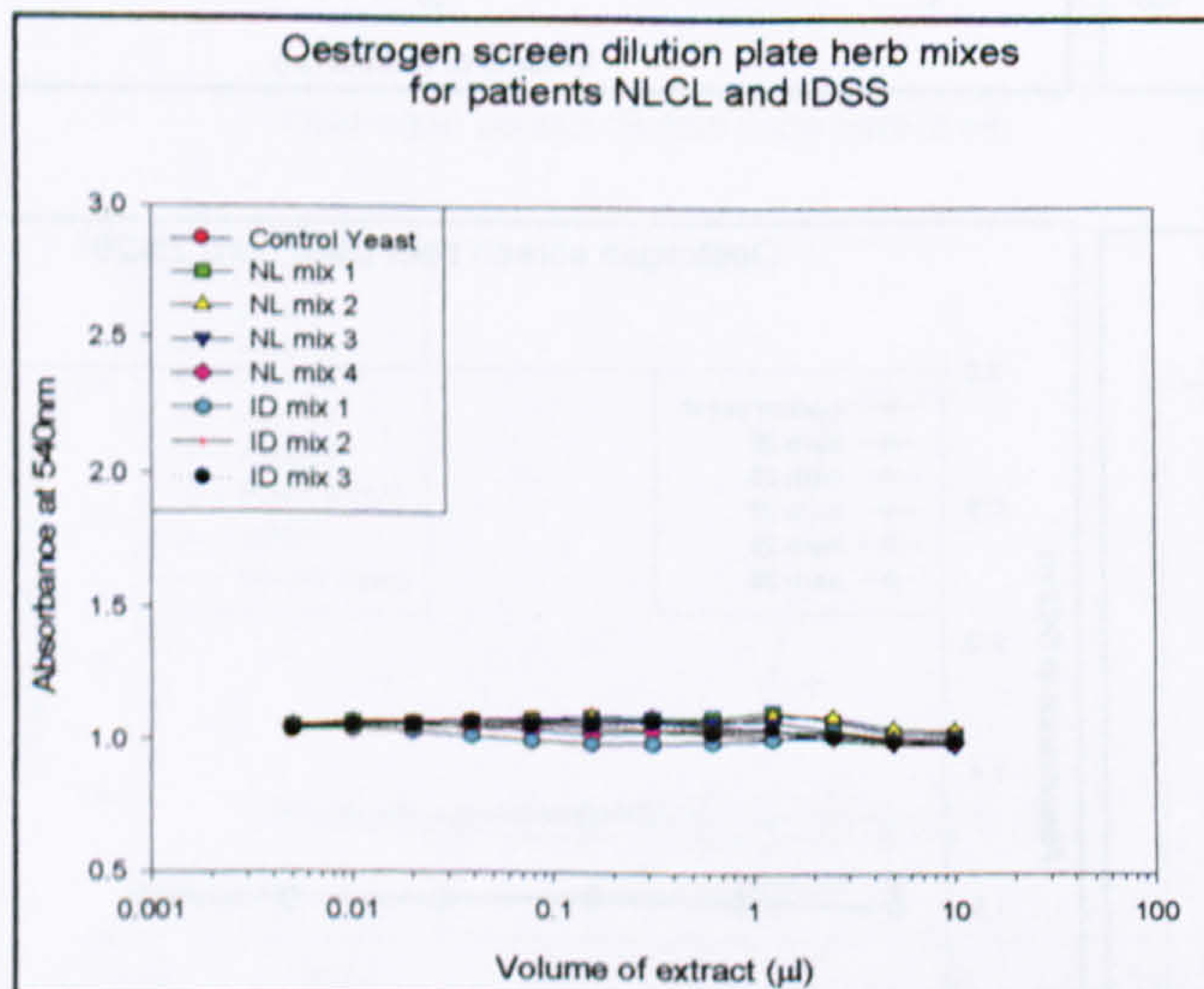
Figure 6.7- oestrogenic activities of herbs 15-29, each herb was assayed at a number of dilutions inluding: $5x10^{-3}$-10ul (dilution plates) and 1.25-40ul (spot plates), responses are compared to those of known concentrations of 17B- oestrodiol











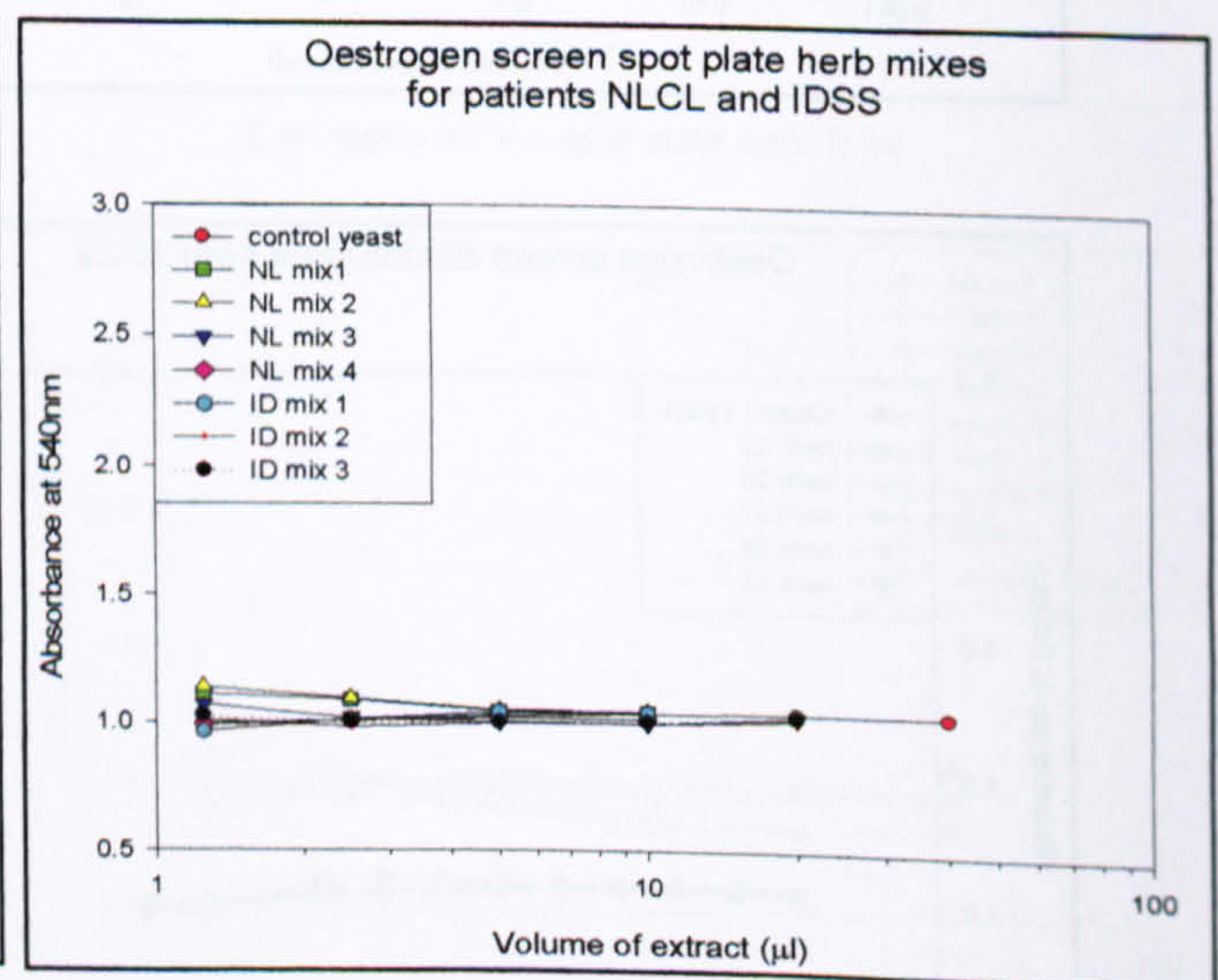


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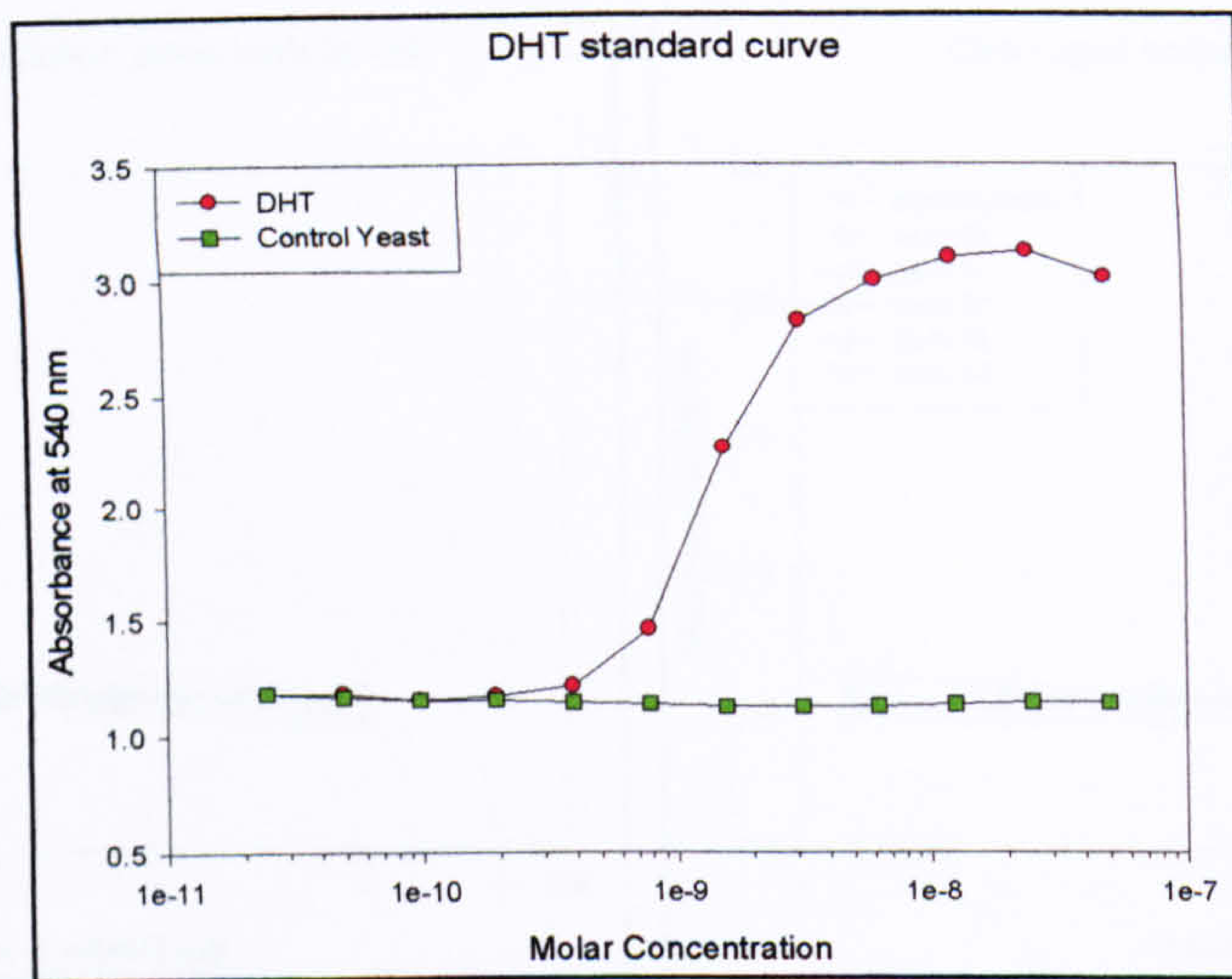
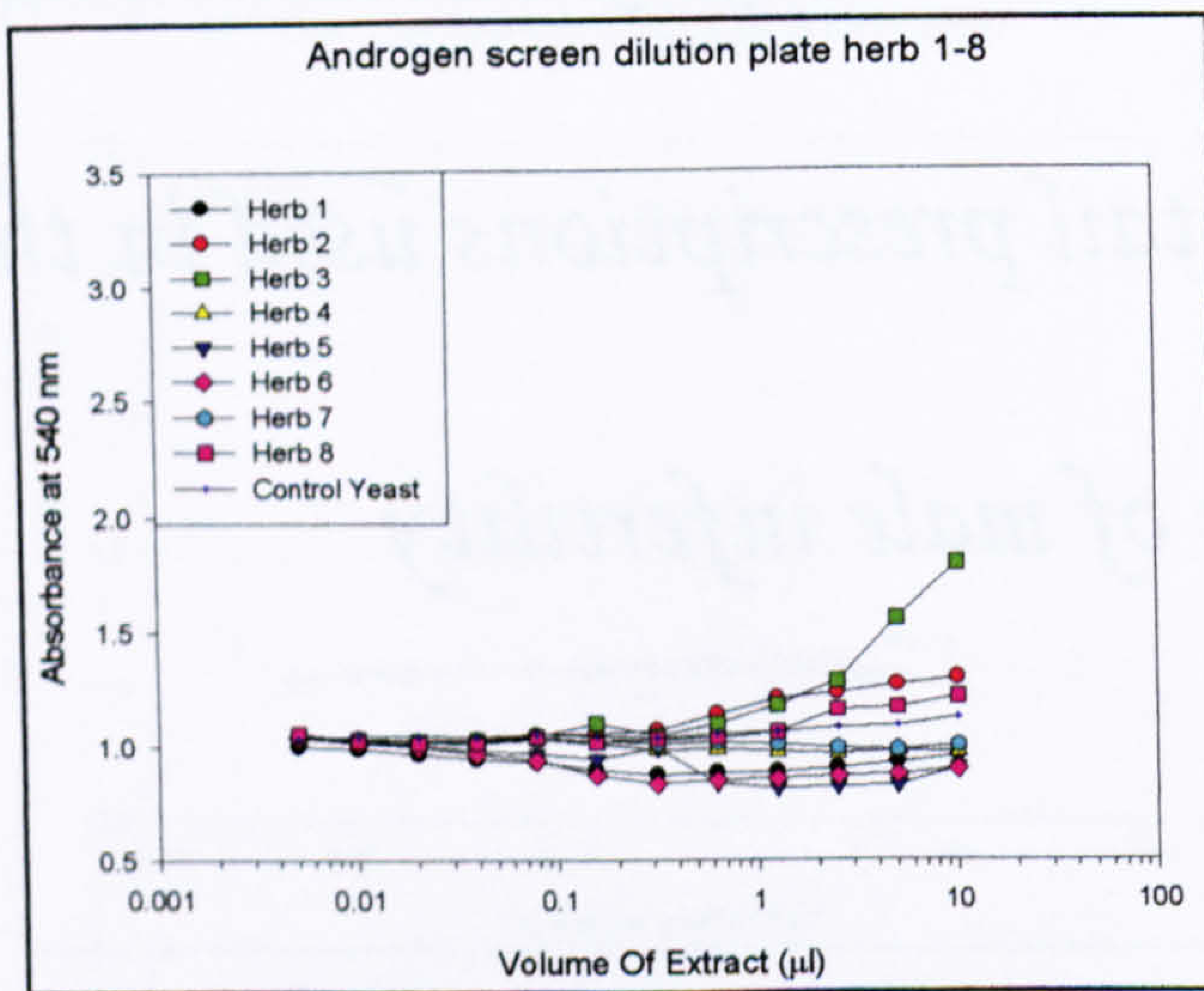
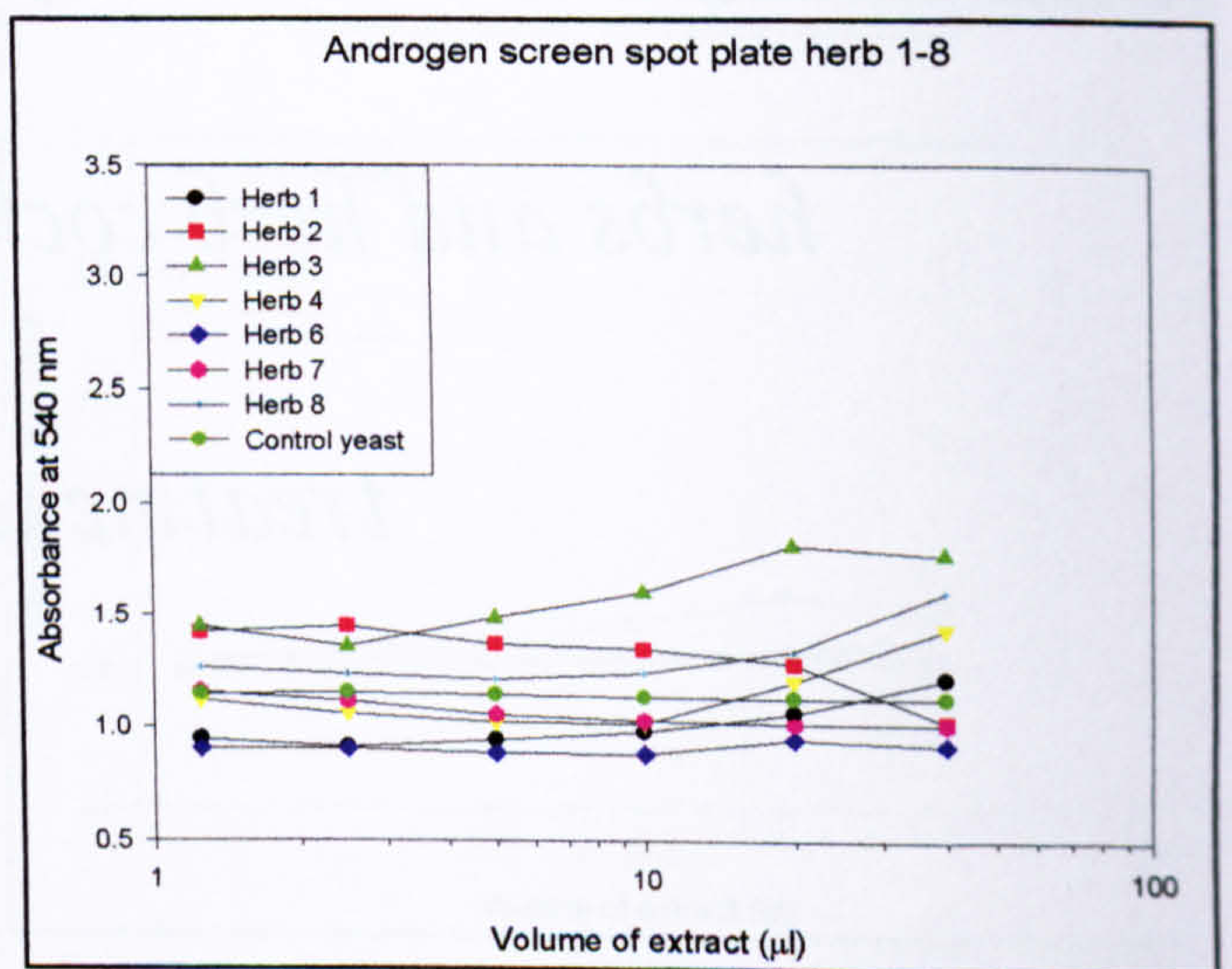
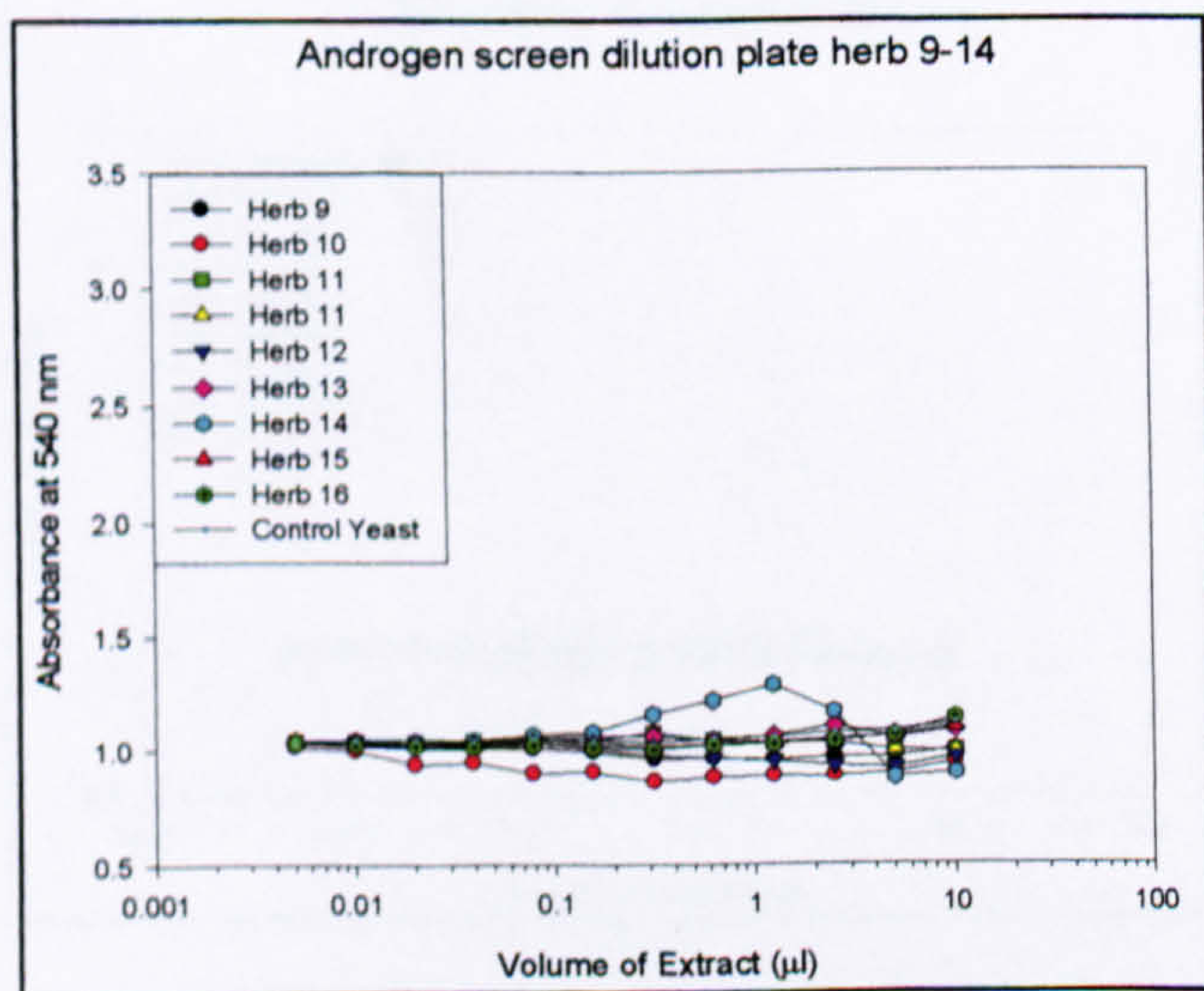
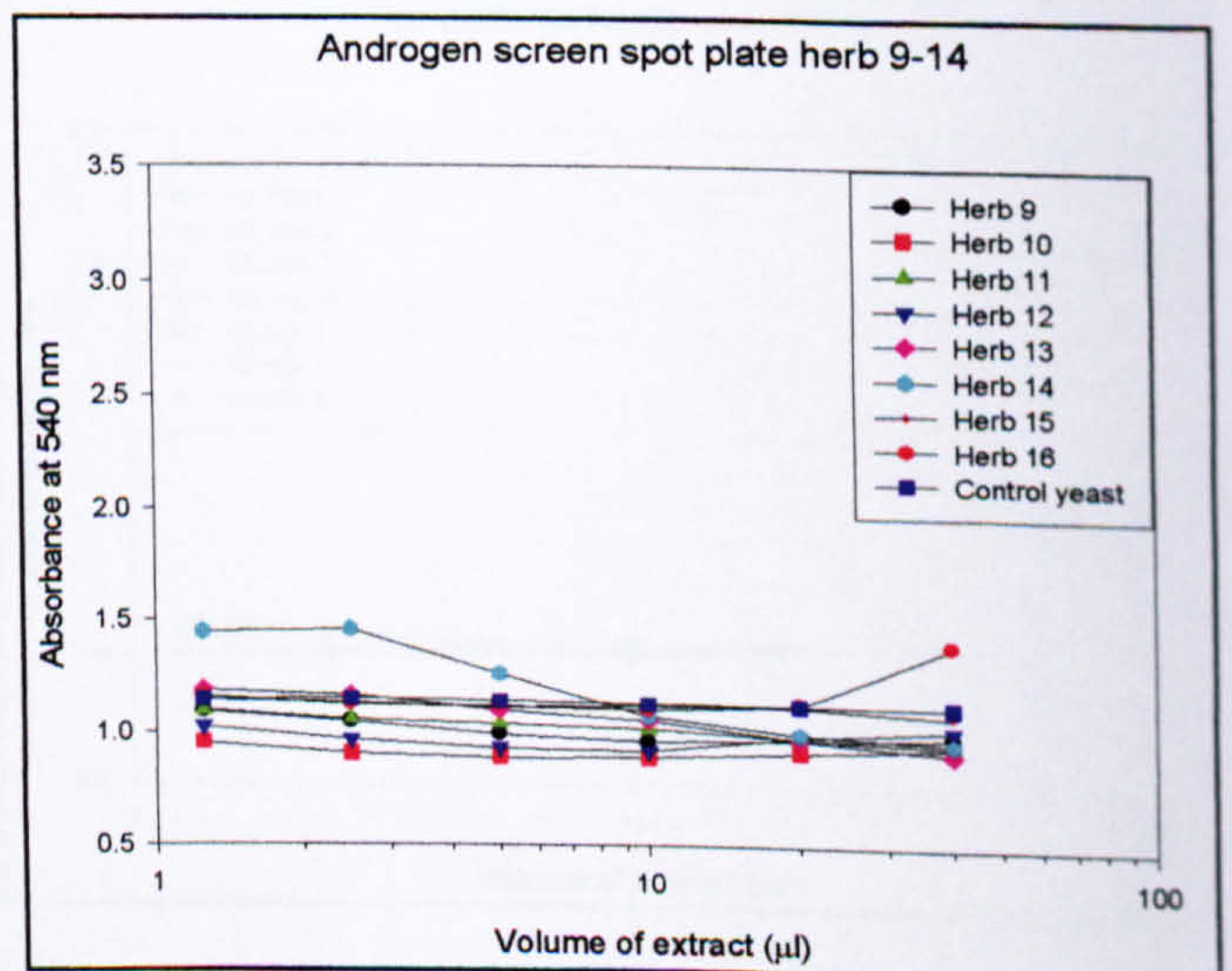
Figure 5.8- androgenic activity of the individual herbs and herb cocktail prescriptions used in the treatment of male infertility











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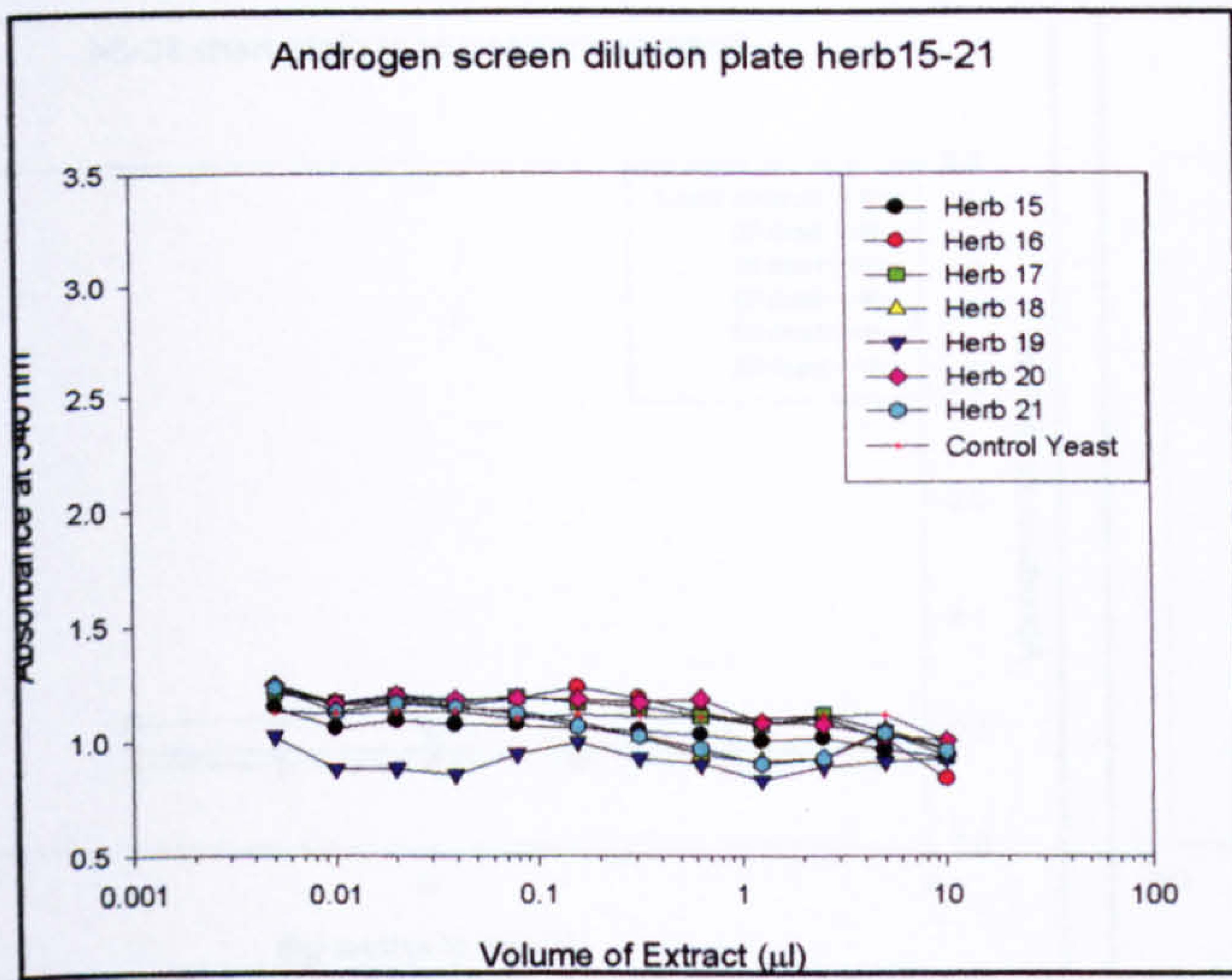

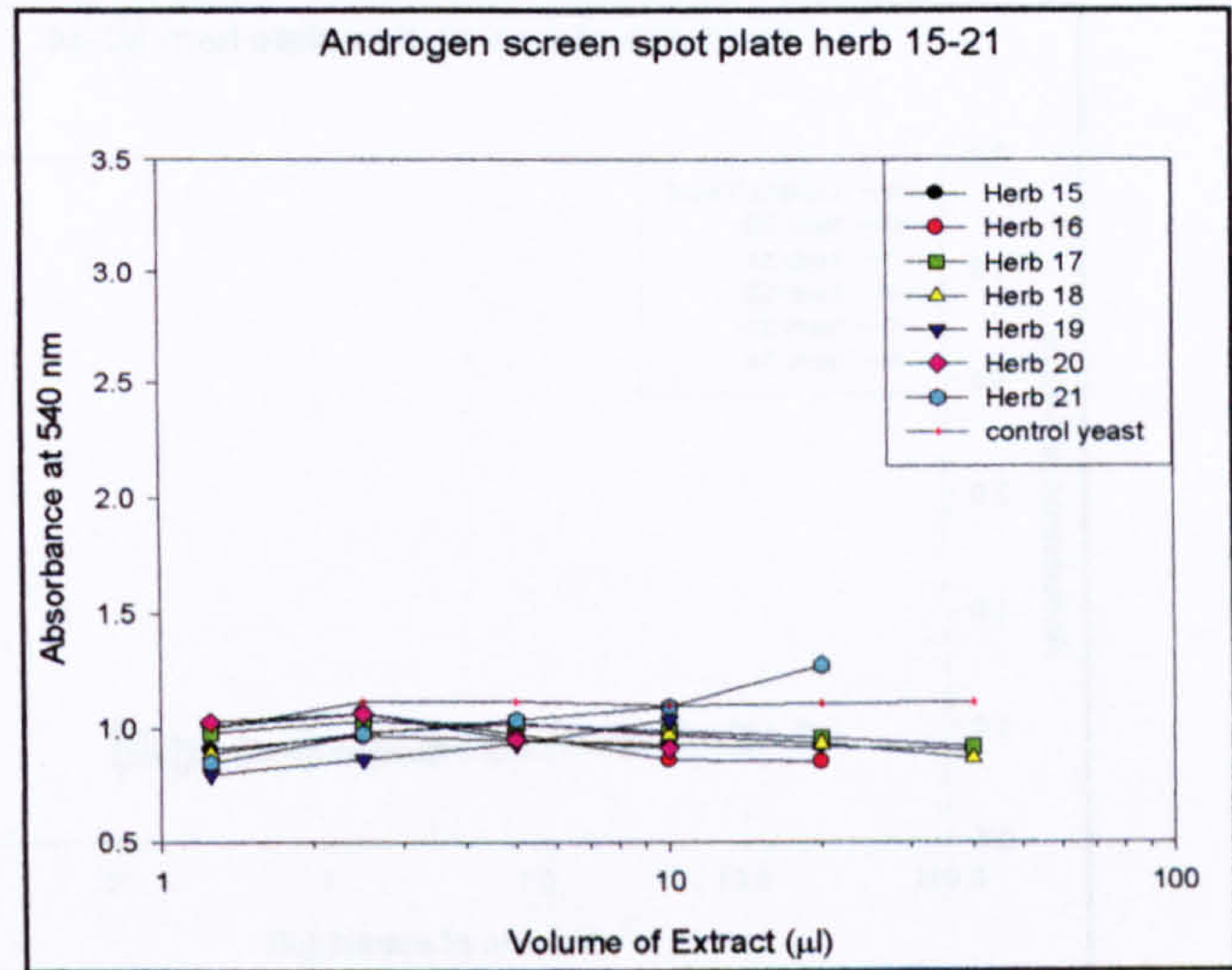

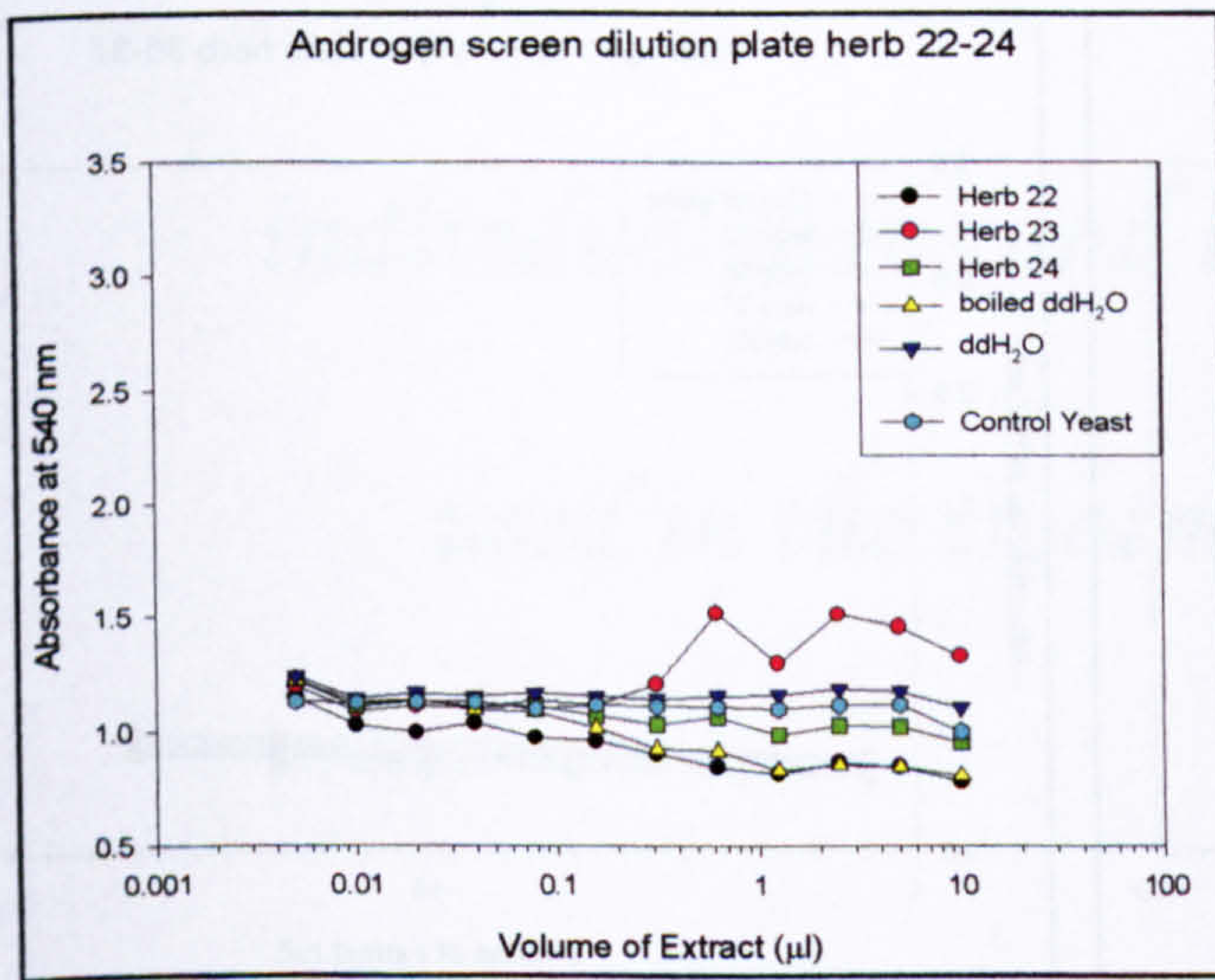

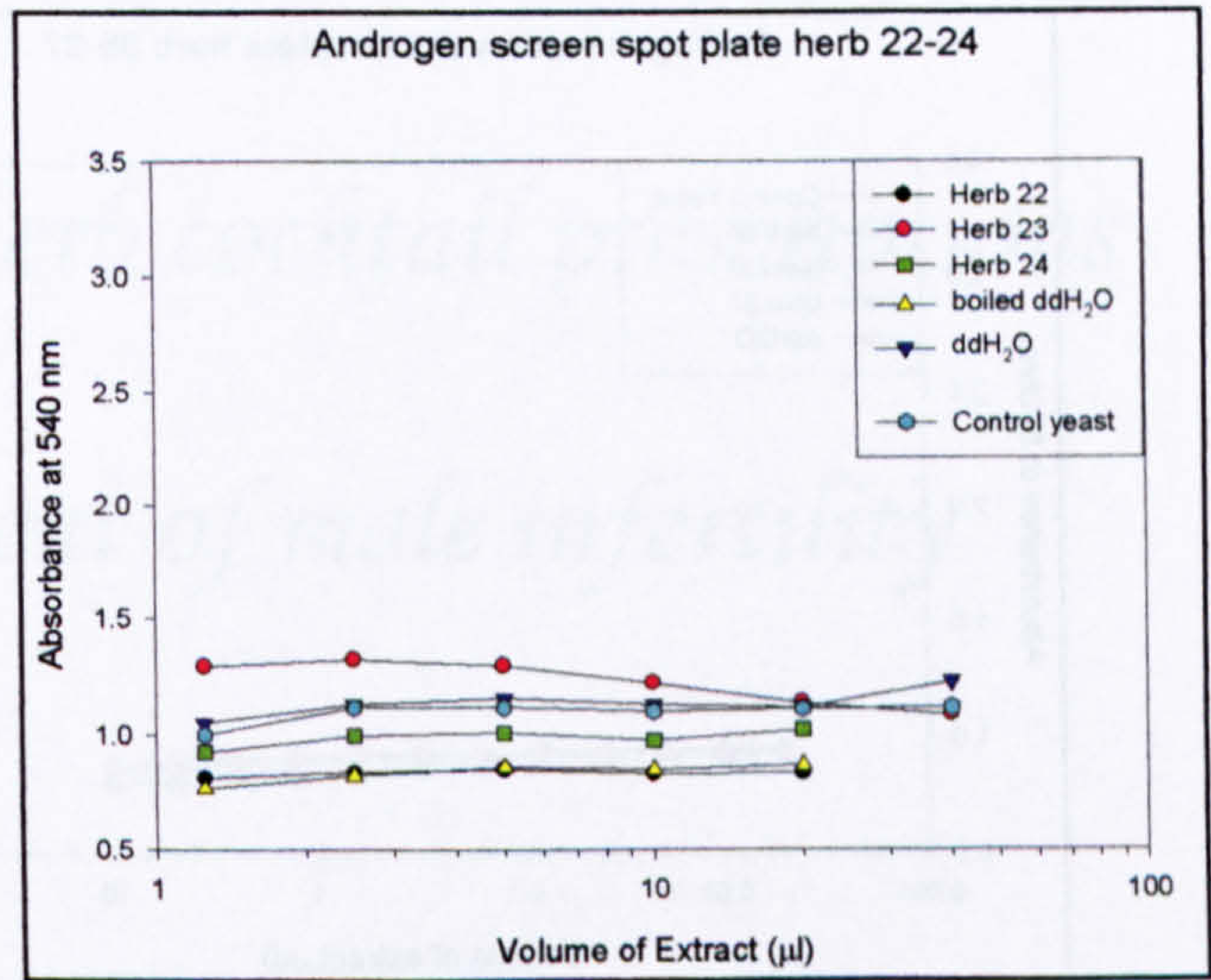

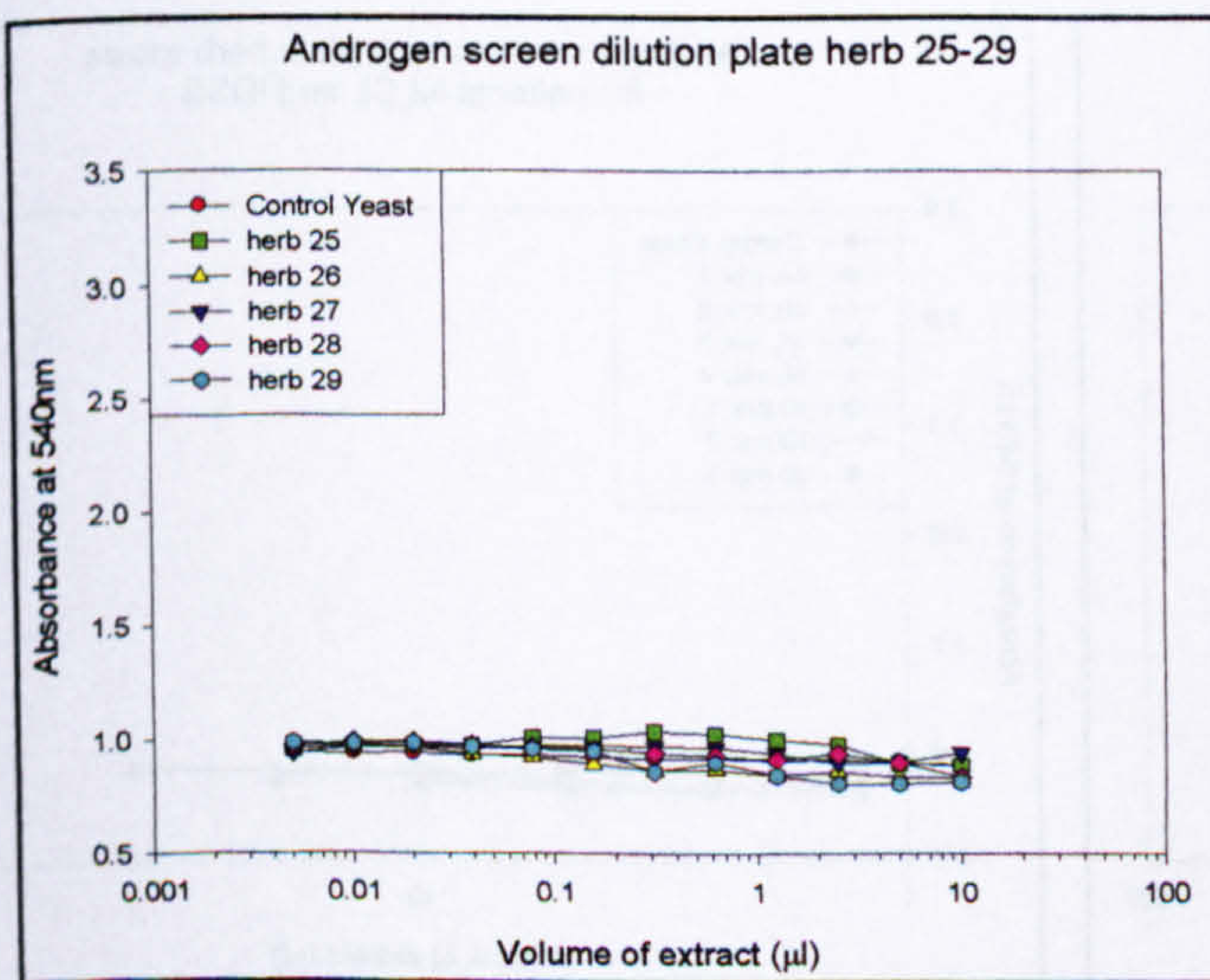

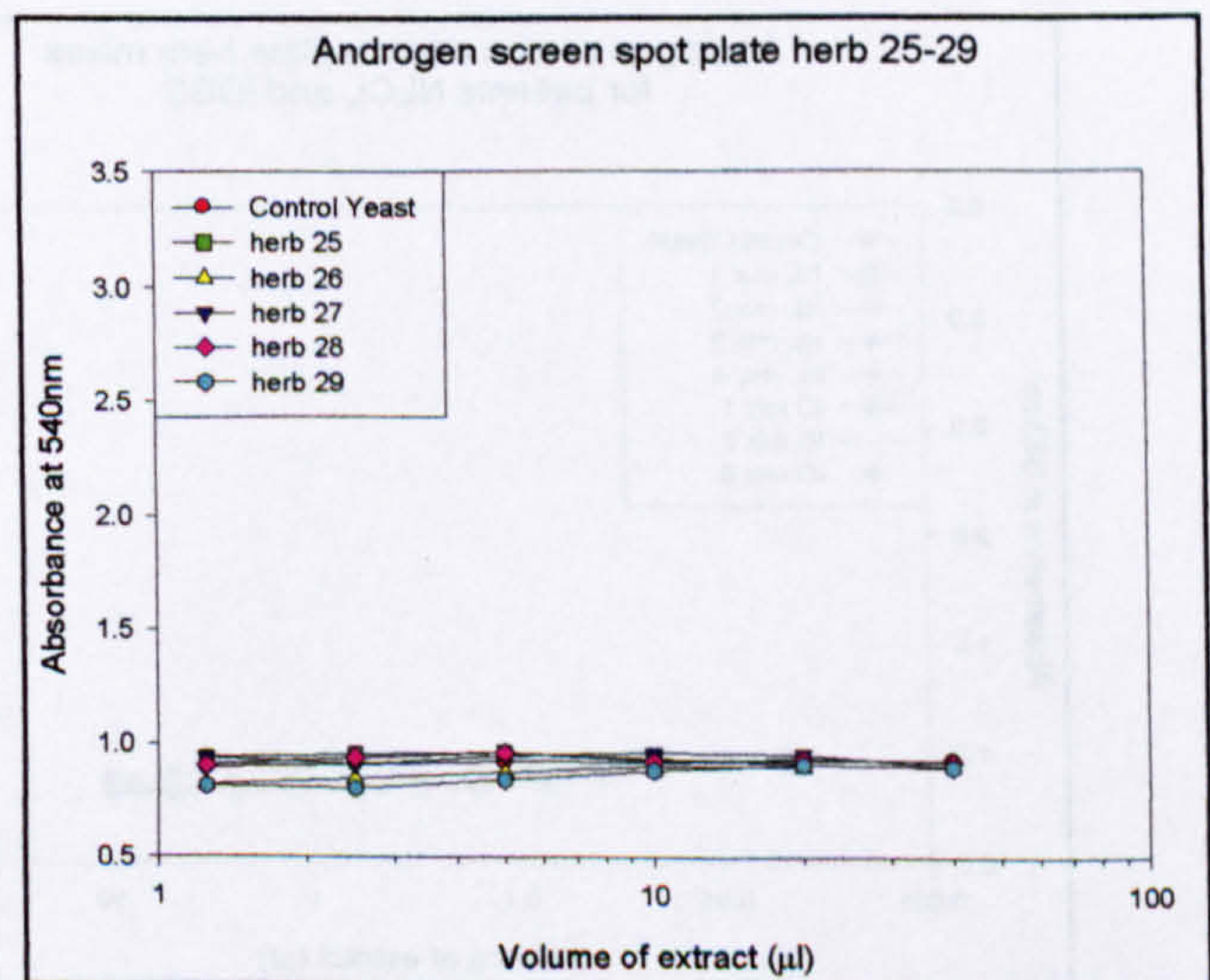

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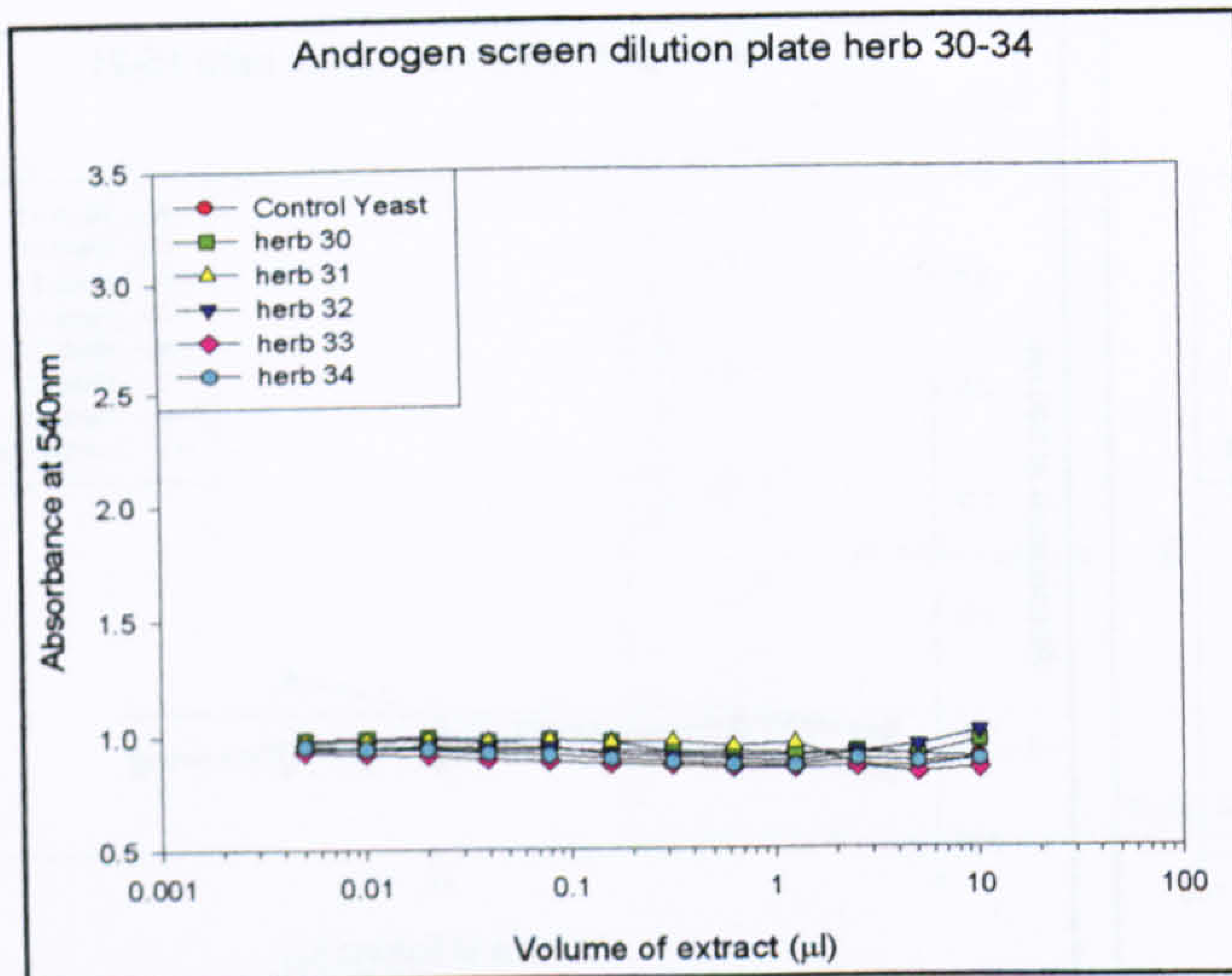

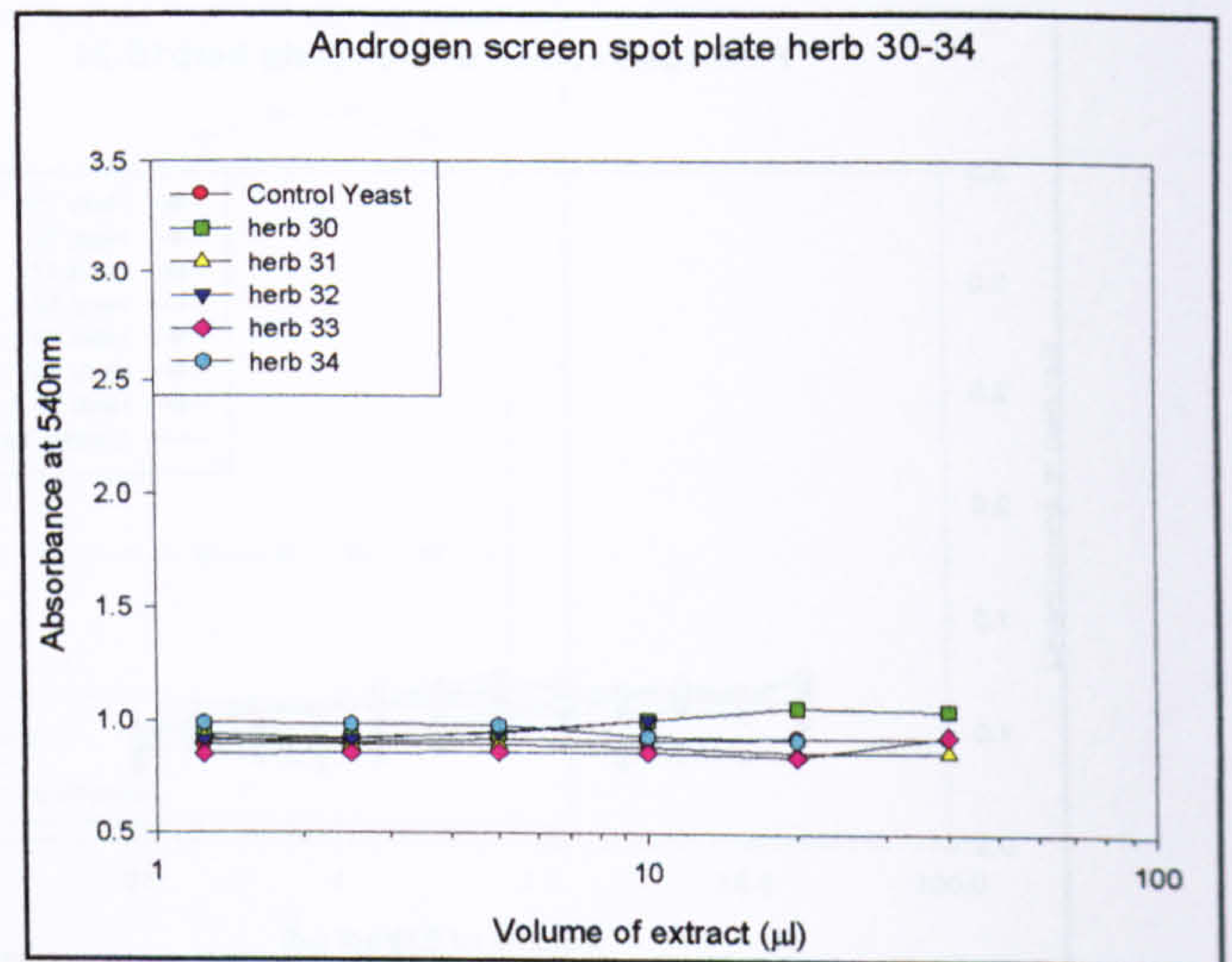

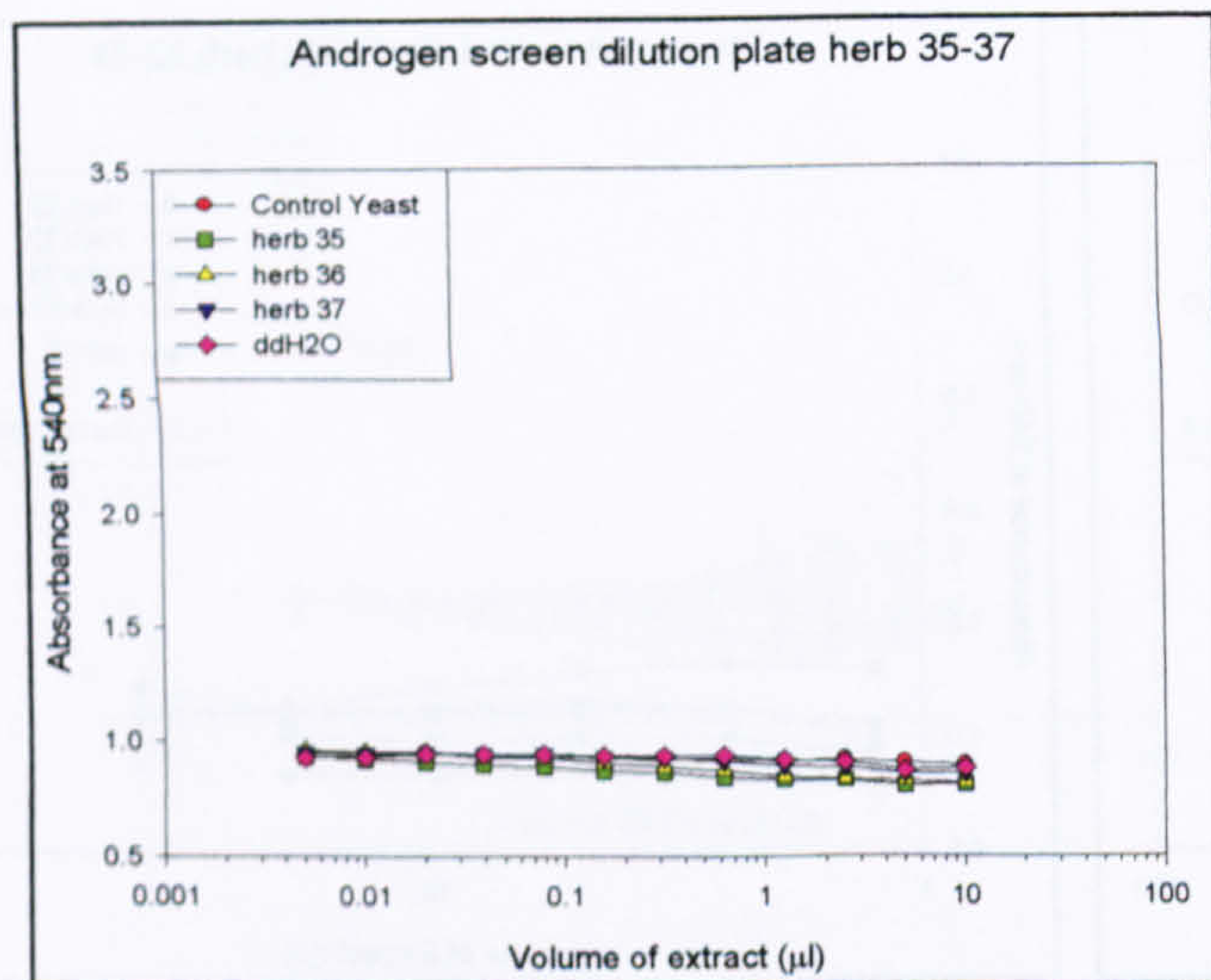

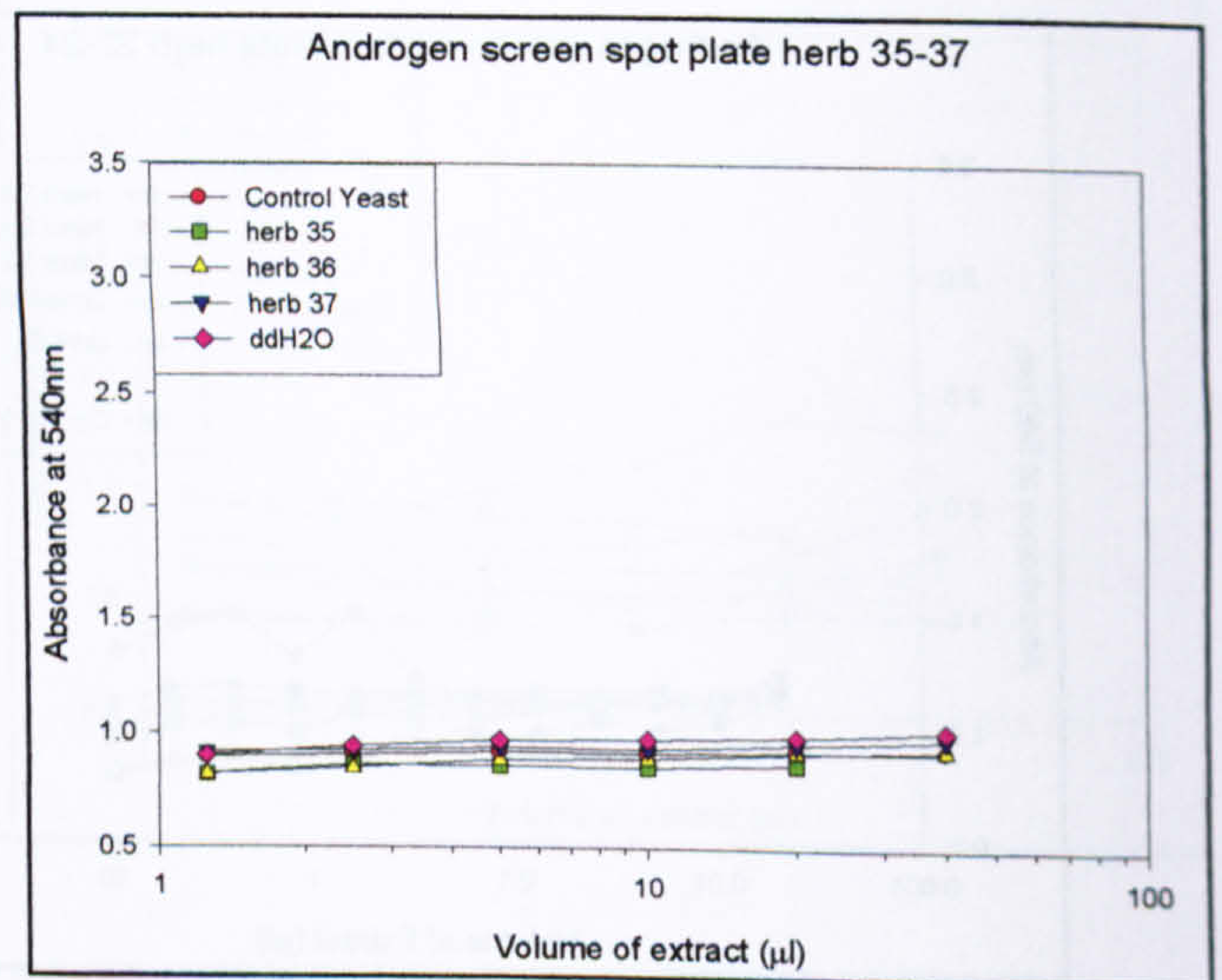

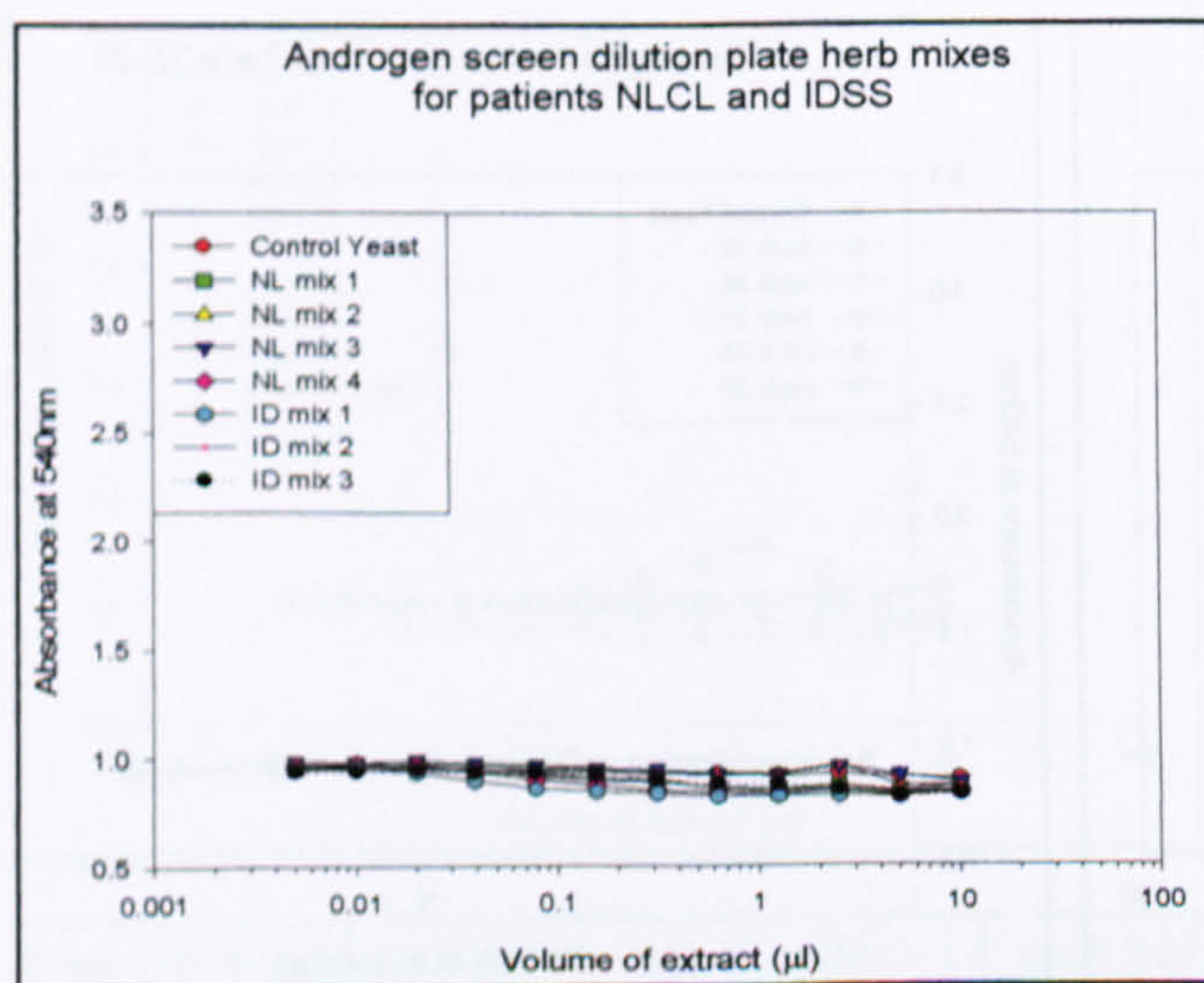

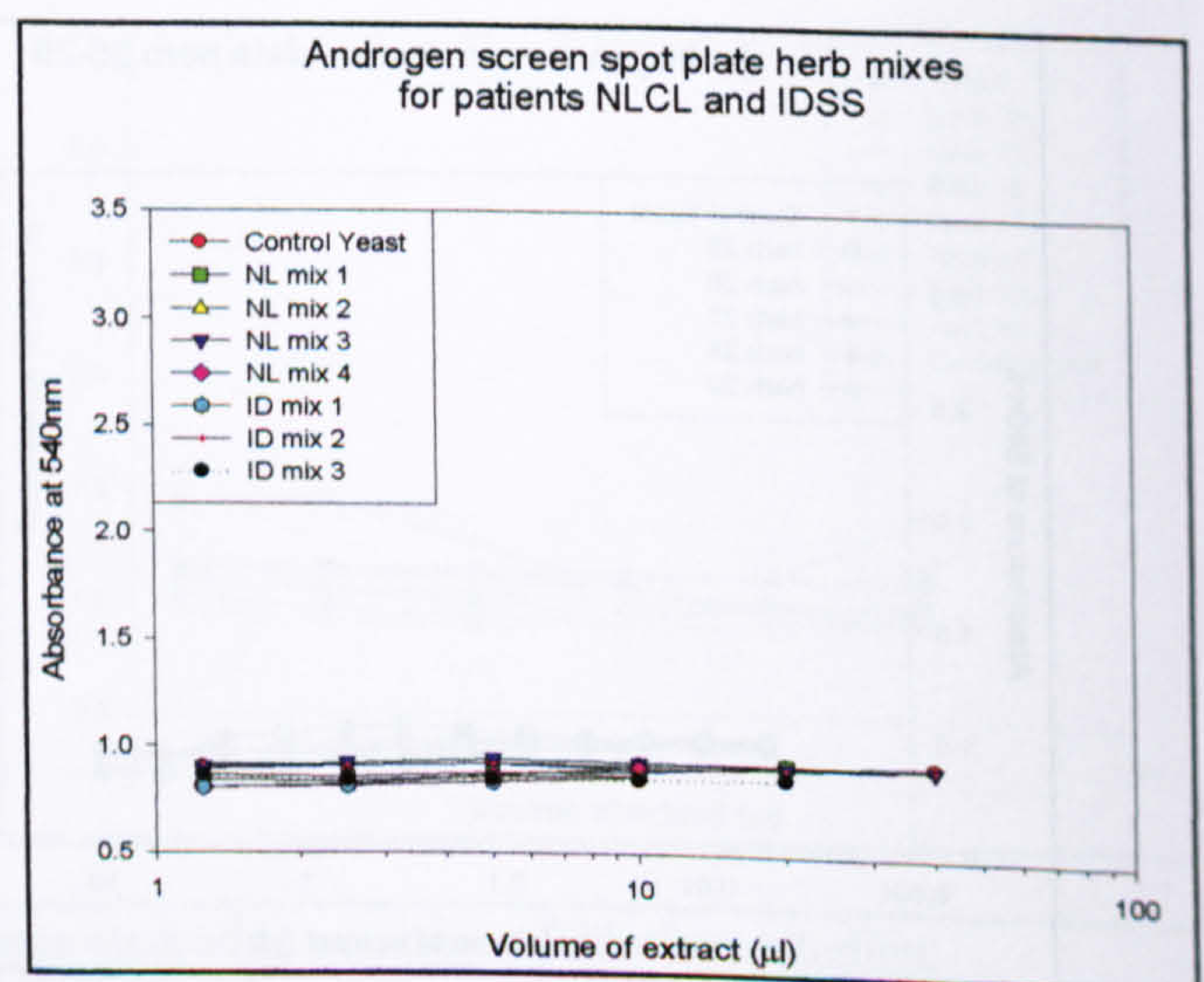

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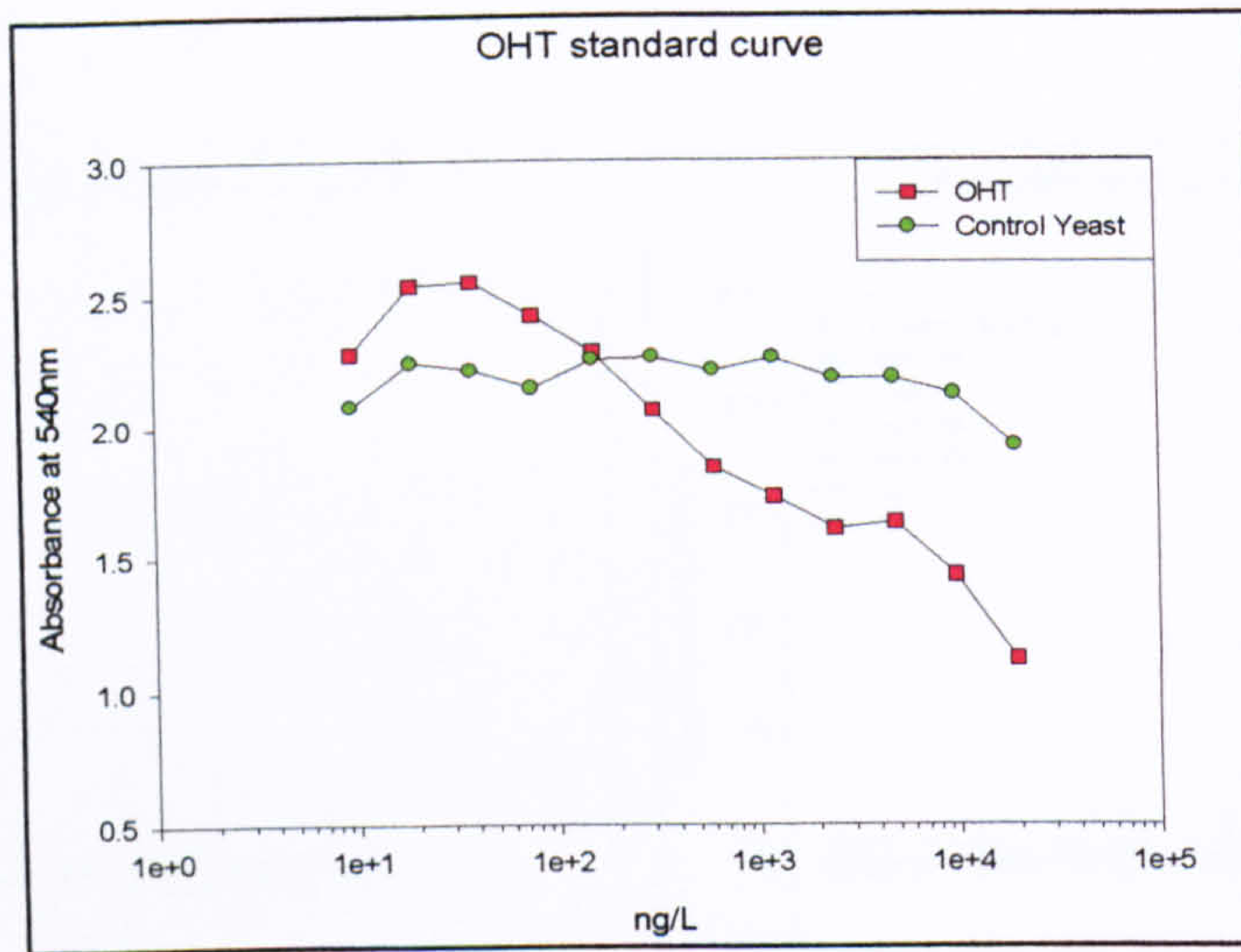
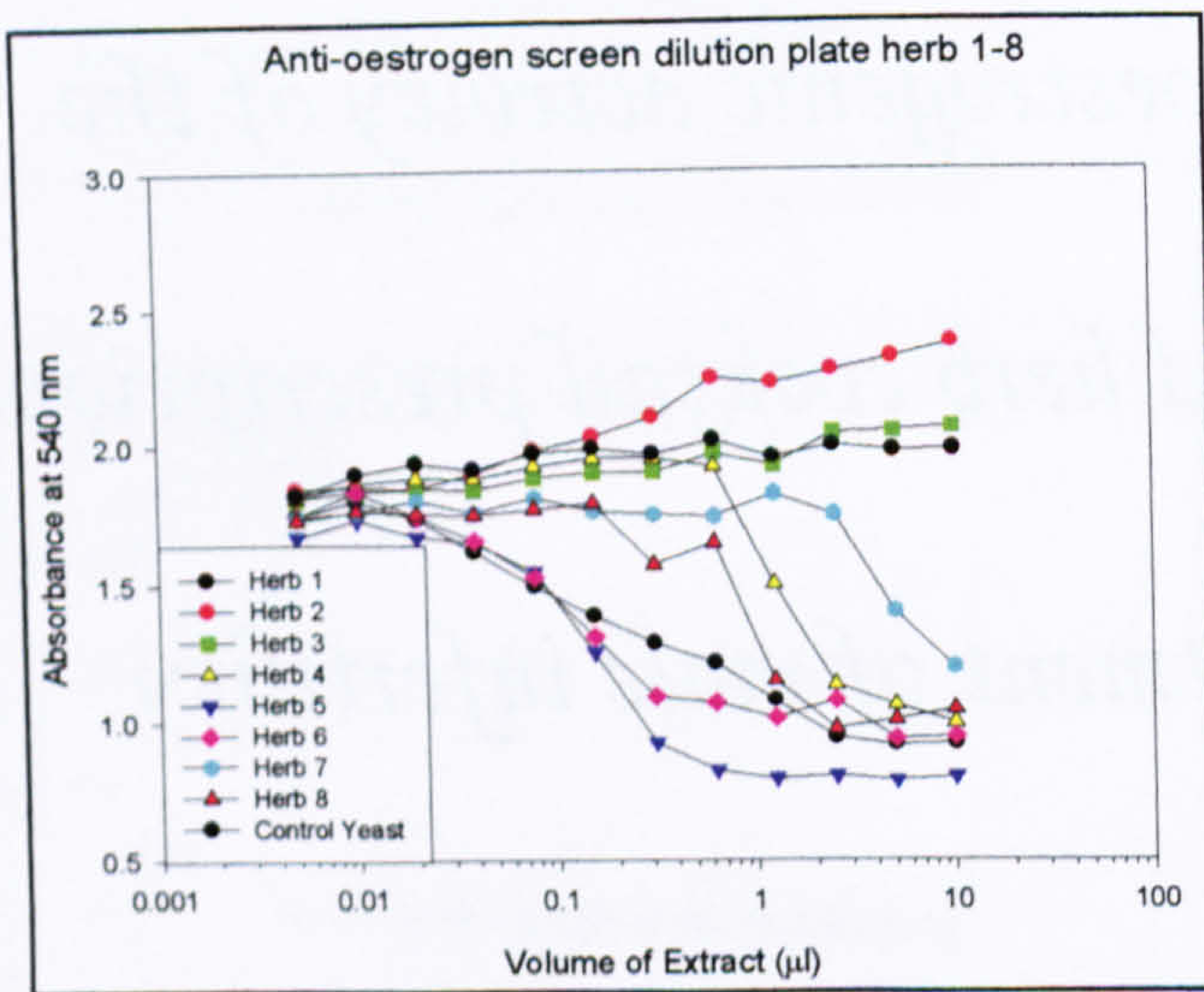
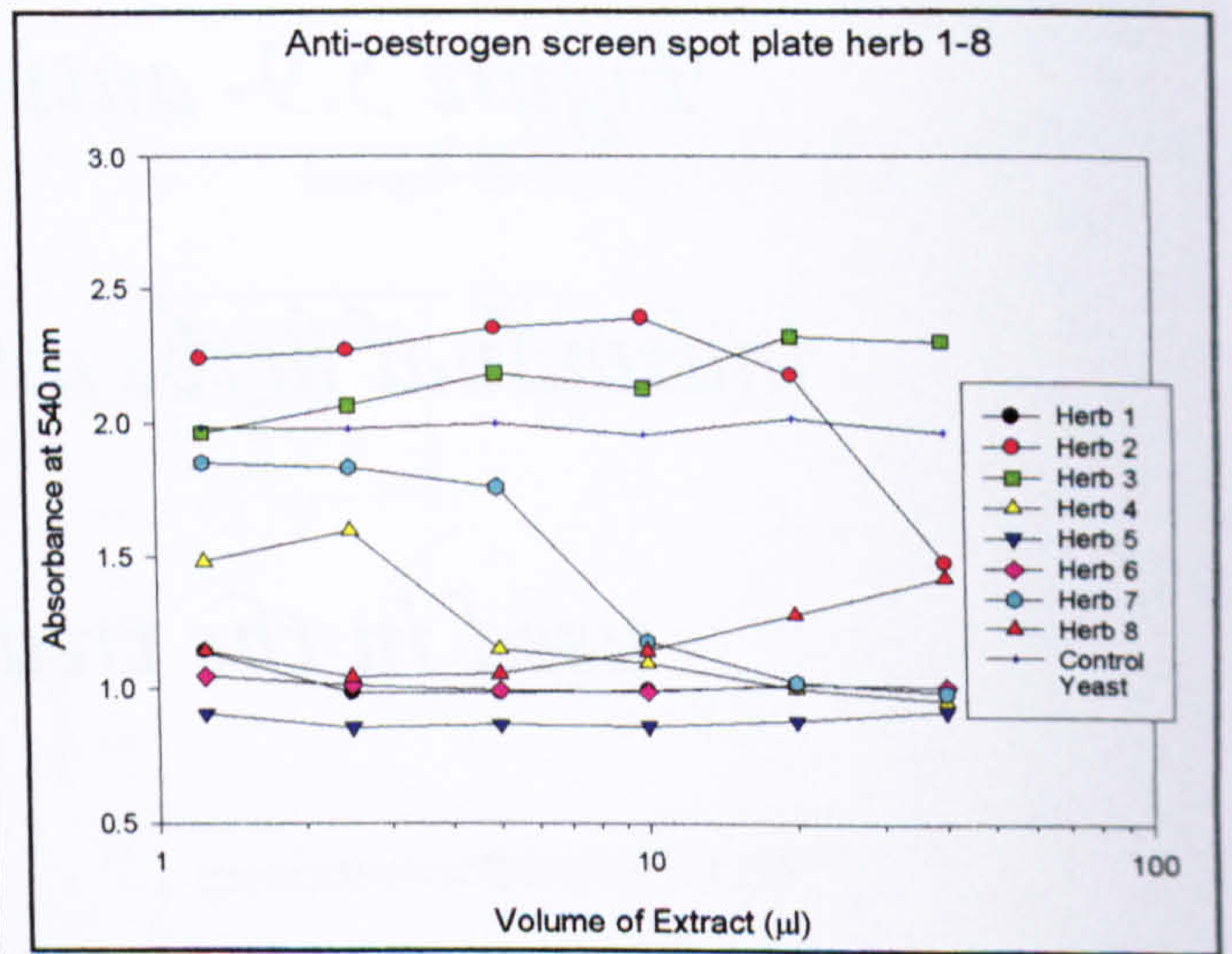
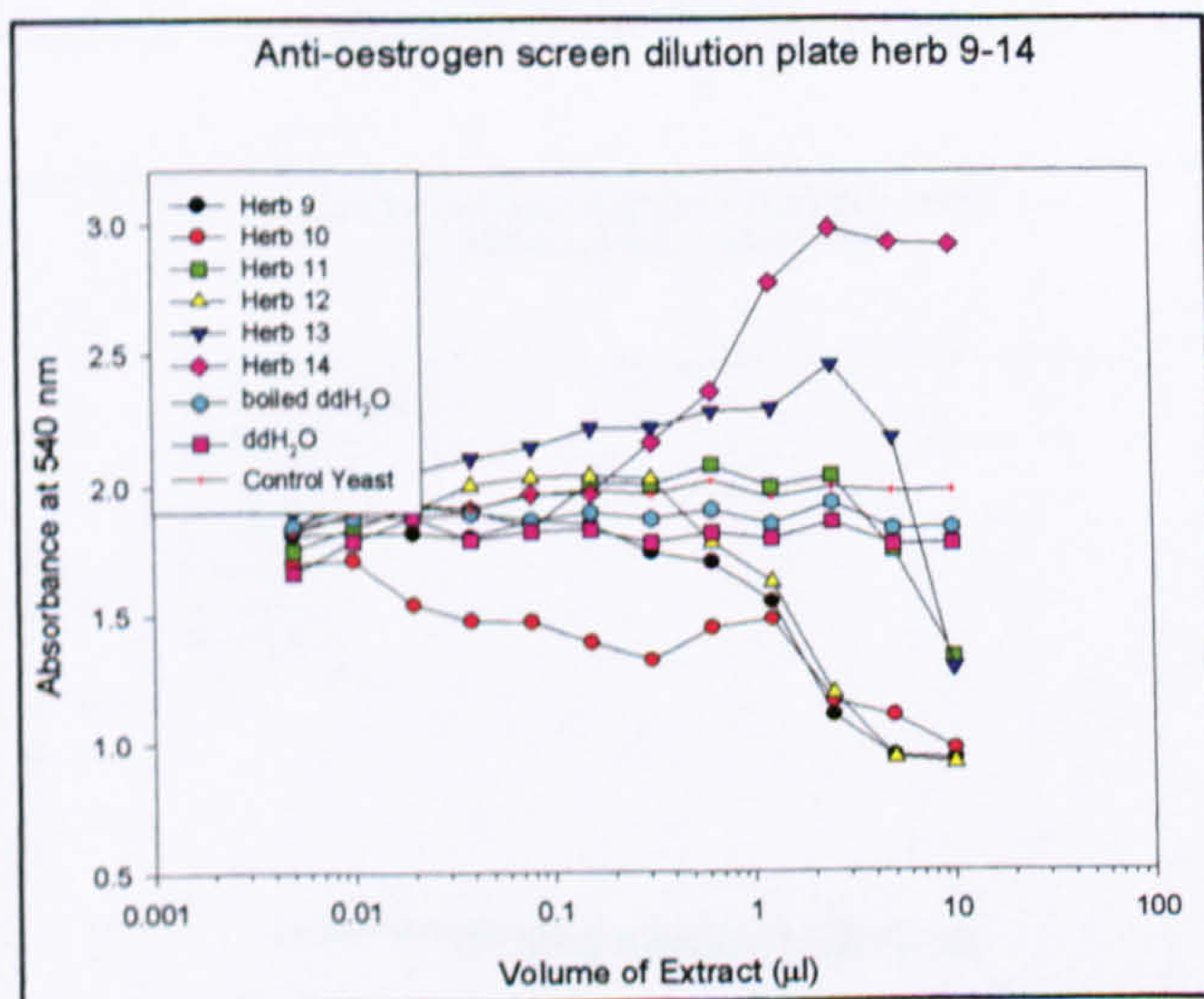
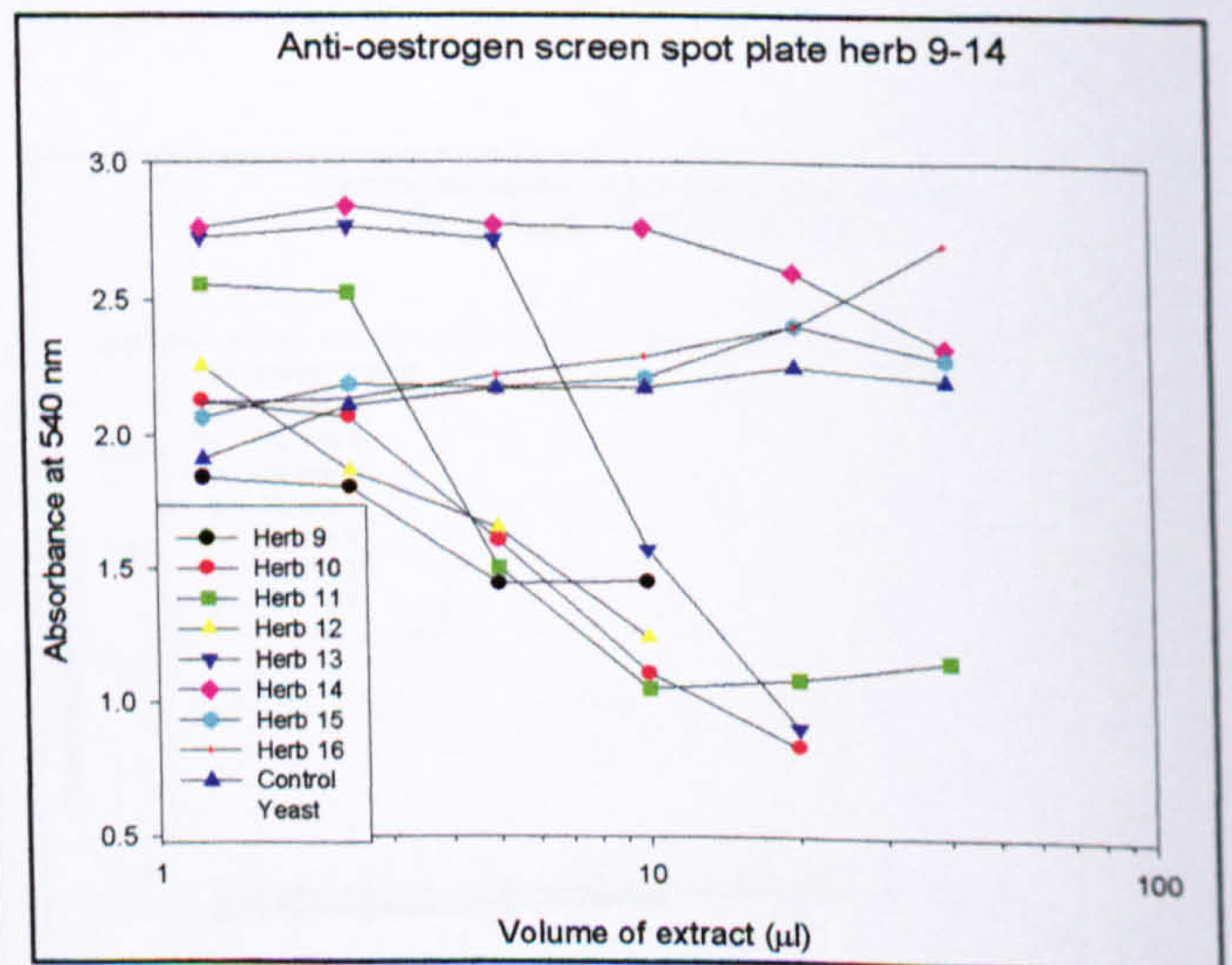
Figure 5.9- anti-oestrogenic activity of the individual herbs and herb cocktail prescriptions used in the treatment of male infertility











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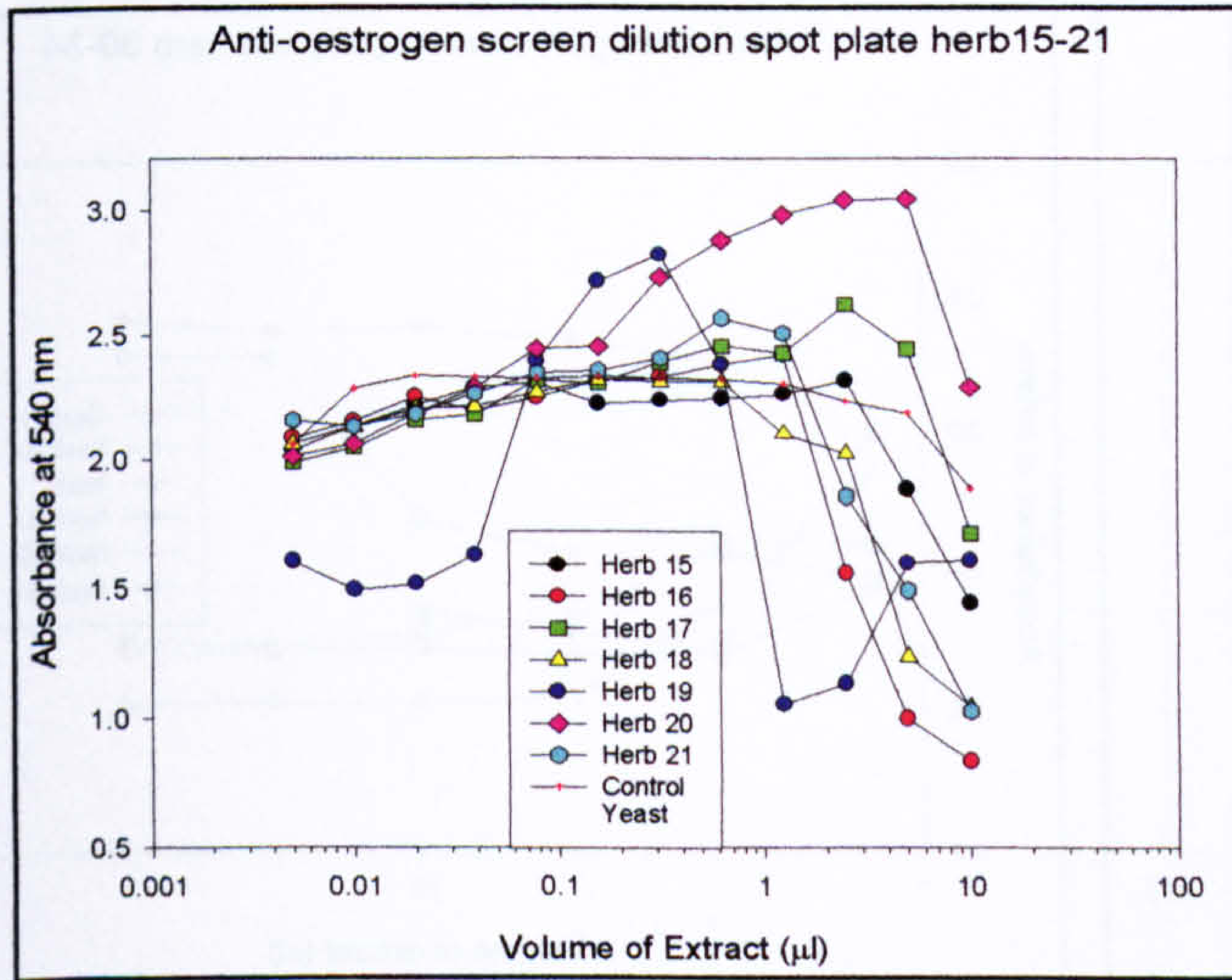

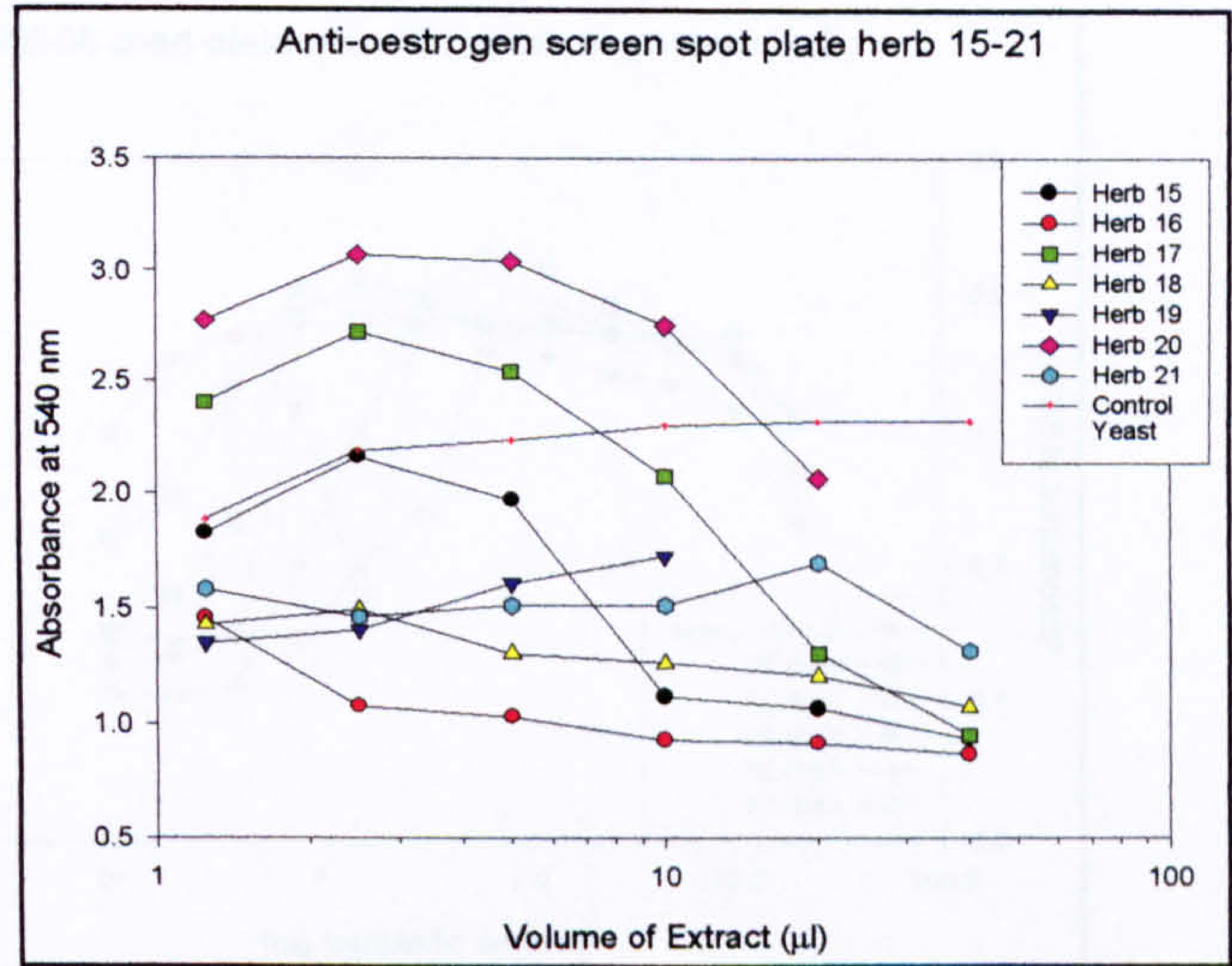

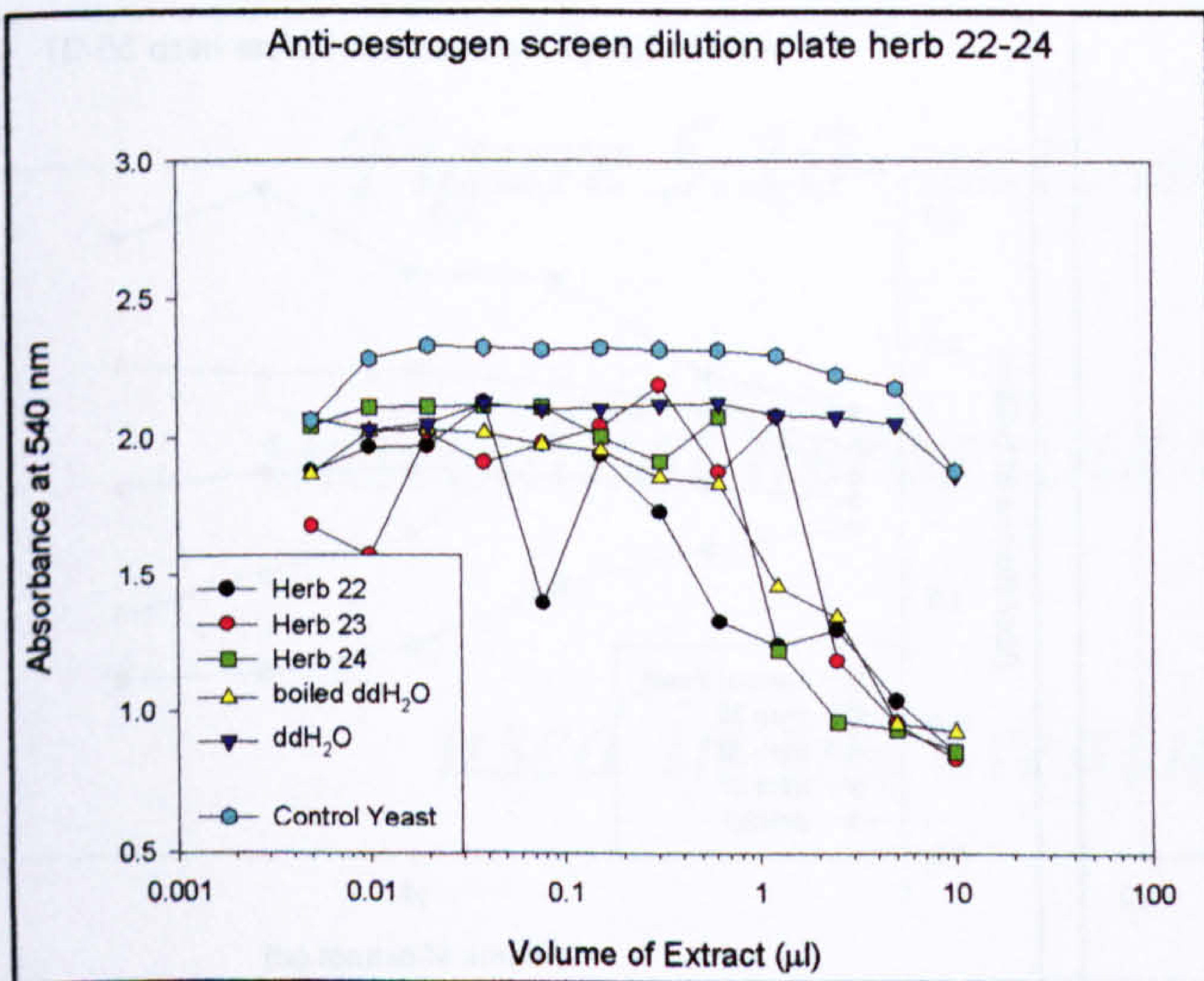

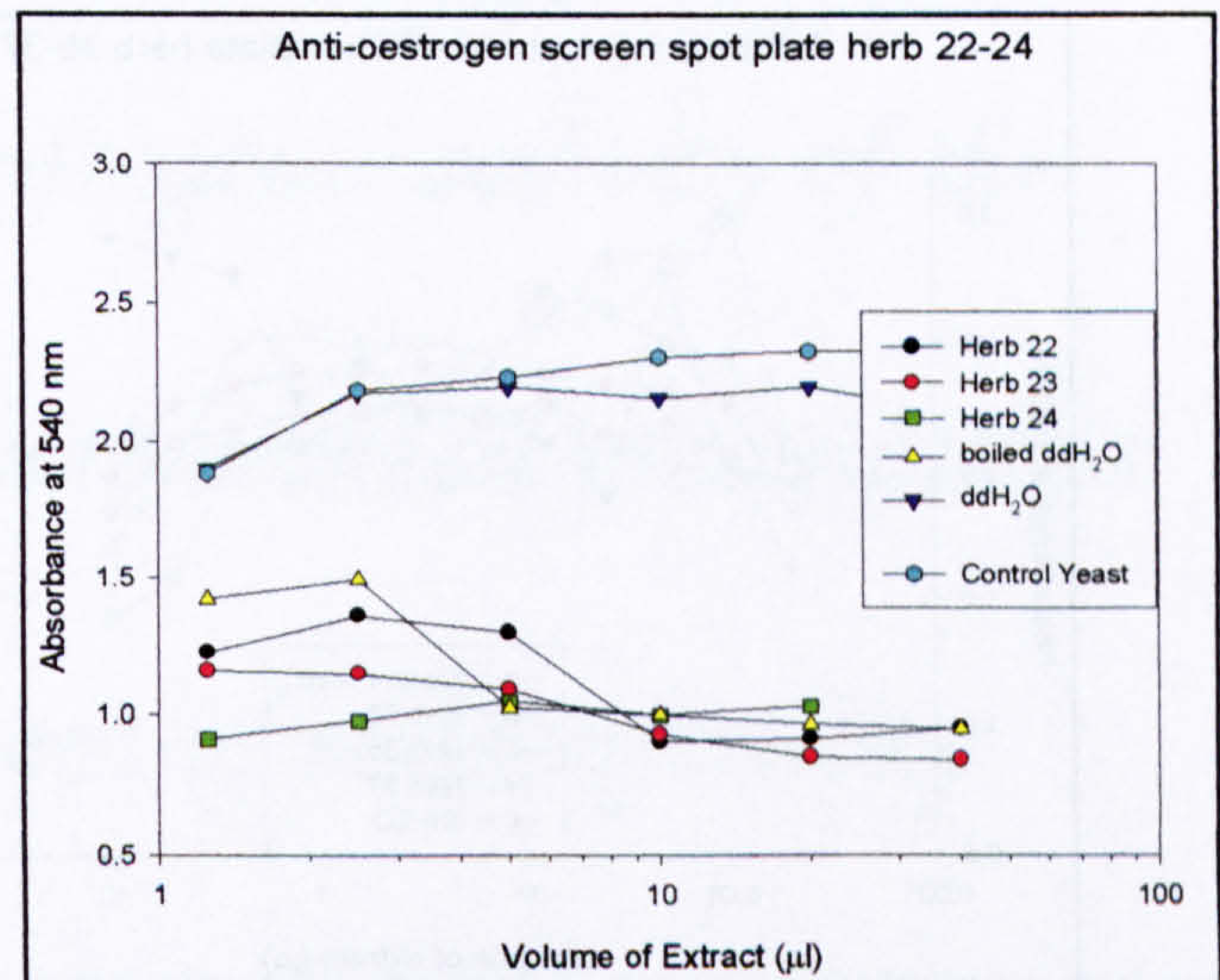

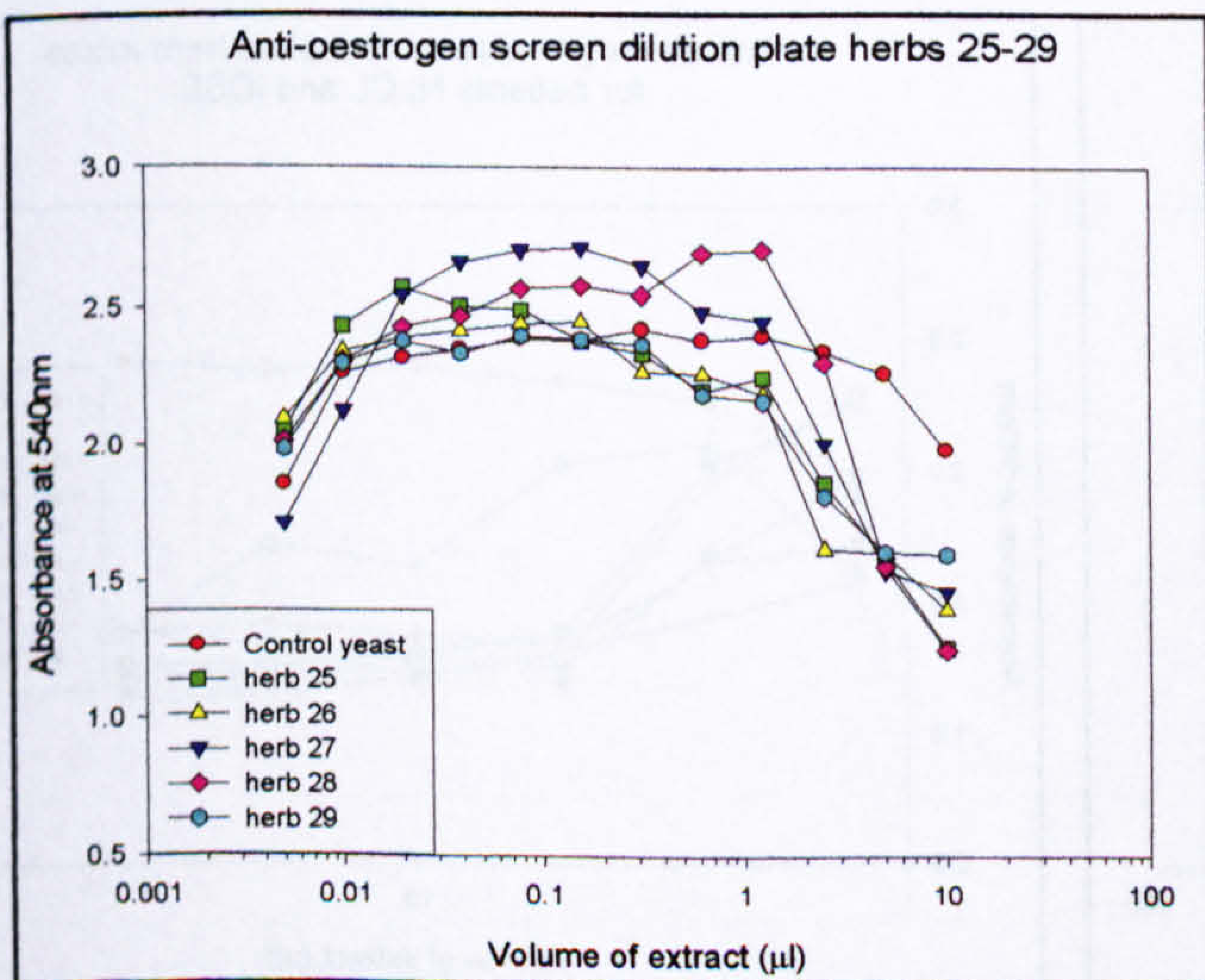

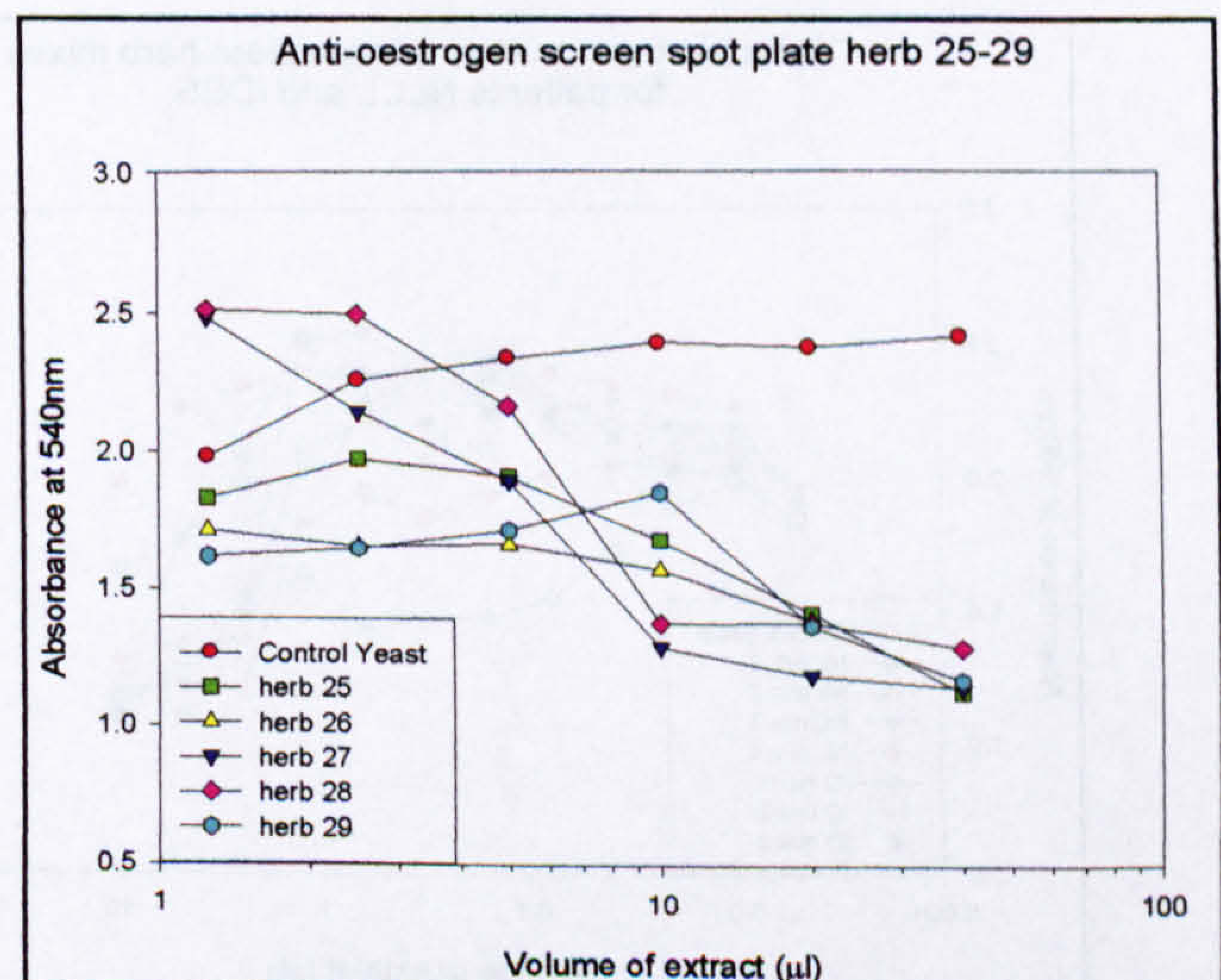

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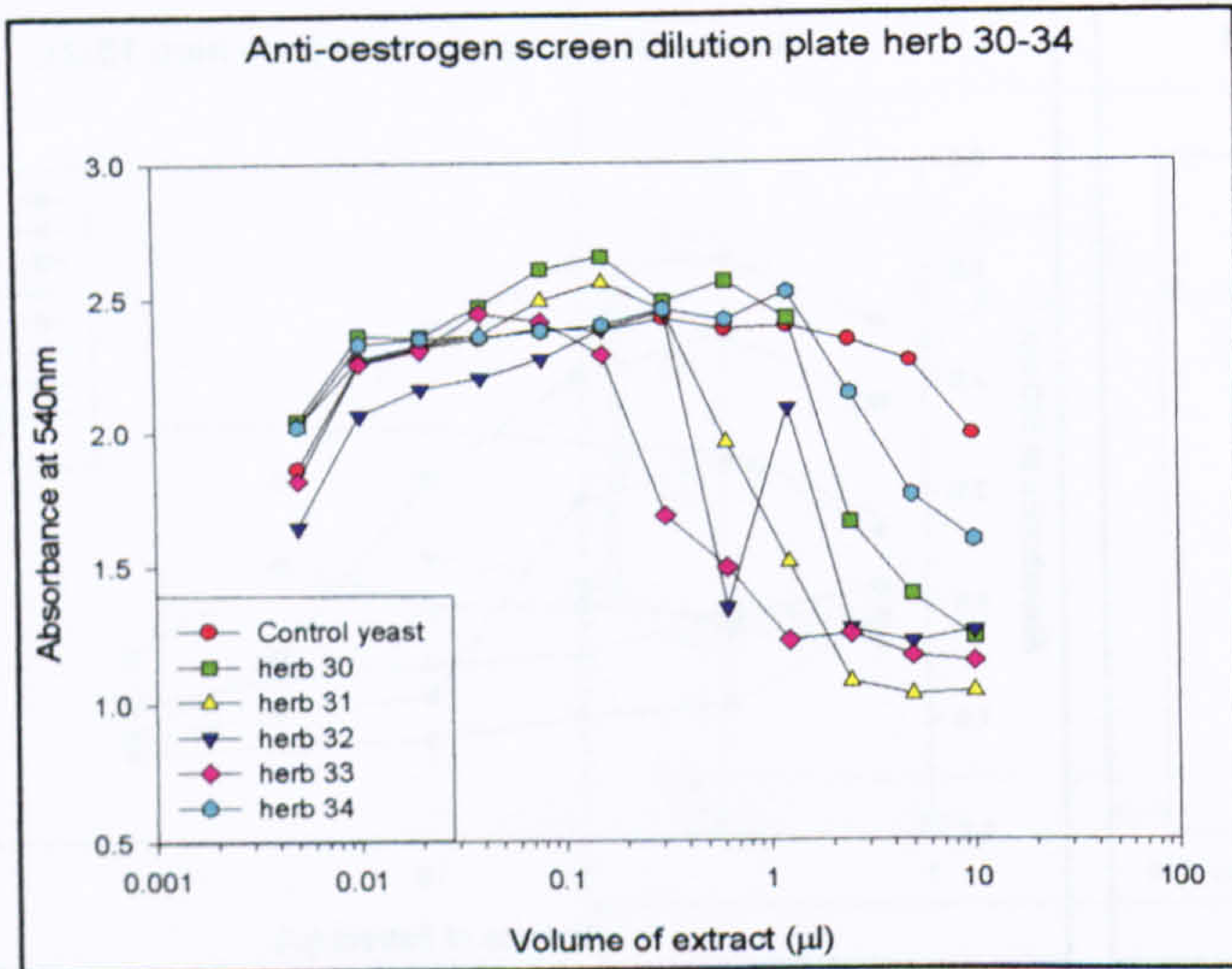

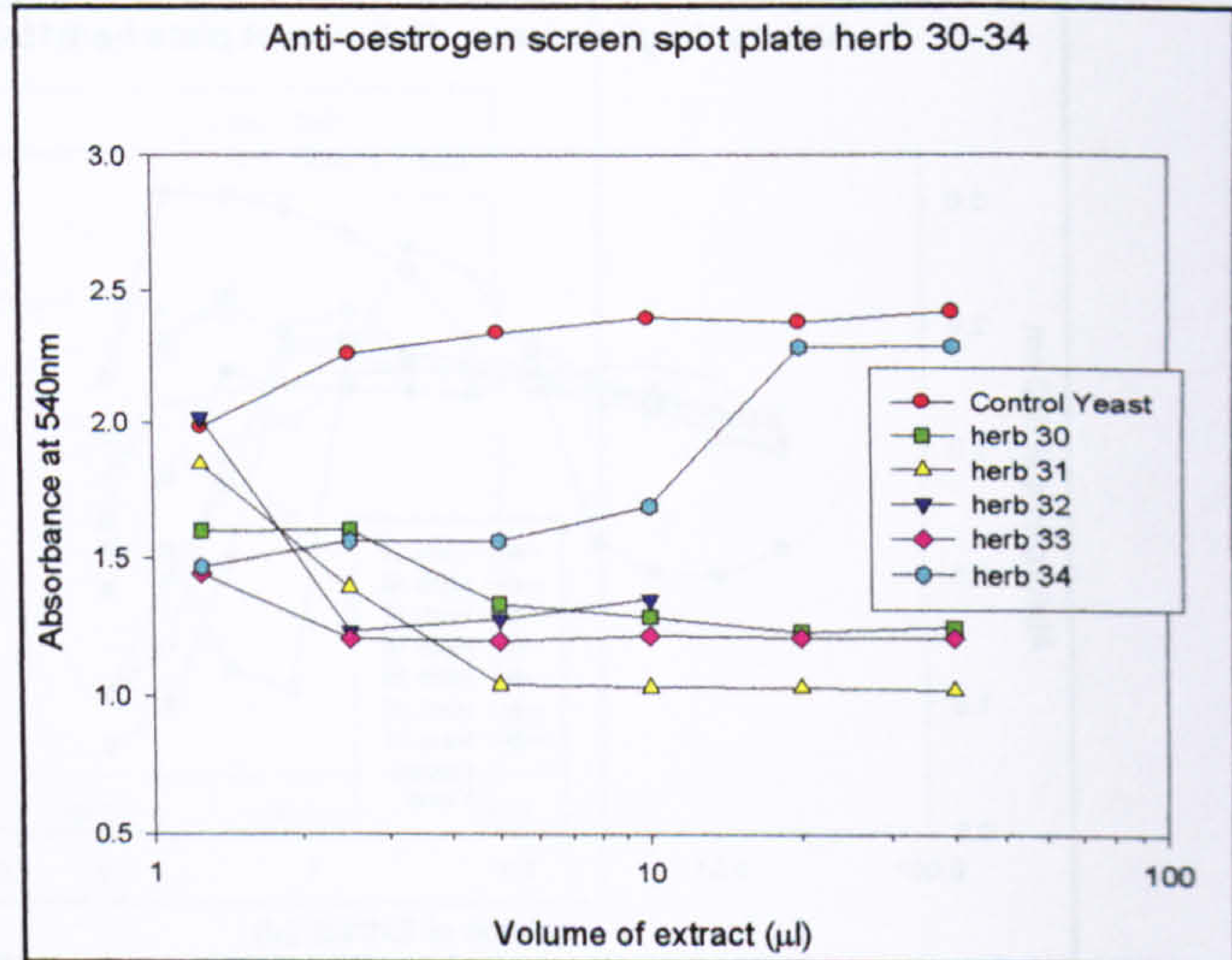

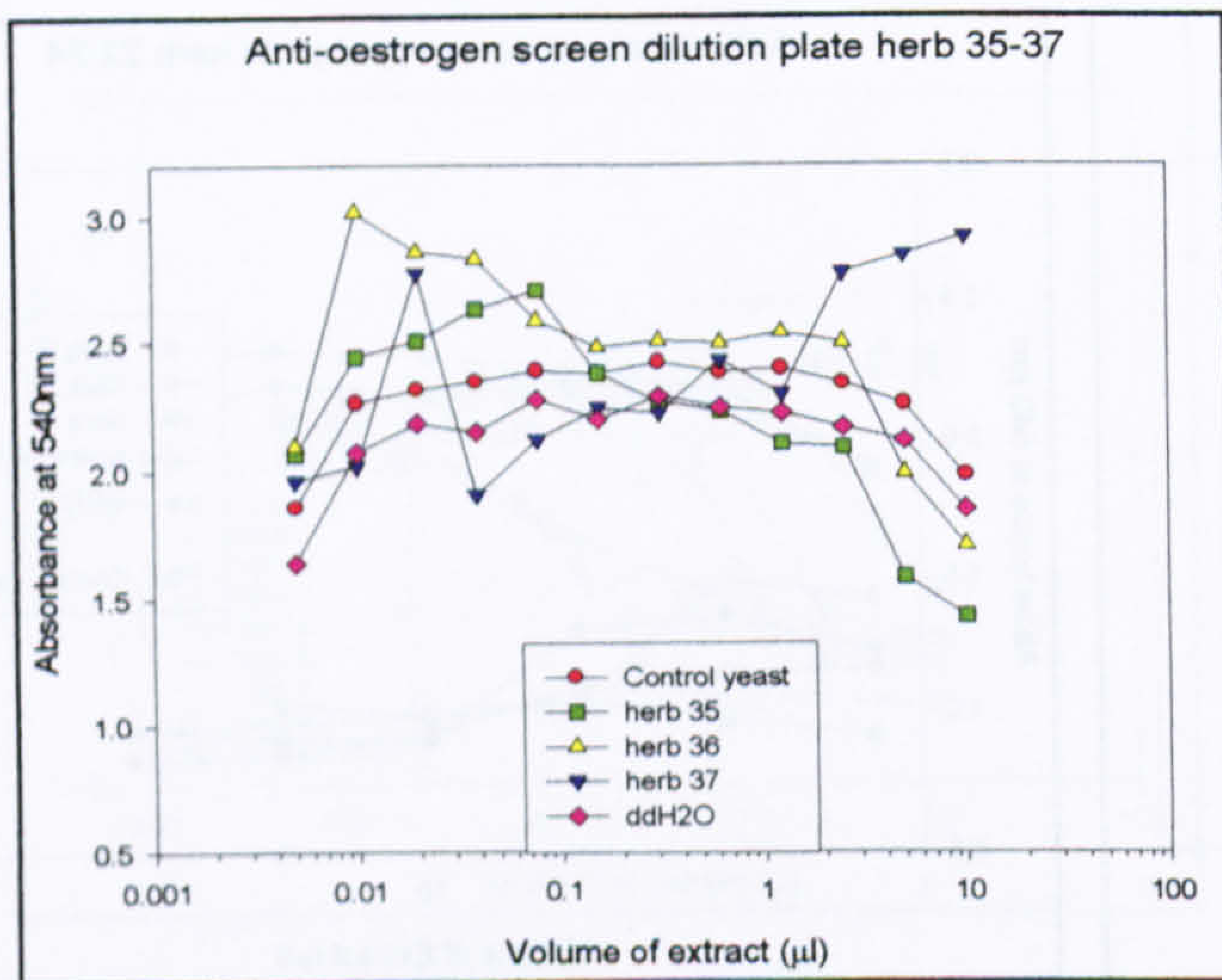

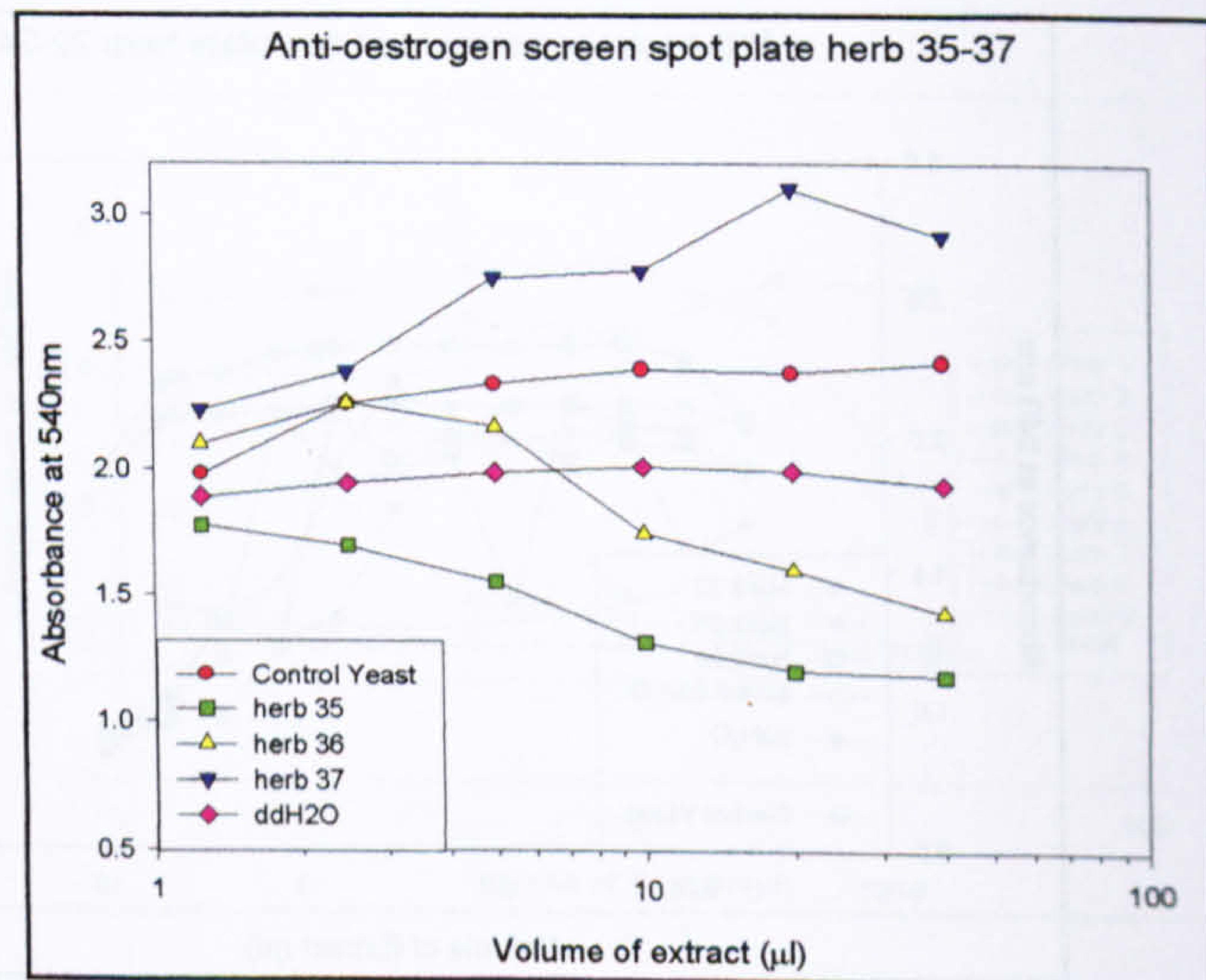

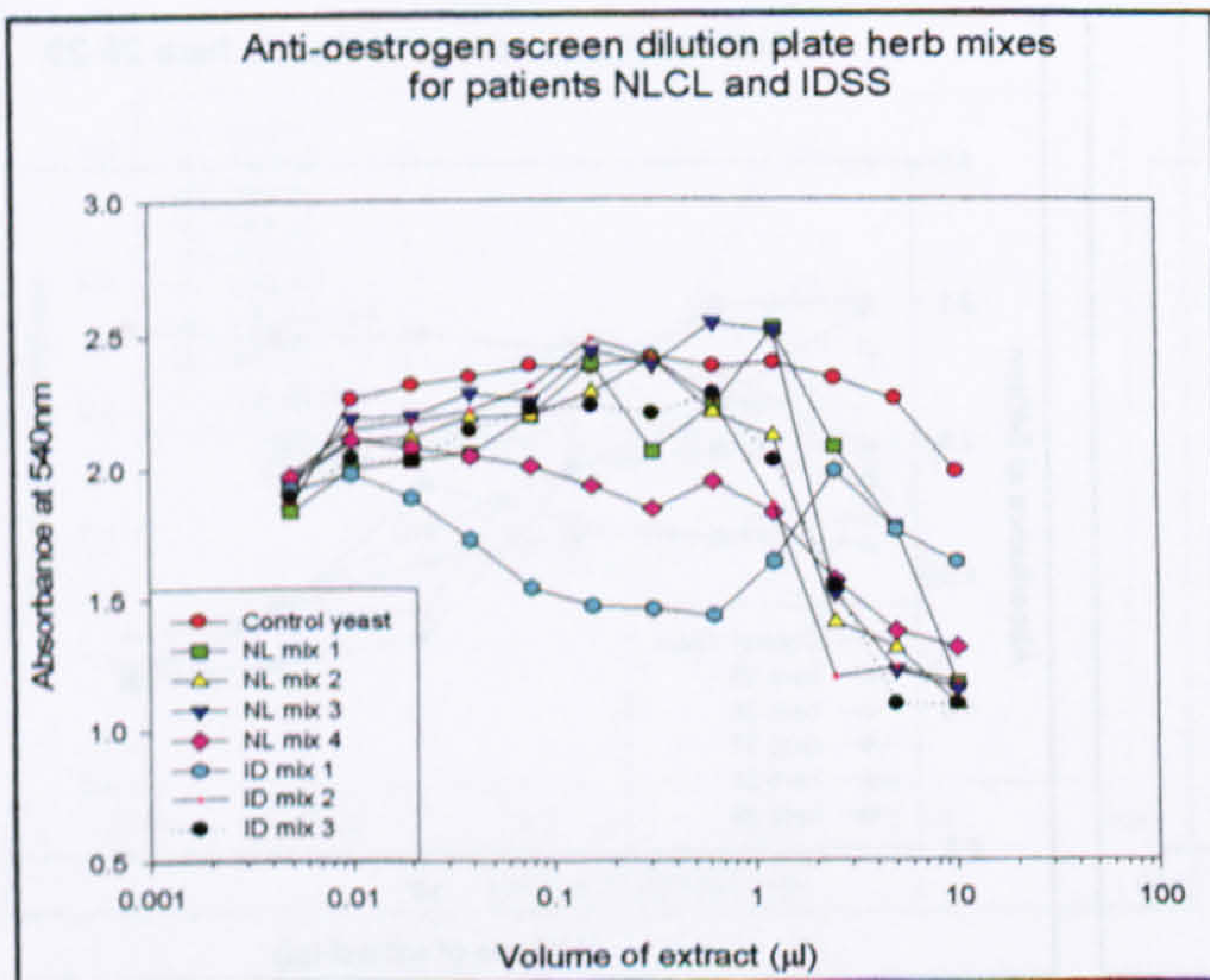

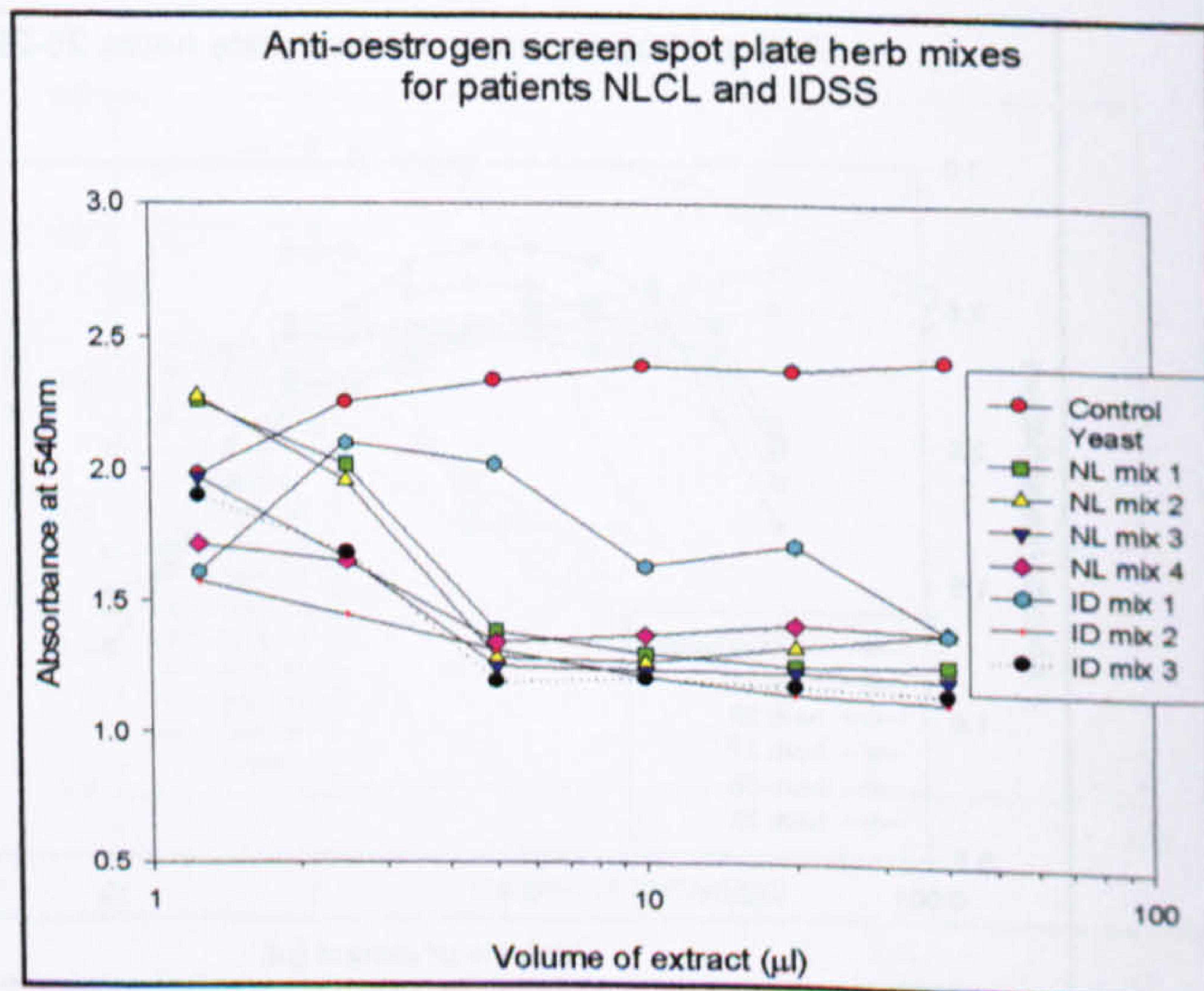

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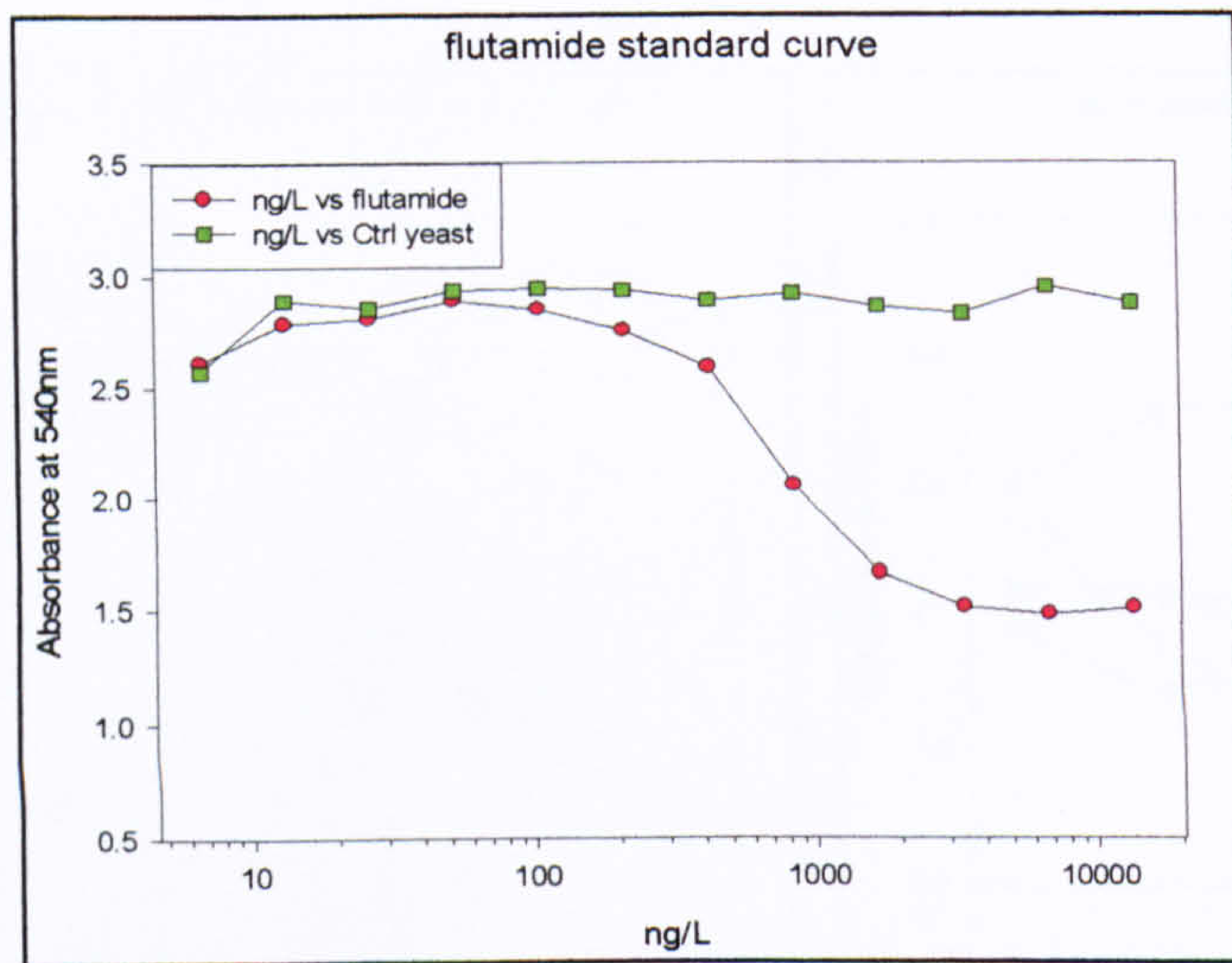
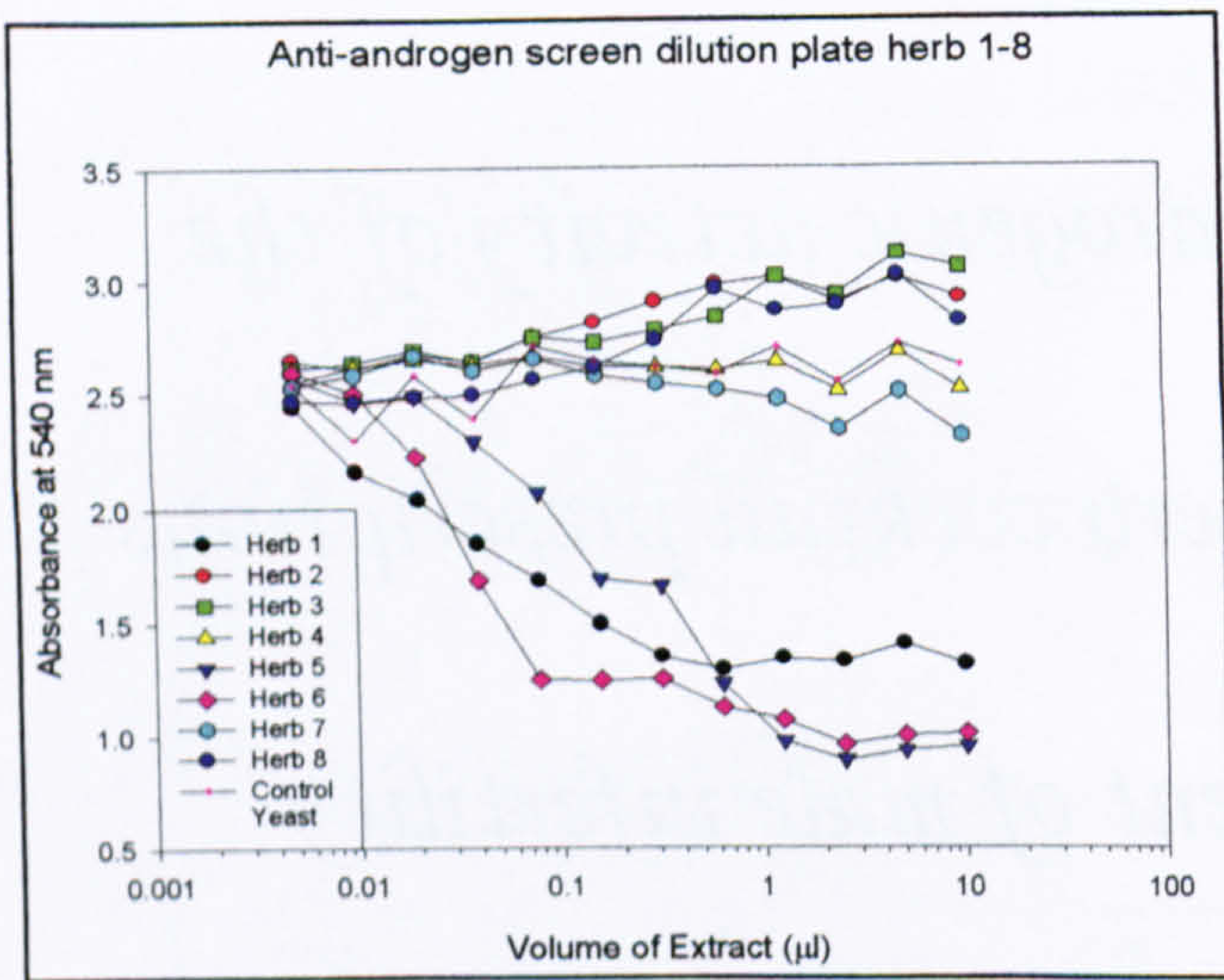
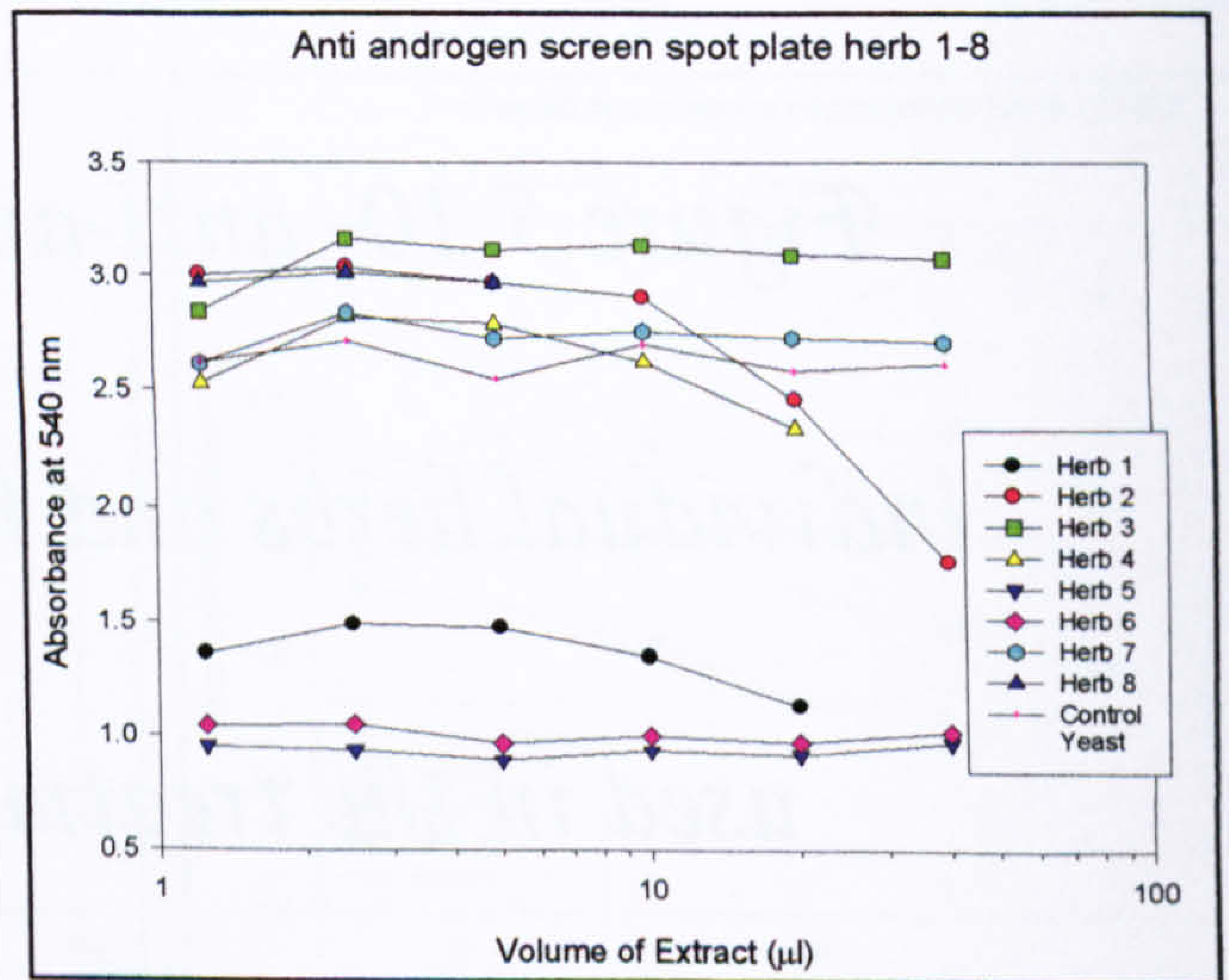
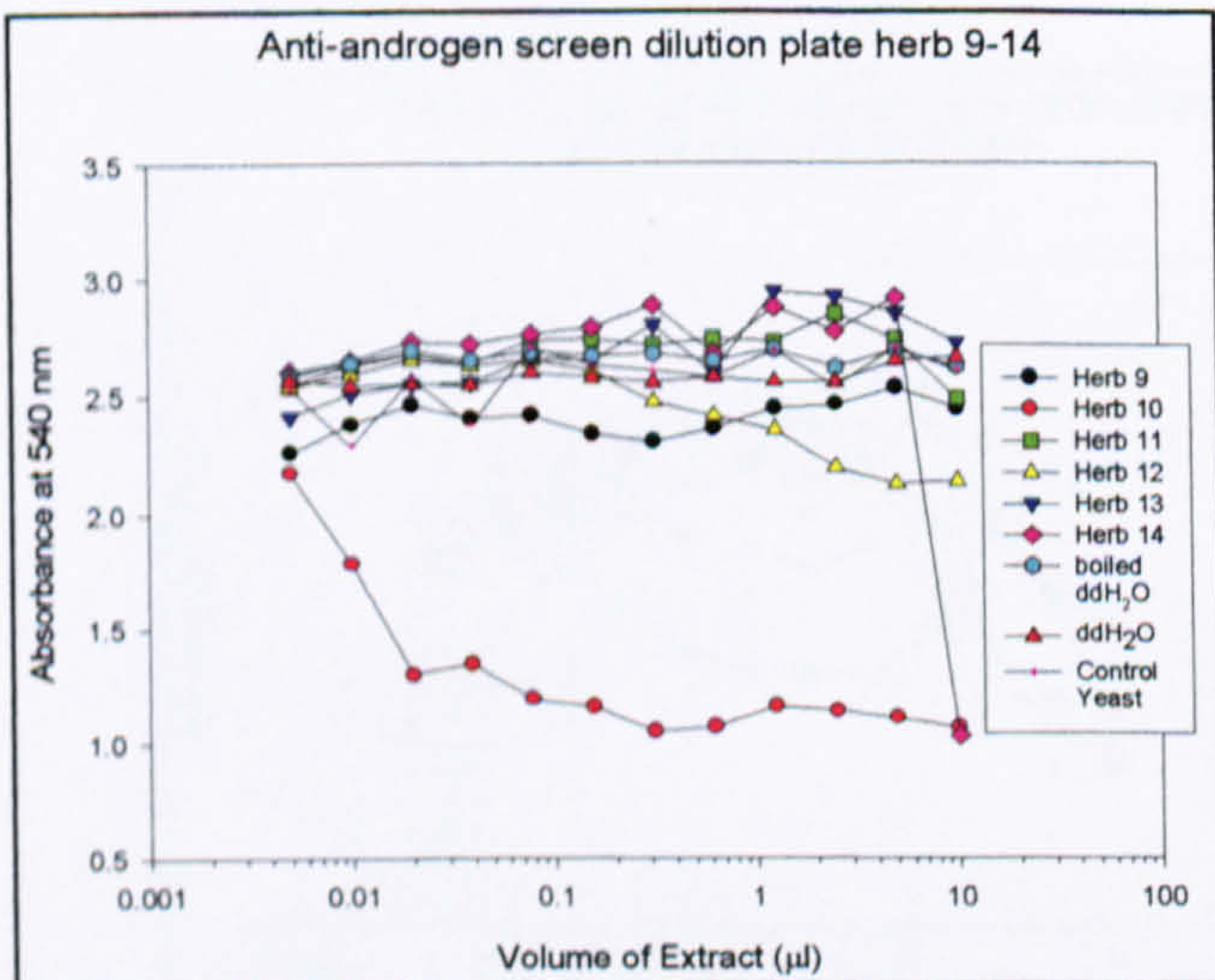
Figure 5.10- anti-androgenic activity of the individual herbs and herb cocktail prescriptions used in the treatment of male infertility









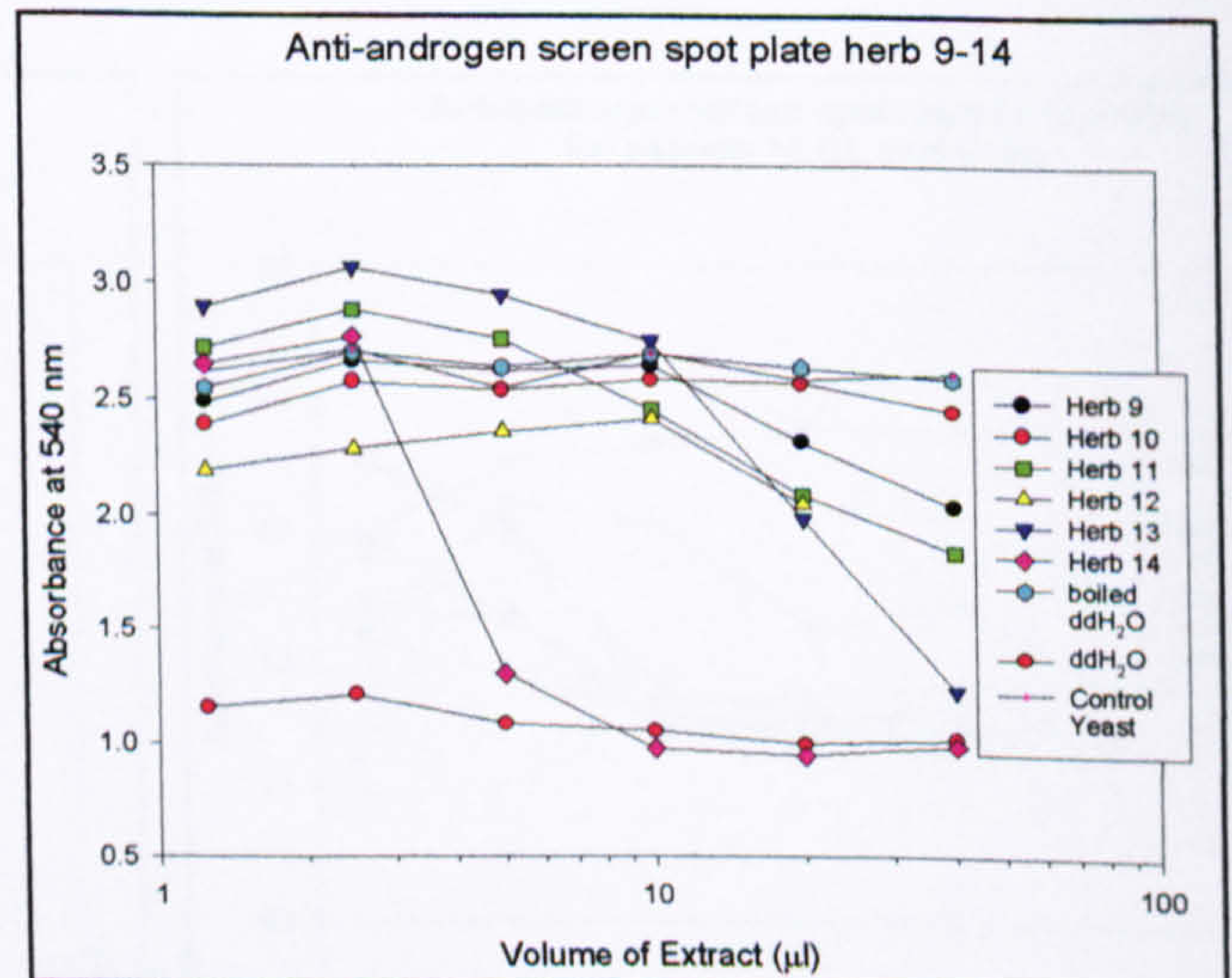
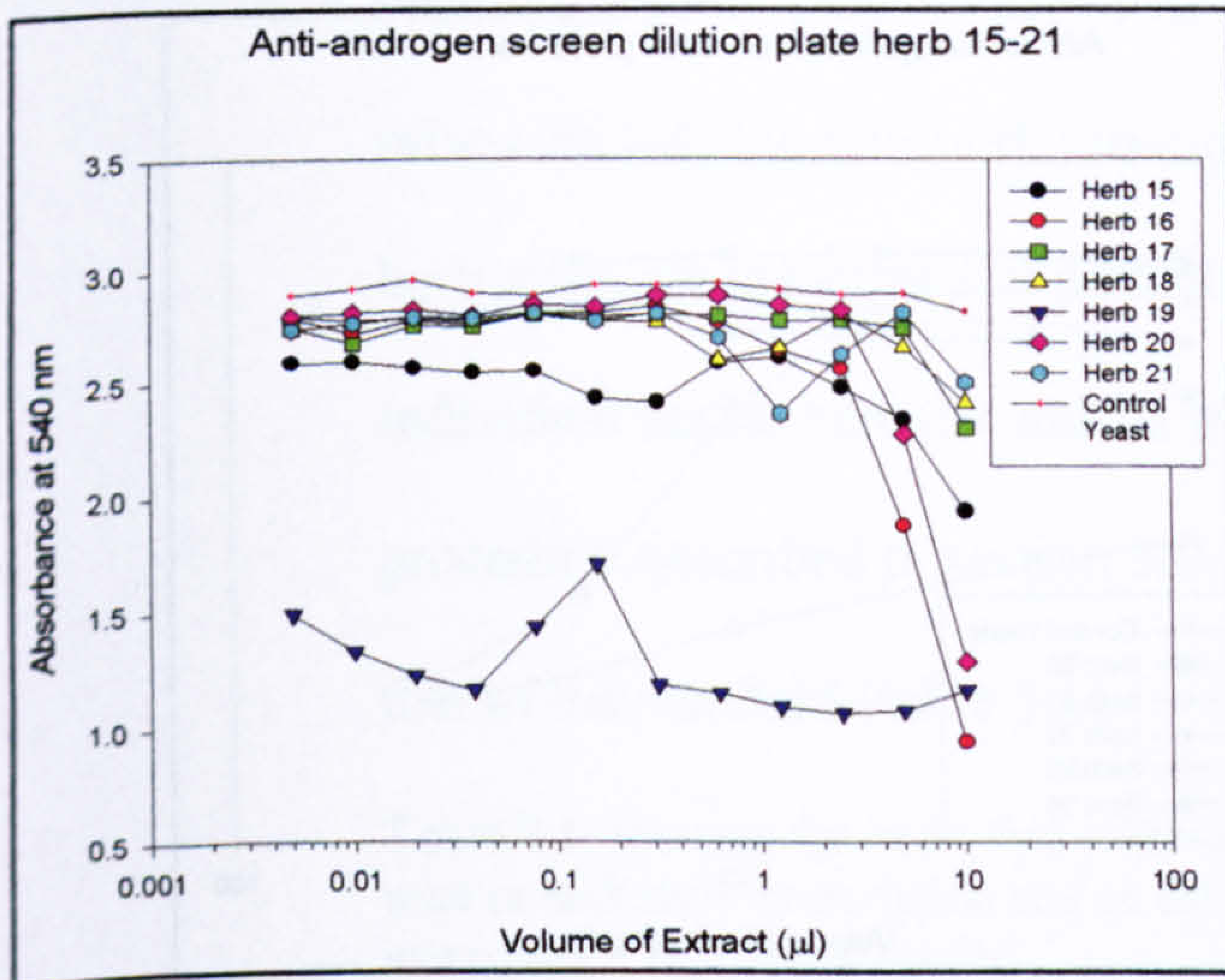
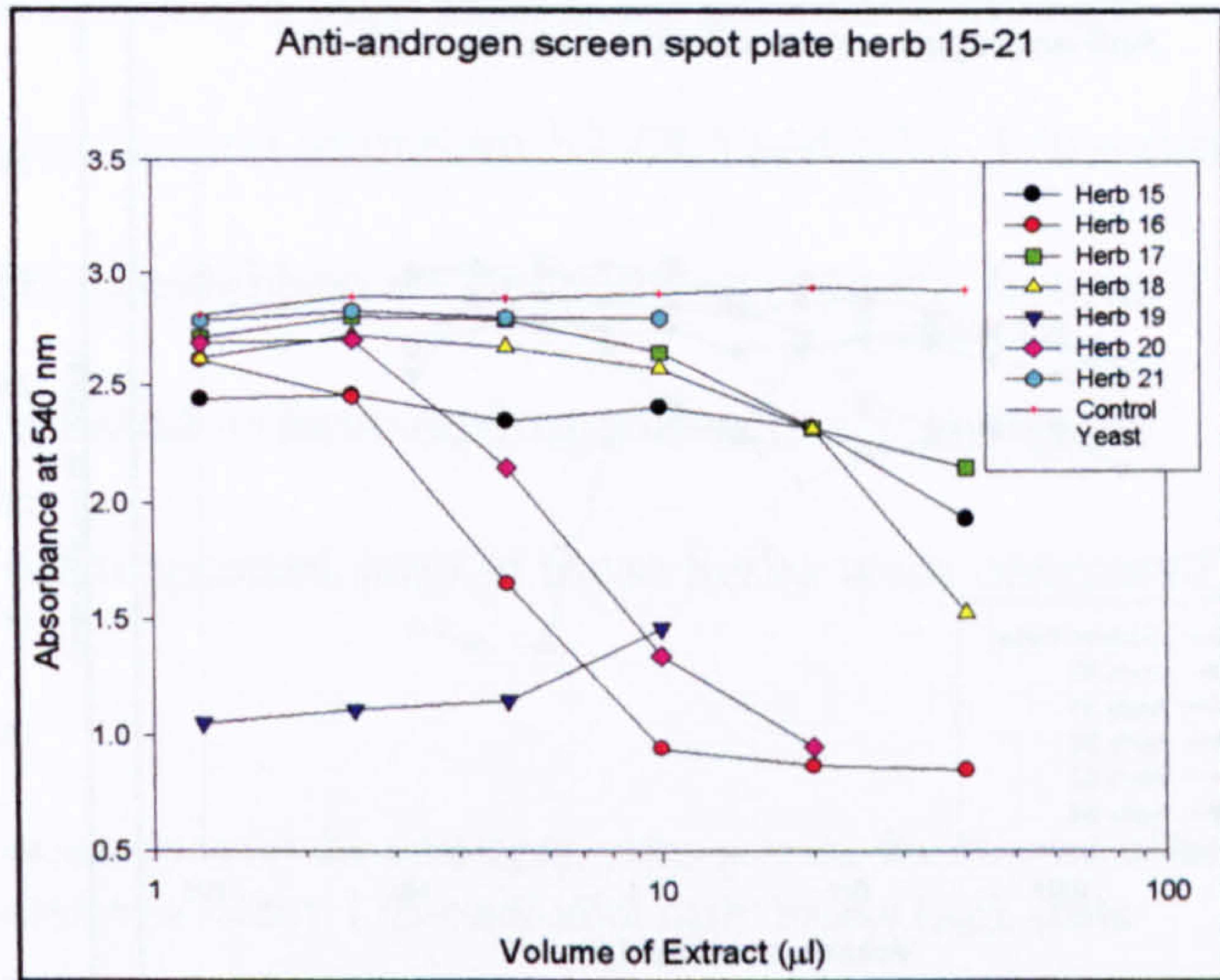
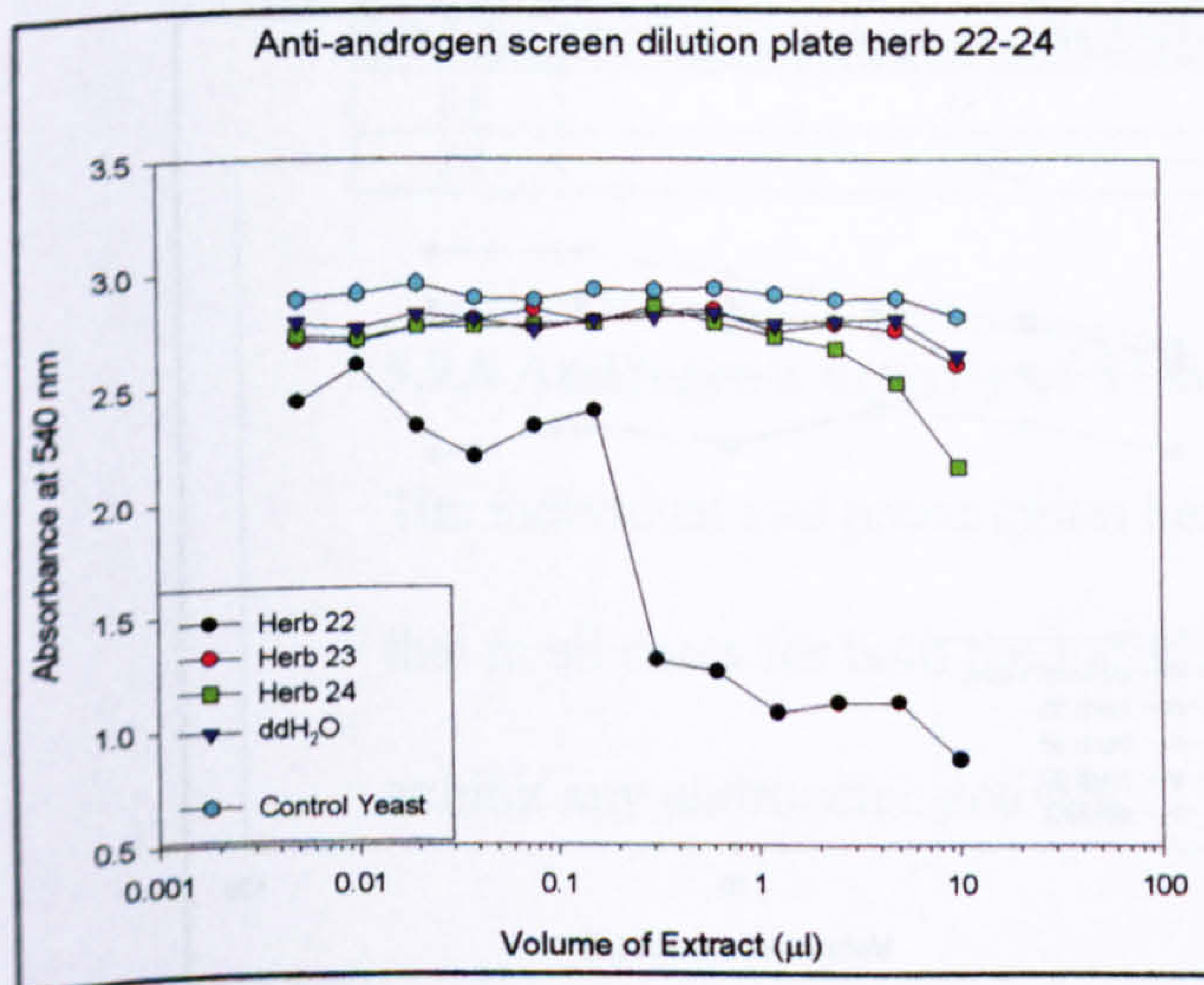
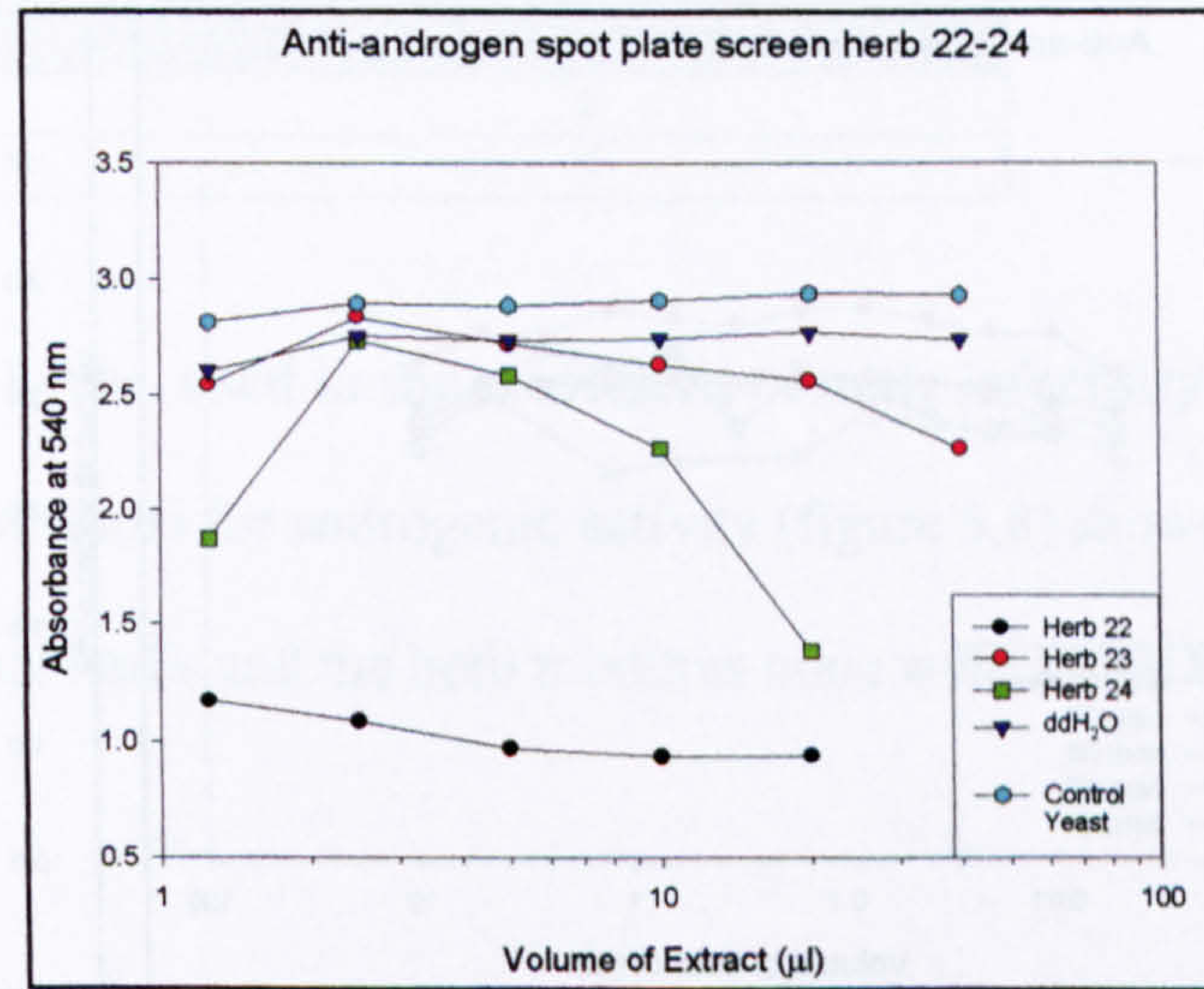


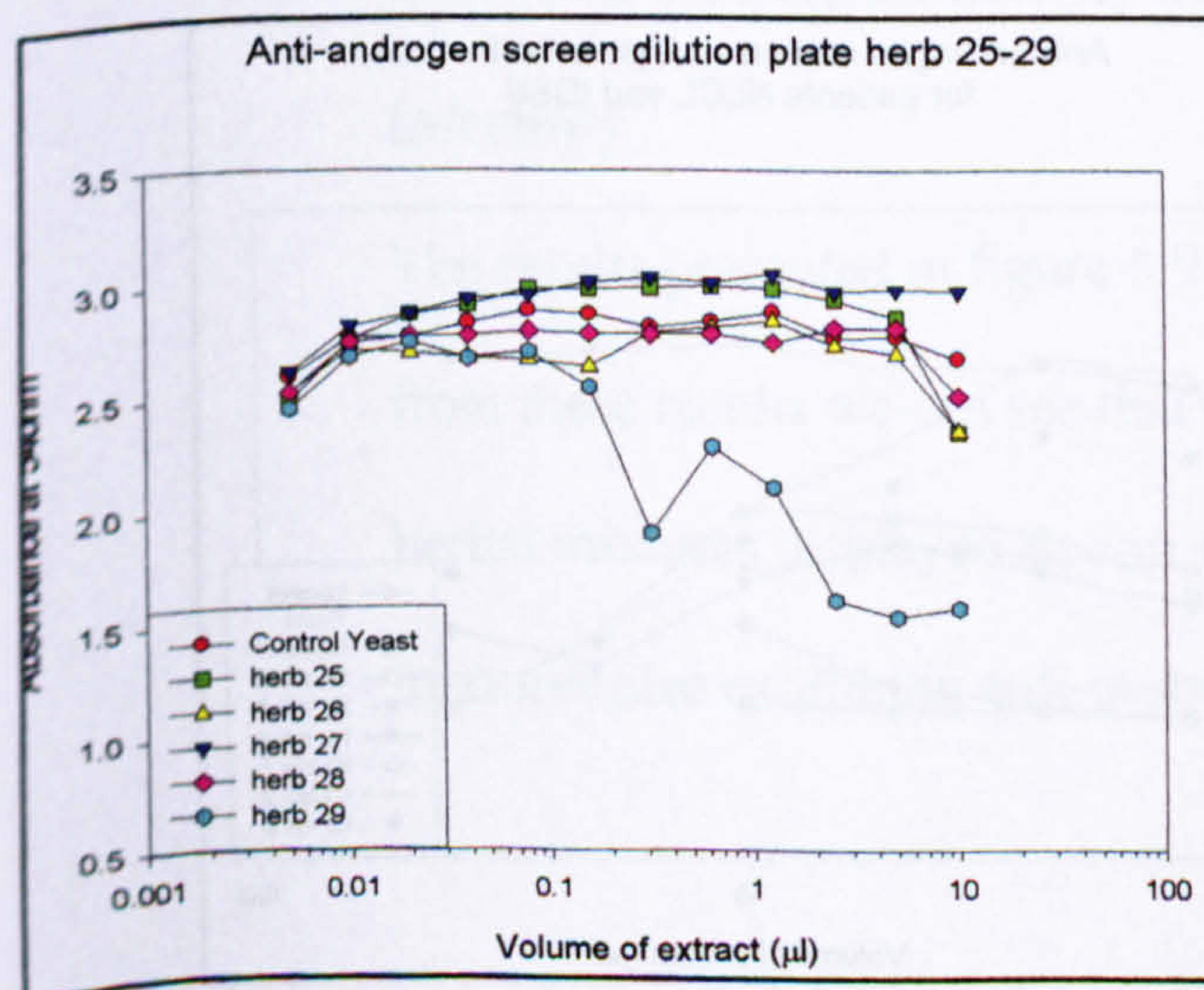
Figure 6.10- anti-androgenic activities of herbs 1-14, each herb was assayed at a number of dilutions inluding: $5x10^{-3}$-10ul (dilution plates) and 1.25-40ul (spot plates), responses are compared to those of known concentrations of Flutamide

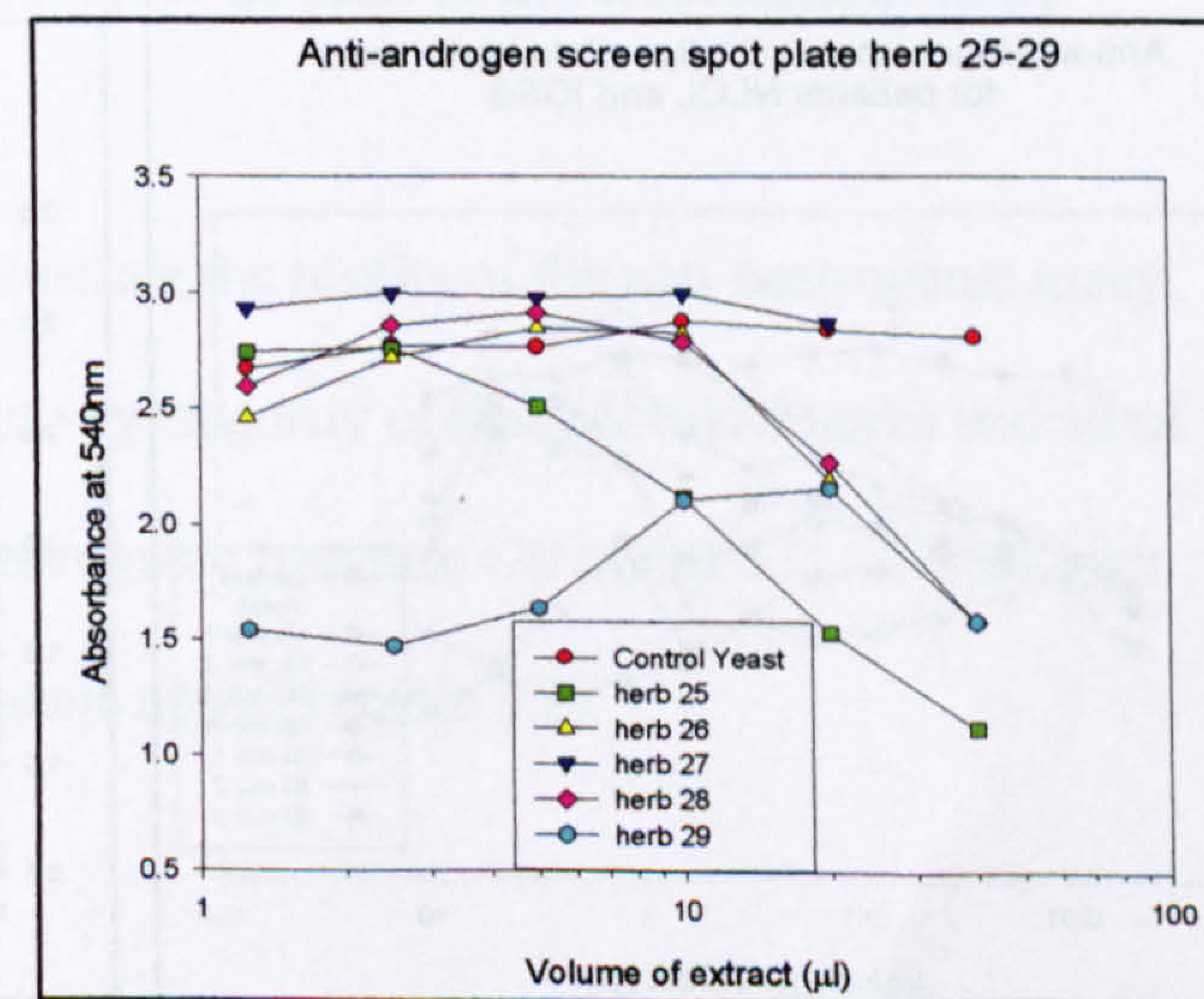












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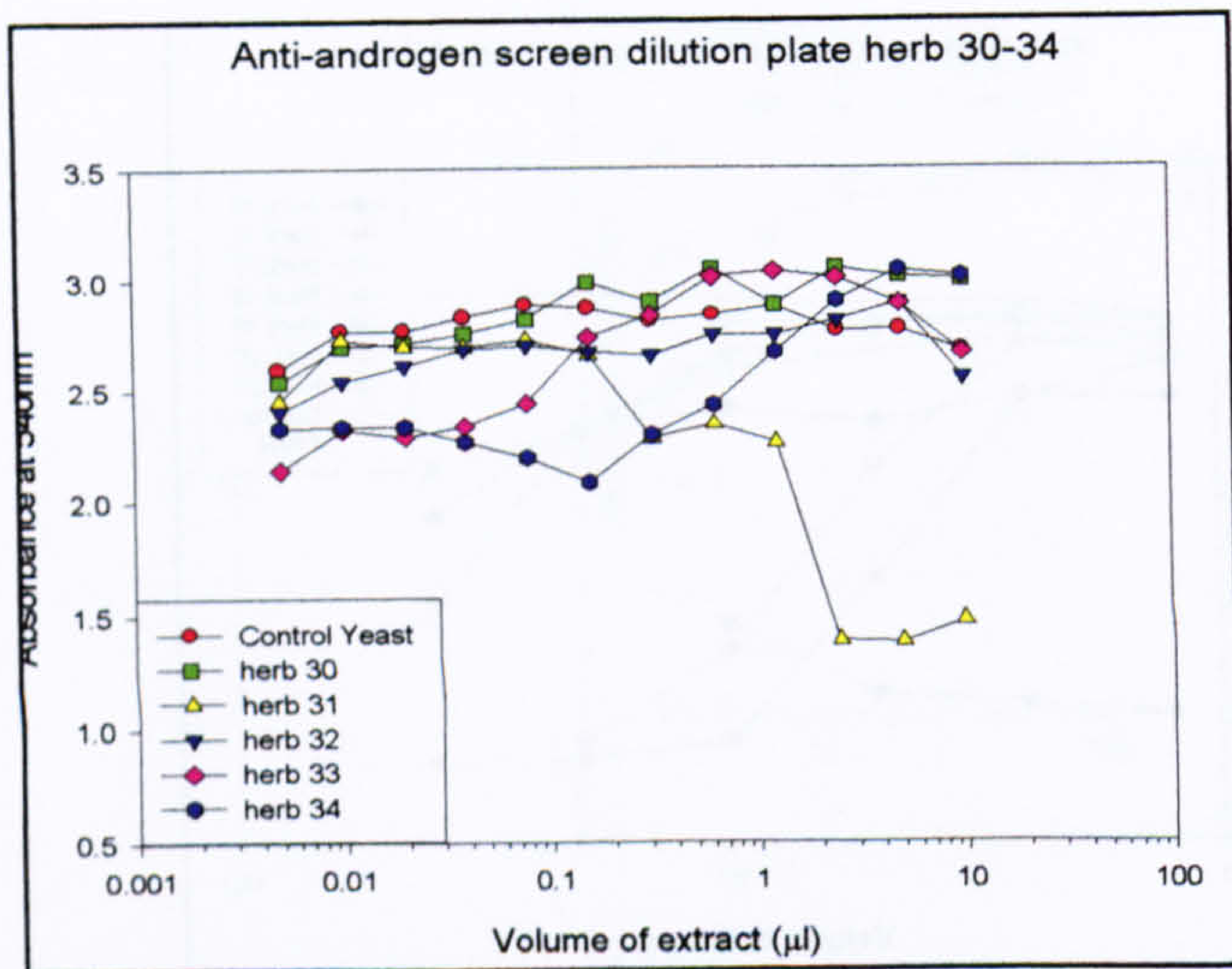

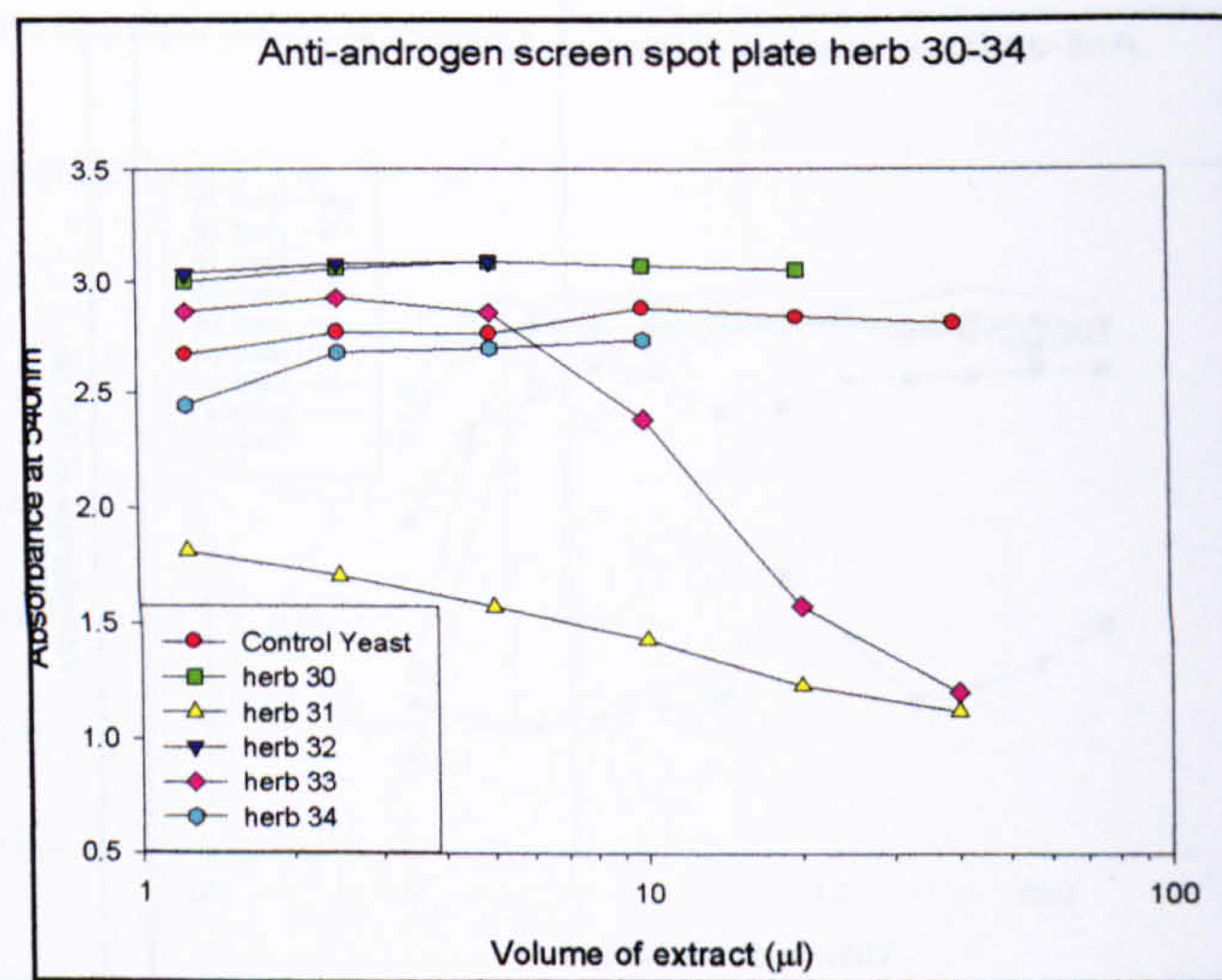

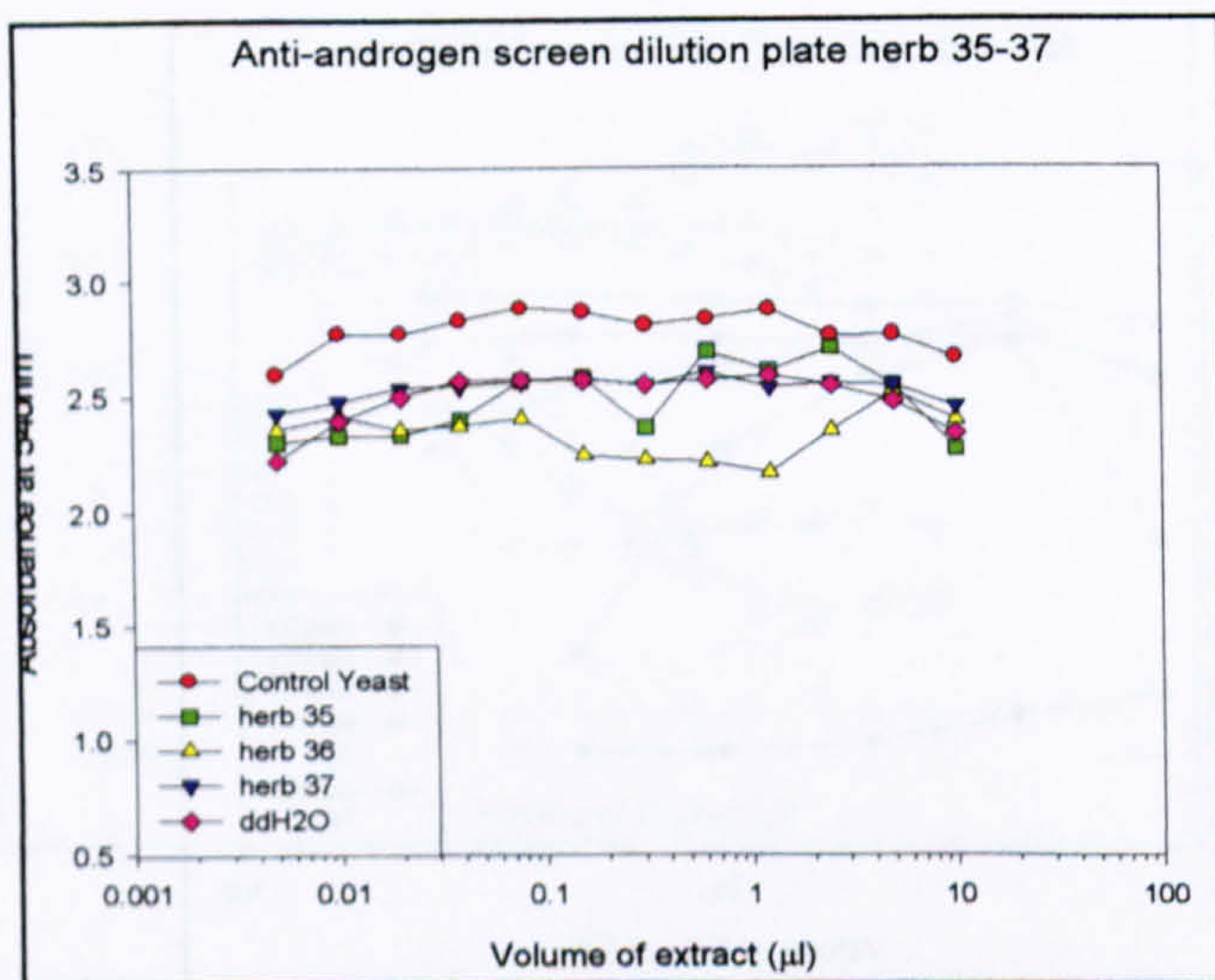

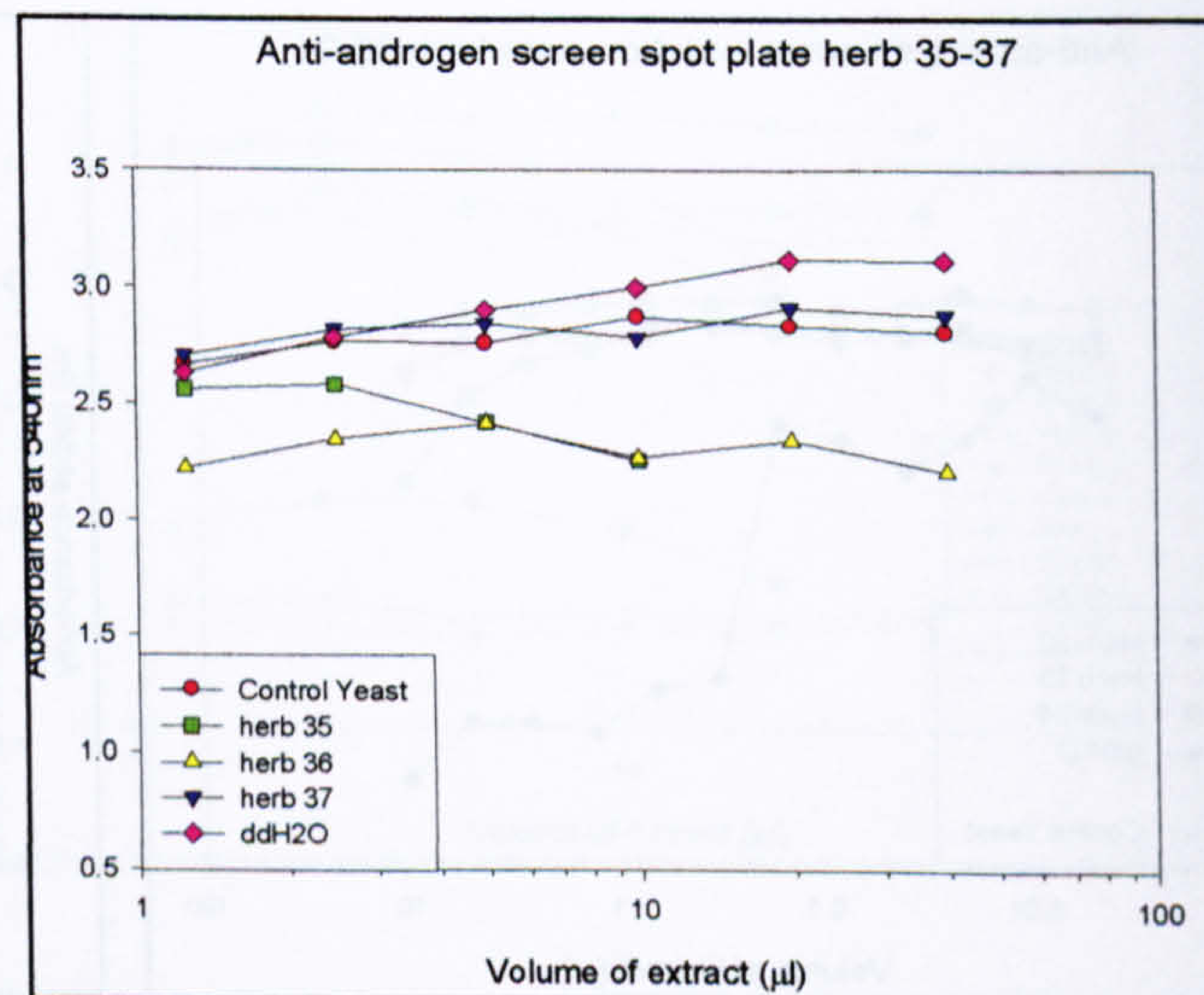

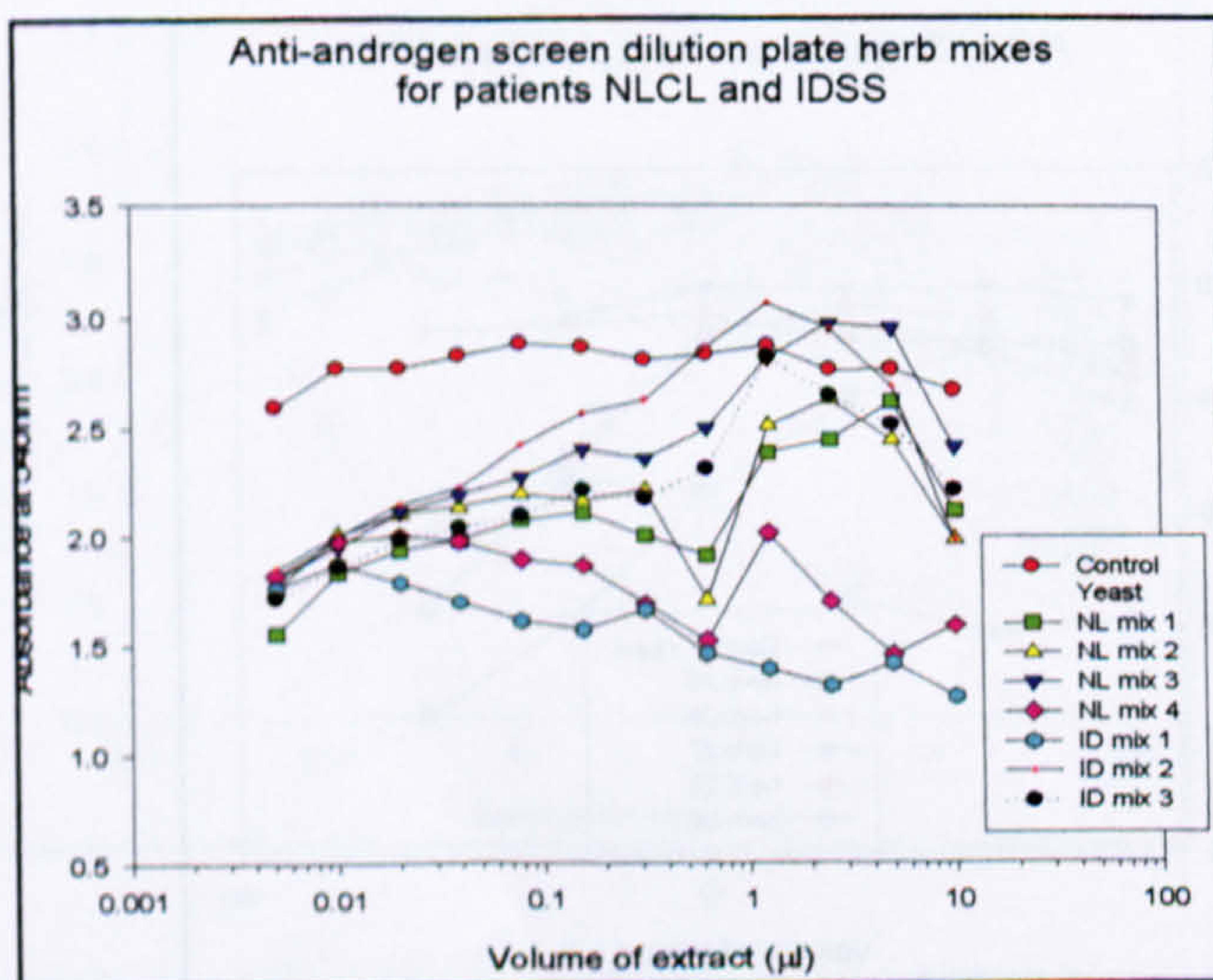

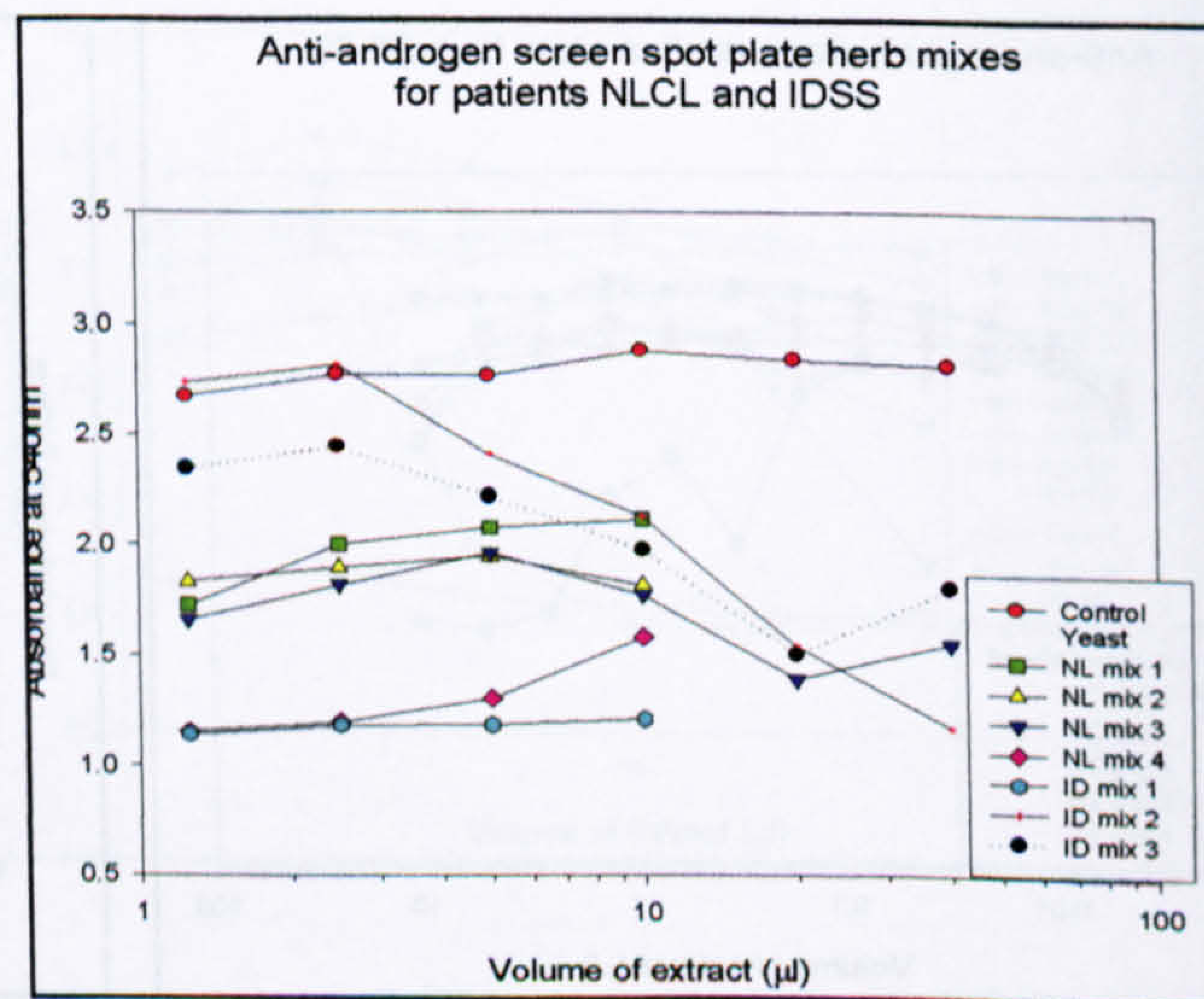

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5.9.5 Oestrogenic activity of TCM herbs used in the treatment of male infertility

From figure 5.7 it can be seen that the majority of the individual herbs and all herb mixes NL1-4 (the four herb mixtures prescribed to patient NLCL) and ID 1-3 (the three herb mixtures prescribed to patient IDSS) exhibited no oestrogenic activity. In total 2 individual herbs, herbs14 and 20 were found to have oestrogenic activity, using the procedure described in section 5.9.3 the responses seen in these herbs were compared to that of the standard (table 5.1).

Table 5.1- showing the herbs that exhibited a response on the oestrogen yeast screen, the amount of herb used in each daily prescription and an estimated equivalent 17β-oestrodiol daily intake (μg), (data extrapolated from assay results).

Herb	*Equivalent daily intake of Oestrodiol (μg)*	*Amount of Herb added to ddH_2O for each prescription (in grams)*
14	0.1	9
20	0.063	5

5.9.6 Androgenic activity of TCM herbs used in the treatment of male infertility

The individual and prescription herbs tested for androgenic activity (figure 5.8) showed that in all cases for both the individual herbs and the herb mixtures none were found to exhibit any androgenic activity.

5.9.7 Anti-oestrogenic activity of TCM herbs used in the treatment of male infertility

The results presented in figure 5.9 illustrate the results of the anti-oestrogenic assay, from these results we can see that the vast majority of the individual herbs and all of the herbal mixtures displayed an anti-oestrogenic response (29 out of 37), with all herb mixtures also exhibiting anti-oestrogenic activity (table 5.2).

Table 5.2- showing the herbs that exhibited a response on the anti-oestrogen yeast screen, the amount of herb used in each daily prescription and an estimated equivalent of OHT daily intake (µg), (data extrapolated from assay results).

Herb	*Equivalent daily intake of OHT (µg)*	*Amount of Herb added to ddH_2O for each prescription (in grams)*
1	0.466	9
4	0.3	10
5	0.16	9
6	1.25	12
7	6.6	9
8	1.06	9
9	3.3	9
10	3.3	9
11	10.8	10
12	2.2	12
13	18.0	6
15	0.053	9
16	3.0	12
17	0.08	12
18	0.024	15
21	0.048	5
22	1.4	12
23	2.4	10
24	1.2	10
27	0.4	12
28	1.06	9
29	1.3	9
30	2.0	6
31	0.8	15
32	2.0	12
33	8.0	6
34	0.3	12
35	1.6	9
36	1.6	6
ID Mix 1	1.6	126
ID Mix 2	0.055	110
ID Mix 3	0.05	128
NL Mix 1	0.8	117
NL Mix 2	0.17	140
NL Mix 3	0.34	105
NL Mix 4	0.18	132

5.9.8 Anti-androgenic activity of TCM herbs used in the treatment of male infertility

Figure 5.10 presents the results generated in the anti-androgenic yeast-based assay, a total of 9 individual herbs displayed anti-androgenic activity (table 5.3).

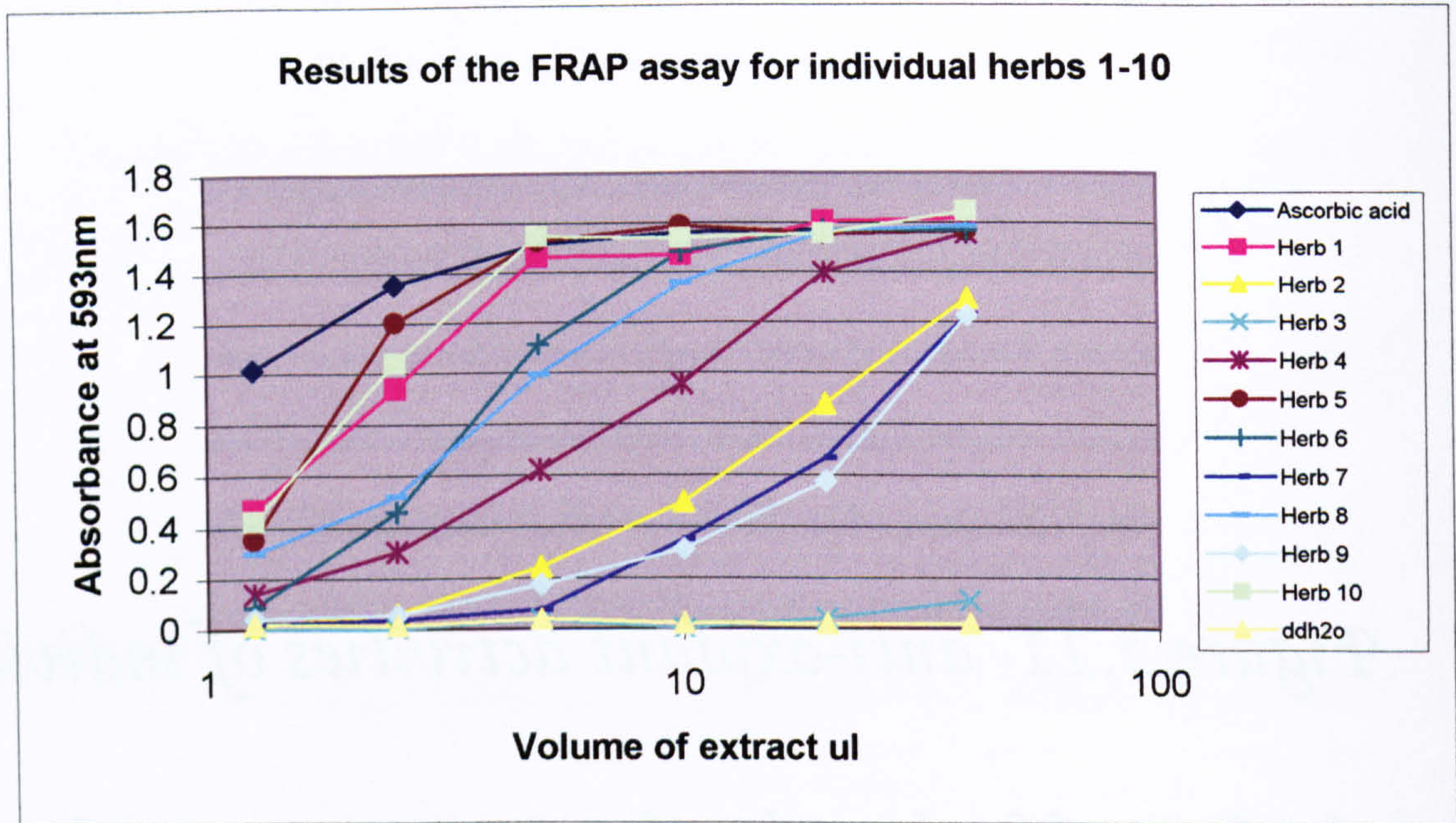
Table 5.3- showing the herbs that exhibited a response on the anti-androgen yeast screen, the amount of herb used in each daily prescription and an estimated equivalent of flutamide daily intake (μg), (data extrapolated from assay results).

Herb	*Equivalent daily intake of flutamide (μg)*	*Amount of Herb added to ddH₂O for each prescription (in grams)*
1	0.07	9
2	10.5	10
5	0.12	9
6	0.006	12
10	0.0006	9
13	10.0	6
14	1.16	9
16	3.0	12
20	3.6	5

5.10.1 Anti-oxidant activity of TCM herbs used in the treatment of male infertility

The anti-oxidant potential of each of the individual herbs and the herb mixtures prescribed to patients NLCL and IDSS were tested using the FRAP assay as described in section 8.6. The herbs tested in this assay covered a range of 1.25-40μl, the activities of these herbs were compared to a known anti-oxidant (ascorbic acid 1mM) covering the same range of 1.25-40μl. Within this assay herbs exhibiting similar responses as ascorbic acid were determined to have anti-oxidant activity, whilst those comparable to the negative control (ddH_2O) were established to not possess anti-oxidant activity. The results produced in this assay are presented in figure 5.11.

Figure 5.11- anti-oxidant activities of individual herbs and herb cocktail prescriptions used in the treatment of male infertility



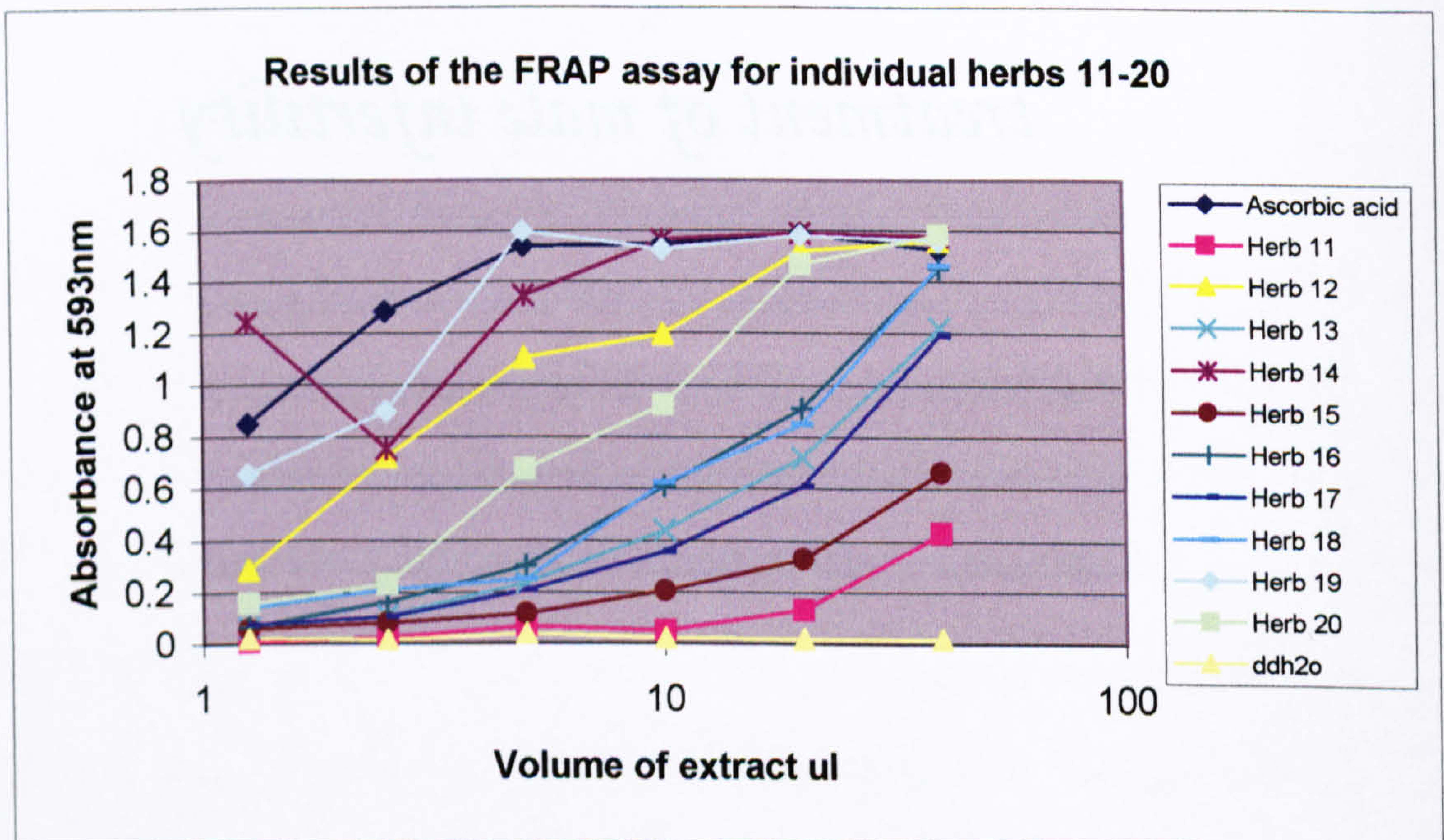
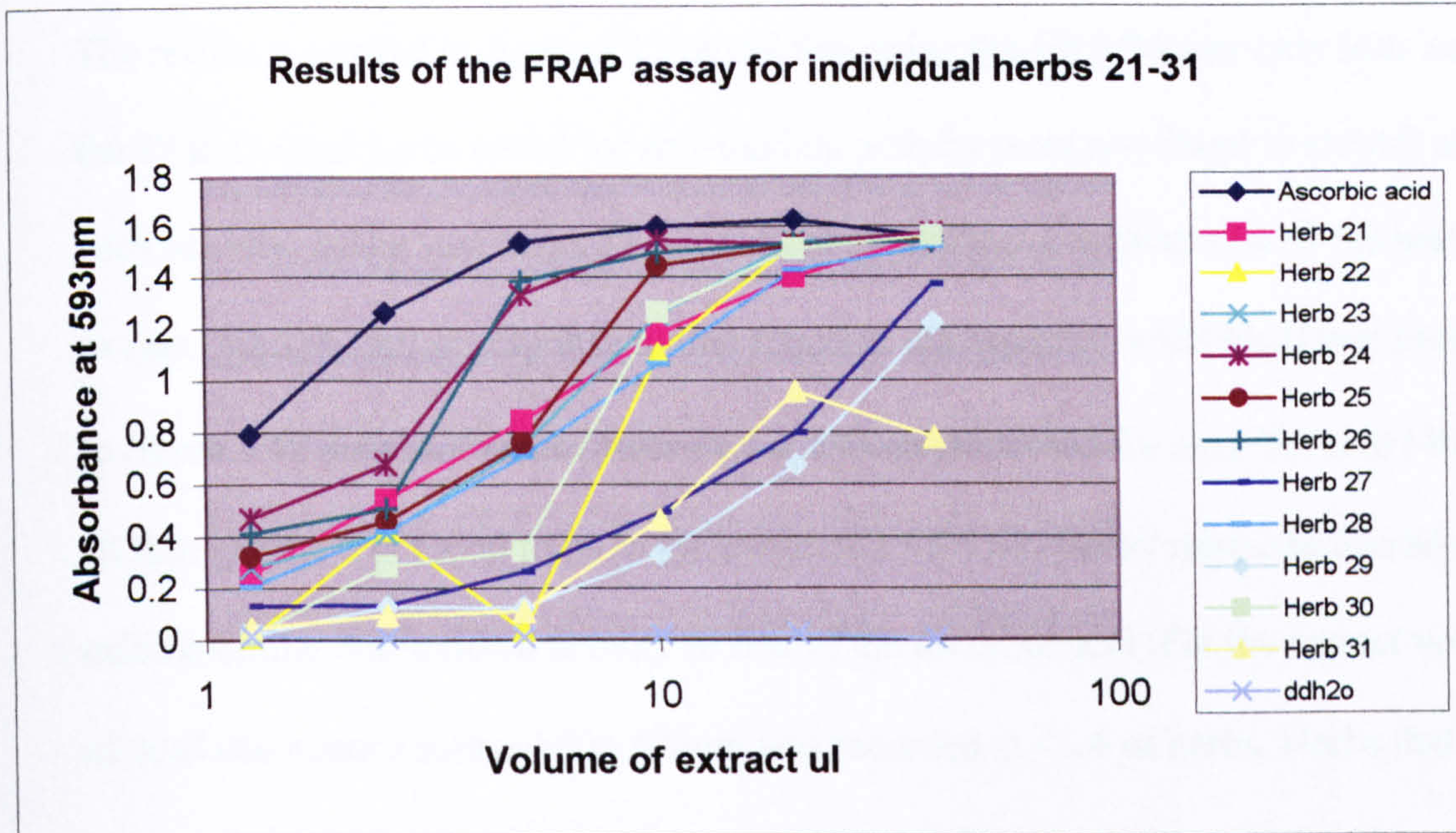


Figure 6.11- results of the FRAP assay for herbs 1-20, ascorbic acid and ddH_2O



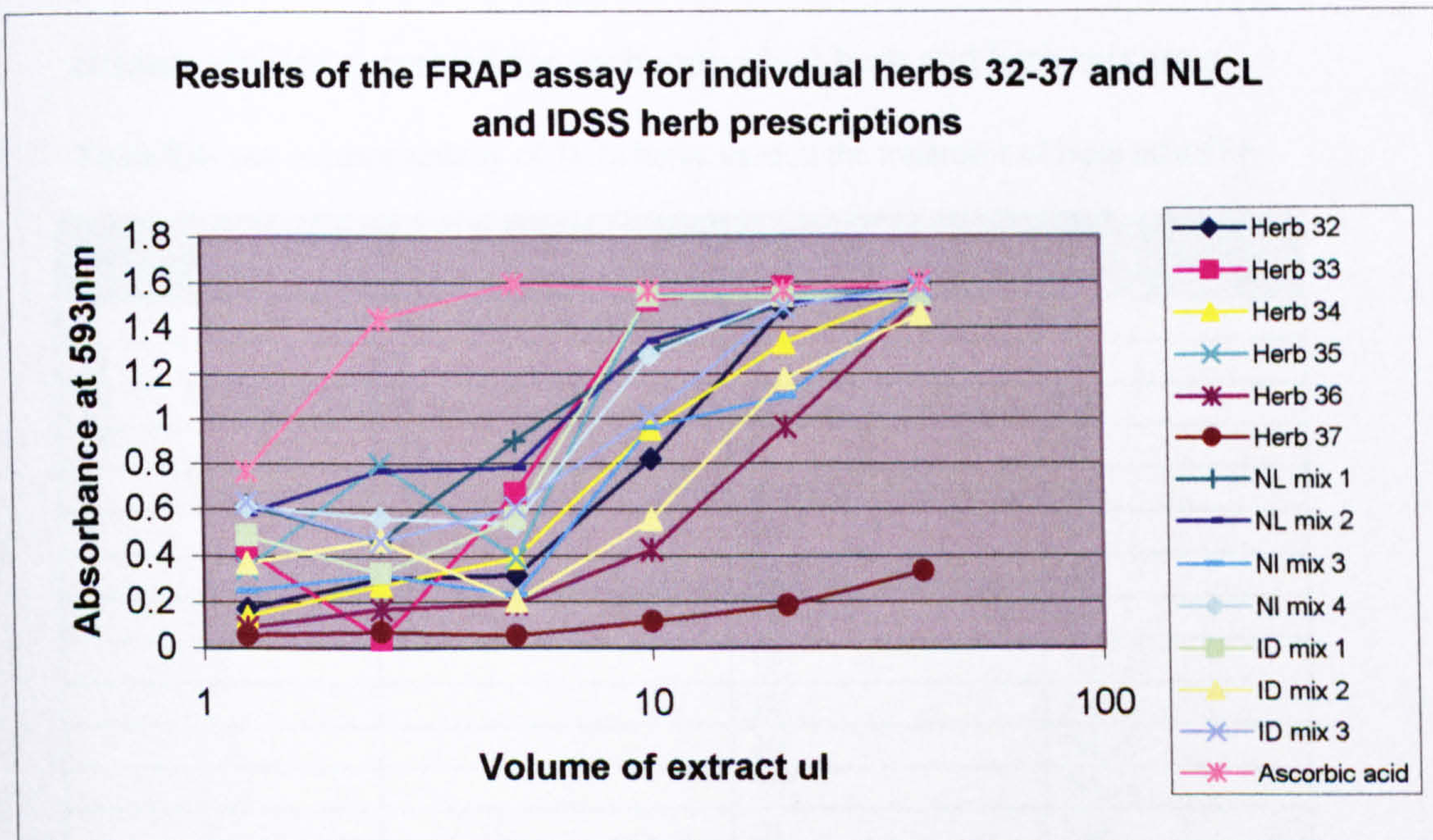


Figure 6.11- results of the FRAP assay for herbs 21-37, herb mixes, ascorbic acid and ddH_2O

5.10.2 Anti-oxidant activity of the TCM herbs used in the treatment of infertility

The results presented in figure 5.12 reveal that using the FRAP assay only four out of the 37 individual herbs tested for anti-oxidant activity were not found to exhibit any such activity. In the vast majority of cases, at the highest concentration of the herbal extract (40μl) a similar response to that found in the ascorbic acid (40μl) was produced. In figure 5.12 the absorbance (measured at 593nm) recorded for ascorbic acid (40μl) in all four graphs was found to be in the range of 1.55-1.62. Herbs were considered to exhibit similar anti-oxidant activity to that of the ascorbic acid if at the extract volume of 40μl absorbance measured at 593nm was recorded at <1.4 of herbs. Herbs that produced activities of >1.4 but <0.8 were considered to be weaker anti-oxidant with herbs producing activities of >0.8 considered as possessing very weak/ no anti-oxidant activity at the investigated concentrations of herbal extract. Table 5.4 indicates the anti-oxidant activity generated for each individual herb and herb mixtures.

Table 5.4- anti-oxidant activity of TCM herbs used in the treatment of male infertility

No anti-oxidant activity	*Weak anti-oxidant activity*	*Comparable activity to ascorbic acid*	
3	2	1	26
11	7	4	28
15	9	5	30
37	13	6	32
	17	8	33
	27	10	34
	29	12	35
	31	14	36
		16	NL-1
		18	NL-2
		19	NL-3
		20	NL-4
		21	1D-1
		22	1D-2
		23	1D-3
		24	
		25	

5.11 Discussion

In this study we report on data generated into the investigation of the activities of TCM herbs used in the treatment of infertile males, for which significant improvements in chromosome 21, X and Y aneuploidy levels in several patients were observed. Specifically we investigated the endocrine and anti-oxidant activity of these herbs. Of the 37 individual herbs commonly used in the TCM prescriptions administered to infertile males by Dr Ping Zhai, the following endocrine activities were found: 2 oestrogenic, 29 anti-oestrogenic and 9 anti-androgenic. Moreover, of the 37 individual herbs, 8 were found to be weak anti-oxidants and 25 were found to be strong anti-oxidants. As part of this study we were also able to investigate the activities in seven of the herb mixtures prescribed to two of the six patients (NLCL and IDSS) at the time of the semen assessments investigated for aneuploidy frequencies (data presented in chapter 4). Of these 7 herb prescriptions (four administered to patient NLCL and three administered to patient IDSS) all were found to be strong anti-oxidants, none were found to be oestrogenic or androgenic, however all prescriptions displayed anti-oestrogenic activity.

We have investigated the endocrine activities, not only that of oestrogenic but also that of androgenic, anti-oestrogenic and anti-androgenic. We are not the first study to report anti-oxidant activity of plants used for medicinal purposes, however in contrast to previous studies we have investigated the anti-oxidant activities in a large number of individual plants. This study has also investigated the anti-oxidant potential of plant mixtures, to the best of our knowledge we are the first to report on the activities of each of the individual herbs analysed but also that of these particular herb mixtures (within the English literature).

There have been a number of studies that have investigated the chemical constituents of a variety of plant matter used in TCM, some of these studies have also investigated the effects of these herbs in in-vitro models to establish potential biological activities that may have a physiological effect on humans. The most commonly investigated herb is that of Ginseng, the constituents of this include triterpene, saponins, essential oils and polyacetylenes (Bisset et al., 1994), animal studies have revealed that it has a potential role as a immunostimulant (Singh et al., 1983; 1984). The majority of studies have investigated the mixtures used in TCM treatment, many of which have revealed anti-thrombotic, anti-inflammatory, anti-allergic, anti-tussive and anti-bacterial effects (Gong and Sucher, 1999).

On the basis that this is a small pilot study, we have determined that these herbs possess biological activity, however given time it is beyond the scope of this study to identify the routes by which these may be acting if the improvement in chromosome aneuploidy and semen parameters is as a result of this treatment. We have suggested possible pathways as to where these may be acting i.e. through endocrine or anti-oxidant activity both of which play key roles in fertility, as any imbalance/ disturbance results in decreased fertility, and tested the herbs for each of these biological activities. Given the results it is clear that a larger study investigating the efficacy of TCM treatment in male infertility needs to be carried out.

On the basis of the evidence provided within this chapter and chapter 4, we feel that there is strong preliminary evidence in the efficacy of TCM treatment in male infertility, specifically improved levels of aneuploidy and that the results provided in this chapter provide evidence of strong endocrine and anti-oxidant activity. Given these findings we

suggest that there is sufficient evidence to warrant progression to a double-blind placebo controlled clinical trial with the aim to determine if these treatments are having an effect or whether the results demonstrated are due to a placebo effect or lifestyle improvements. Such a trail however, would not be simple to perform as there are a number of difficult issues to resolve in initiating a clinical trial involving TCM these include:

- The chemical constituents of these herbs must be identified and the herbs used in the trial standardised, this is of particular relevance given the results of the study carried out by (Drew and Myers, 1997; Yuan and Yin, 2000) analysis of the chemical constituents of herbs harvested at different times of year and within different geographical regions all herbs revealed very different results.
- Whether the skill of the TCM practitioner in tailoring the herbal treatments to each patient and the individual visits have a significant effect on the outcome, or whether a standardised treatment would be sufficient in the majority of patients. If improvements seen are as a result of the tailor made treatments, it would not be suitable for pharmaceutical development; a standardised treatment for such development would be vital. It is also clear that future studies require a larger control group.

Chapter 6

Investigations into the genome organisation of chromosomes 18, X and Y in human sperm

A manuscript containing the results from this chapter has been invited for submission by the Editor of Cytogenetics and Genome Research and is in preparation.

Chapter 6: Investigations into the genome organisation of chromosomes 18, X and Y in human sperm

6.1 Introduction

Within recent years there have been a number of studies published that have investigated the organisation of the genome within the interphase nucleus, these studies suggest that in interphase individual chromosomes tend to occupy discrete territories rather than intermingling with each other (reviewed by Bridger and Bickmore, 1998). To date there have been several studies that have investigated the organisation of chromosomes (i.e. chromosome position) within interphase nuclei of different cell types (including lymphoblastoid cells and dermal fibroblasts). These studies have provided evidence that the genome is highly organised within the nucleus and that correct organisation is critical for normal nuclear function. In humans, for example it has been demonstrated that gene-rich chromosomes tend to be located towards the interior with gene-poor chromosomes found in a more peripheral location in the nucleus (Bridger and Bickmore et al., 1998; Boyle et al., 2001; Croft et al., 1999). There is evidence that the organisation seen within human nuclei is conserved through evolution, as reported by Tanabe et al, (2002) who have undertaken genome organisation studies within primates. It has been suggested that the position of chromosomes within nuclei may play a vital role in gene expression and function, and that any change in chromosome location within the nucleus may be significant in some diseases (Greaves et al., 2001). As a result chromosome positioning is being used to investigate genome organisation in normal and diseased individuals to determine if there is a link between abnormal cell function and altered genome organisation. In one such study (Meaburn and Bridger, 2001) investigated the genome organisation in individuals found to have missing or mutated proteins implicated in genome organisation, specifically individuals presenting

with Emery Dreifuss Muscular Dystrophy (EDMD), evidence was provided for altered genome organisation in EDMD patients compared to normal controls.

Several studies have provided also evidence of preferential localisation of chromosome X within the spermatozoa of humans (Luetjens et al., 1999; Sbracia et al., 2002), monotremes (Watson et al., 1996) and marsupials (Greaves et al., 2001). The results published within these studies suggest that the position of chromosome X within the sperm nucleus corresponds to the point of first contact with the egg at the time of fertilisation (Greaves et al., 2001). This observation has lead several authors to believe that this may explain the increase of sex chromosome aneuploidy seen within ICSI derived conceptions (Luetjens et al., 1999; Sbracia et al., 2002; Terada et al., 2000). Terada et al. (2000) demonstrated that during ICSI the sperm nucleus undergoes atypical decondensation at the anterior end of the sperm nucleus upon injection within the egg. This is hypothesised to be due to the fact that in natural conception the acrosomal cap (located at the anterior of the sperm nucleus) begins to break down upon incorporation into the egg. This atypical decondensation is believed to hinder the onset of the first mitosis in the zygote, leading to an increased incidence of sex chromosome aneuploidy in ICSI derived embryos (Luetjens et al., 1999).

As detailed within section 1.3, correct chromosome segregation is vital for both fertility and survival, and failure of chromosome segregation to proceed normally is the leading cause of mental retardation and pregnancy loss within humans. During gametogenesis this process of chromosome segregation is highly organised (refer to figure 1.1), thus it would seem reasonable to suggest that it is likely that genome organisation plays a role in ensuring that correct segregation takes place and that genome organisation may also have a critical role in the post-fertilisation stages of development (Greaves et al., 1999).

6.2 Objectives

Given the above information the purpose of this study was to investigate whether there is evidence of a relationship between infertility phenotypes and genome organisation in male germ cells.

- In order to do this we investigated the position of chromosomes 18, X and Y in spermatozoa of 3 control males with semen parameters all within the normal range and 4 patients with reduced semen parameters.
- We investigated whether chromosomes 18, X and Y are preferentially located in sperm nuclei as they are in other somatic cells.
- In order to address these questions we carried out two types of analysis:

1) We investigated the position of chromosomes 18, X and Y in sperm nuclei relative to the centre of the nucleus in both patient and control groups.
2) We analysed the position of chromosomes 18, X and Y in sperm nuclei relative to the polarity of the sperm nucleus (in reference to head middle or tail.

6.3 Materials and Methods

The sperm FISH protocol and detailed explanation of the method of analysis (Bridger, personal communication) can be found within the materials and methods section (8.7.4.1).

Chromosome positioning analysis was undertaken for chromosomes 18, X and Y within the sperm of three control individuals with semen parameters within the normal range (refer to section 1.1) and four individuals with compromised semen parameters. For

each investigated chromosome (18, X and Y) a minimum of 100 spem nuclei (50 nuclei analysed on two different slides, and are referred to as repeat samples from herein), were analysed per individual, to determine if there is a relationship between infertility phenotypes and genome organisation in sperm cells.

6.3.1 Statistical analysis carried out for the investigations into the location of chromosomes 18, X and Y

Data produced from this study was analysed using the students t-test, this test was choosen due to its ability to determine any significant differences between the population means of two samples. Data was analysed using the Microsoft Excel statistics package. For all FISH experiments for each chromosome investigated and each individual enrolled in the study were carried out in duplicate (50 nuclei were scored on each occasion). In order to determine whether there was a significant difference between the results gathered in the first sample and the repeat sample a t-test (two-sample) assuming equal variance was utilised (as the two samples being tested were sperm cells from the same person). T-tests were also performed for each investigated chromosome, comparing each of the control males to each other and each of the patients to each other, to establish if there was any significant difference in the location of the chromosome territories within the control and that of the patient group. In each patient the results were also compared against each of the controls to determine any significant difference in chromosome territory location between the control population and the patients. When controls were compared against other controls, patients against other patients and controls against patients a t-test (two-sample) assuming unequal variance was utilised as the sperm cells were dervived from different

individuals. For both t-tests assuming equal or unequal variances two-tailed tests were utilised with cut off point for significance set at <5%.

6.4 Results

6.4.1 Genome organisation relative to the centre of sperm nuclei for chromosomes 18, X and Y in the control group

All analyses were repeated to ensure results were representative of the sample population. Statistical analysis for each control revealed no significant difference between the two sets of data collected for each chromosome, (results are presented in figure 6.1). Statistical analysis was also performed to establish if the location of individual chromosomes were significantly different between the control individuals. Statistical analysis revealed that for chromosomes 18, X and Y there was no statistical difference in the location of the chromosome territory between C1, C2 or C3, except for chromosome 18 for which a statistical difference was identified between C1 and C3 ($p=0.001$; t-stat= -3.39) with C1's mean chromosome 18 position located more towards the periphery compared to that found in C3. The data recorded was also investigated to establish if there was any difference in the location of the chromosome territory within the sperm nucleus (in terms of whether territories are located more towards the periphery or interior) for the investigated chromosomes 18, X and Y. This was determined by comparing the location of chromosome 18 to that of X and Y, and the location of X to Y in the same control patient. Analysis of the location of each chromosome in each control revealed that for C1 there was a significant difference in the location of chromosome 18 and Y ($p=2.6 \times 10^{-5}$; t-stat= -4.44) and that of 18 and X ($p=2.34 \times 10^{-7}$; t-stat= -5.56). No difference in location however, was identified between

chromosome X and Y, with chromosomes X and Y found more towards the interior than that of chromosome 18. For C2 no significant difference was established between chromosome 18 and Y or chromosome X and Y, however chromosome X was found to be located more towards the interior than that of chromosome 18 (p=0.00398; t-stat= -2.97). In the case of C3 no significant difference in chromosome location was observed between any of the investigated chromosomes.

6.4.2 Genome organisation relative to the centre of sperm nuclei for chromosomes 18, X and Y in the patient group

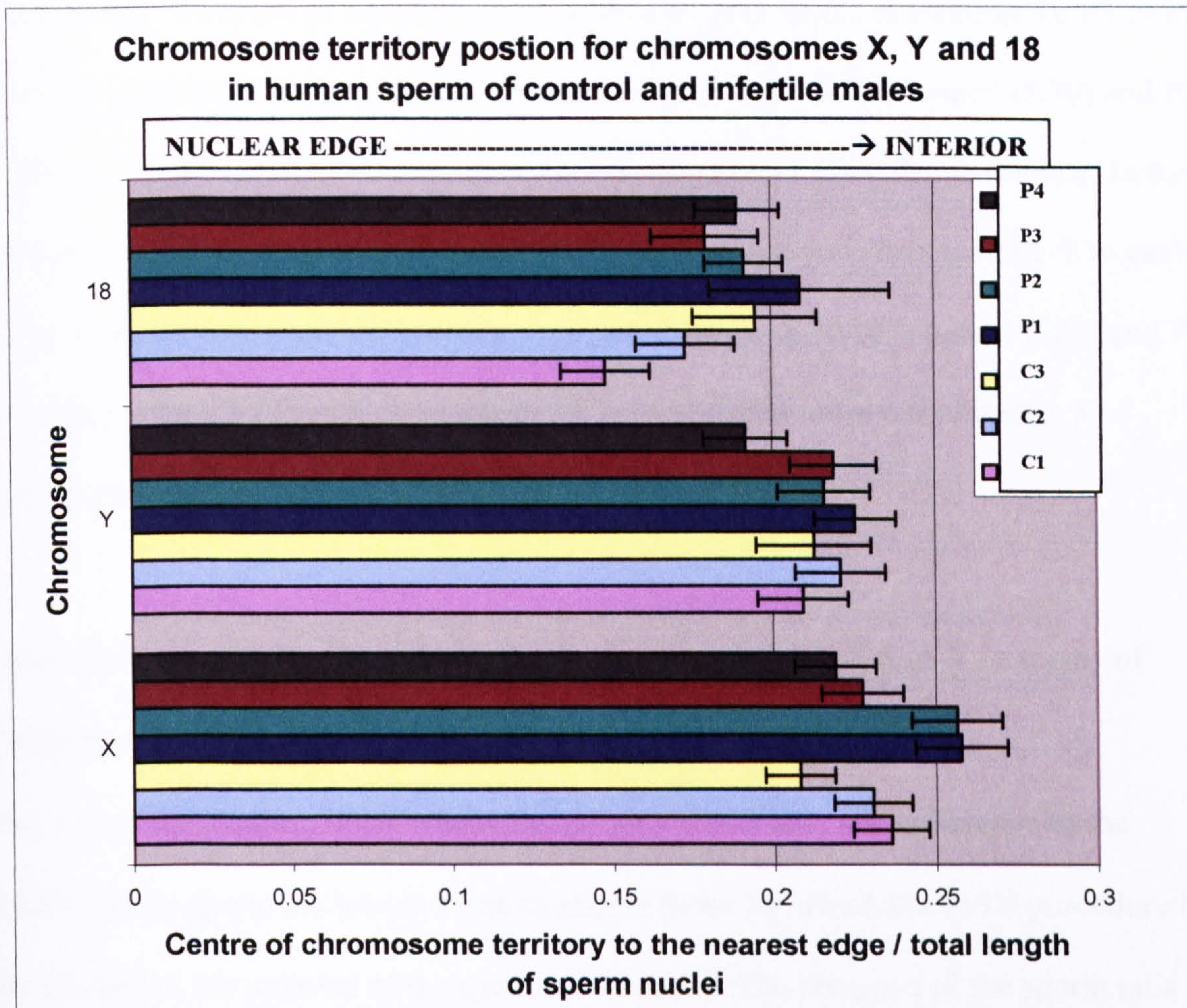
As in the control group all sperm analysis for the investigated chromosomes were repeated in the four patients investigated (P1, P2, P3 and P4). As with the control group the repeat data was compared to the first set of data obtained. For all four patients no significant difference was observed between the recorded data of the initial samples and that of the repeat samples. Statistical analysis was also performed to establish if the location of individual chromosomes were significantly different between the patients. Statistical analysis revealed that in the case of P1 the location of chromosome 18 was significantly different from that of P3 (p=0.019; t-stat= 2.39) and P4 (p=0.008; t-stat= 2.69) (both of which were found to be more peripheral) however, no significant difference was found in any other patients. Analysis of the data generated for chromosome X revealed a significant difference of territory position between P1 and P4 (p=0.013; t-stat= 2.55) and P2 and P4 (p=0.017; t-stat= 2.43), with P4 located more peripherally than that of P1 and P2. No significant difference was found in the location of the Y chromosome territory between patients with the exception of P1 and P4 (p=0.036; t-stat= 2.13) with P1 located more towards the interior. Analysis of the data was also investigated to establish if there was any difference in the location of the

chromosome 18, X and Y territory within the sperm nucleus between each of the patients (in terms of whether territories are located more towards the periphery or interior). The results of the statistical analysis found that there was no significant difference in the location of chromosome 18, X or Y in P1 or P4, however significant differences were identified in P2 and P3. The location of chromosome X was found to be more towards the interior compared to that of chromosome 18 in both P2 ($p=0.011$; t-stat= -2.60) and P3 ($p=0.003$; t-stat= -2.99).

6.4.3 Comparison of chromosome territory location relative to the centre of sperm nuclei found in controls versus patients

Statistical analysis was also carried out to compare the location of chromosome territories of chromosome 18, X and Y in controls and patients to determine if there is any significant difference in territory position between males with normal and compromised semen parameters. The three control individuals were grouped together and compared to each patient using a t-test to test for any significant differences in the location of chromosomes 18, X and Y. The results of the statistical analysis revealed that there was no significant difference in the location of chromosome Y in sperm nuclei between the control group and the four patients investigated. In the case of chromosome 18 a significant difference in chromosomal location was identified between the control group and P1 ($p=0.007$; t-stat= -2.74), however no other patients were found to exhibit any significant differences. Comparisons of the X chromosome between the control group and patients revealed no significant difference for P1, P2 or P3 however a significant difference was observed in P4 ($p=0.041$; t-stat= 2.07).

Figure 6.1 the location of chromosome 18, X and Y territories in the sperm nuclei of controls and patients (error bars represent SEM).



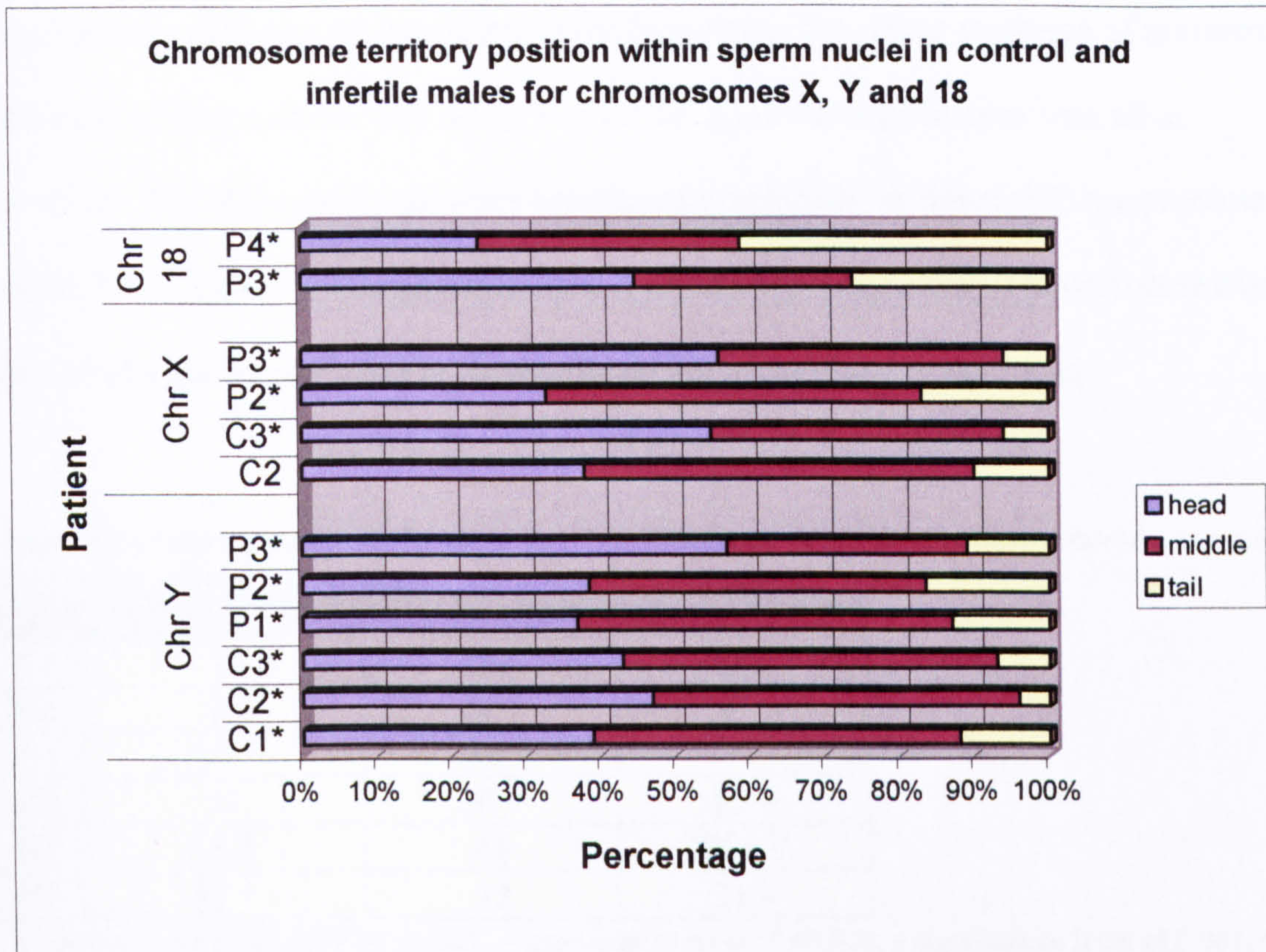
In order to determine if there was any difference in the location of the chromosomes in the patients (in terms of peripherally or interiorly located) compared to the control group the following were compared and analysed using t-test statistics: chromosome 18 (grouped controls) to chromosome X for each patient; chromosome 18 (grouped controls) to chromosome Y in each of the patients and chromosome X (grouped controls) to chromosome Y in each patient. The results revealed significant differences in chromosome location between chromosome 18 in the controls and chromosome X in P1 ($p=1.11 \times 10^{-5}$; t-stat= -4.66), P2 ($p=8.98 \times 10^{-6}$; t-stat= -4.69) and P3 (p=0.0003; t-stat= -3.67). With chromosome 18 within the control group located more peripherally than that of chromosome X with the exception of P4 for which no significant difference

was identified. When comparing chromosome 18 data from the control group to chromosome Y in each of the patients no difference was found in location for P3 or P4, however, significant differences were recorded for P1 ($p=0.0002$; t-stat= -3.79) and P2 ($p=0.044$; t-stat= -2.04), with chromosome Y located more towards the interior. In the comparisons between chromosome X in the control group and chromosome Y in each of the patients, significant differences were noted for P3 ($p=0.027$; t-stat= 2.24) and P4 ($p=0.008$; t-stat= 2.71) with chromosome Y being located more peripherally. No significant difference was observed in P1 or P2.

6.4.4 Chromosome territory position for chromosome 18, X and Y in terms of polarity, within the control and patient groups.

Analysis of the location of chromosome territory was made, with reference to the polarity of the sperm nucleus in some cases, (in those for which the FISH procedure had not resulted in the removal of the spermatozoa tails). The presence of the sperm tails enabled further analysis of the chromosome territories to be carried out (as described in section 8.7.4.1.2) these sperm nuclei were split into three regions (head, middle and tail). The location of the majority of the chromosome territory within these three regions were recorded for 50 sperm nuclei and duplicated in all investigated individuals with the exception of chromosome X in C2 for which only one slide was able to be analysed (due to the absence of sperm tails in the repeat sample). The results of these studies are presented in figure 6.2.

Figure 6.2- the location (in terms of head, middle or tail) of chromosome 18, X and Y territories in the sperm nuclei of controls and patients (* denotes the mean of two samples).



6.4.4.1 Statistical analysis of chromosome 18, X and Y position in sperm nuclei in terms of polarity

Chi squared statistical analysis was performed in each of these individuals for all chromosomes to determine if chromosome territories were randomly positioned within sperm nuclei (hence equally distributed throughout), or preferentially located within the regions investigated. Chi squared analysis was utilised to determine which situation pertained, to do this we assumed that if chromosome territories were equally distributed throughout sperm nuclei we would expect a third (33.3%) located in the head, a third (33.3%) located in the middle and a third (33.3%) located in the tail region. In the chi-squared calculations we therefore used 33.3 as the expected frequency, therefore if the observed frequencies were not found to be significant we would accept the hypothesis

of equal distribution within the nucleus. If observed frequencies were found to be significantly different we would reject the hypothesis providing evidence of preferential location of chromosome territories. The cut off point for significance was set at p=<0.05. The observed frequencies (displayed graphically in figure 6.2) are presented in tables 6.1, 6.2 and 6.3 (for chromosomes Y, X and 18 respectively). In each case any observed frequencies found to be significant are highlighted in bold text.

Table 6.1 displaying the results for the analysis of the location of the Y chromosome territory in terms of polarity expressed as a percentage

	Head (%)	***Middle (%)***	***Tail (%)***
C1	39	**49 ***	**12 ~**
C2	47	**49 ***	**4 ^**
C3	43	**50 ***	**7 ^**
P1	37	**50 ***	**13 ~**
P2	38.5	45	**16.5 ***
P3	**57 ^**	32	**11 •**

* denotes significance level of <0.025; ~ significance level of <0.005; • significance level of 0.001; ^ significance level of <0.0005.

The results from the Chi-squared analysis have revealed that, in the case of chromosome Y (table 6.1) there was evidence to suggest that the Y chromosome territories were not equally distributed throughout the nucleus. Controls C1, C2 and C3 and patient P1 were found to have significantly more chromosome territories within the middle region, and significantly less within the tail region if territories were equally distributed. P2 was also found to have significantly less chromosome territories within the tail region. Significantly greater and less proportions of the chromosome territories were found in the head and tail region respectively in P3.

Table 6.2 displaying the results for the analysis of the location of the X chromosome territory in terms of polarity expressed as a percentage

	Head (%)	*Middle (%)*	*Tail (%)*
C2	38	52 #	10 ^
C3	55 •	39	6 ^
P2	33	50 *	17 *
P3	56 ^	38	6 ^

* denotes significance level of <0.025; # significance level of <0.01; • significance level of 0.001; ^ significance level of <0.0005

Chi-squared analysis revealed that in the case of chromosome X, in all individuals analysed there was evidence to suggest that X chromosome territories are not equally distributed throughout the sperm nucleus. C2 and P3 were found to contain significantly higher and lower proportions of chromosome territories in the head and tail region and C2 and P2 were found to have significantly higher and lower proportions within the middle and tail region respectively, than would be expected if chromosome position was equally distributed throughout the sperm nucleus.

Table 6.3 displaying the results for the analysis of the location of the chromosome 18 territory in terms of polarity expressed as a percentage

	Head (%)	*Middle (%)*	*Tail (%)*
P2	45	29	26
P3	24	35	41

Statistical analysis of the location of chromosome 18 within the sperm nucleus in terms of polarity has found no evidence to suggest that the position of chromosome 18 within the sperm nucleus is not equally distributed throughout the three regions investigated.

Table 6.4 summarises the results of the investigations into the position of chromosomes 18, X and Y with regards to the two types of measurements carried out, (with reference to the centre and with reference to the polarity of the sperm nuclei).

Table 6.4- summarises the results of the investigation into the location of chromosome territories within sperm nuclei in terms of relation to the centre an polarity.

Chromosome	*Position relative to the head and tail*	*Position relative to the centre of the nucleus*
18	Equally distributed throughout the nucleus	Found to be the most peripherally located of the three investigated chromosomes
Y	Evidence to suggest preferential location within the head and middle region	Location similar to that of 18 with evidence of more interior location in several individuals
X	Evidence to suggest preferential location within the head and middle region	Location of chromosome X was found to be the most interiorly located chromosome compared to Chromosomes 18 and Y

6.5 Discussion

In this study, we found only limited evidence for our initial hypothesis that men with compromised semen parameters have increased levels of genome disorganisation for the chromosomes examined. With significant differences in the location of chromosome 18 and X (in P1 and P4 respectively) compared to the data obtained in the control group. Due to the small sample size for both the control group and infertile group, it is possible that these may be attributted to normal variation in genome organisation. Nevertheless, even in such a small sample population these results provide some evidence to support the hypothesis that in some individuals with compromised semen parameters there may be perturbed levels of genome organisation. Clearly further studies need to be performed with the recruitment of a much larger sample population and, in particular, a correlation between the level of sperm aneuploidy and the degree of genome organsiation for a wider range of chromosomes needs to be established. Our results indicate, that as in lymphoblastoid cells, and human dermal fibroblasts there appears to be some degree of genome organisation (Boyle et al., 2001) and preferential location for chromosomes 18, X and Y within sperm nuclei.

Further analysis of chromosome territory position was also made possible in sperm cells retaining there tails after FISH analysis. This enabled the location not only to be determined as peripherally or interiorly located, but also in terms of head middle or tail position. The results from these studies suggest that the sex chromosomes were not equally distributed throughout all three regions. In the case of the Y chromosome, significantly higher levels were found in the middle region and significantly lower levels in the tail region in all three controls, the same situation pertained in P1. In P2 significantly lower levels were found in the tail region than would be expected if chromosome Y was equally distributed with the remainder of territories roughly equally spread over the head and middle regions. Whilst in P3 significantly higher and lower levels were found in the head and tail region respectively. Analysis of chromosome X in the four individuals analysed revealed that in two cases significantly higher levels were found in the head region with the remaining two cases having significantly higher levels within the middle region. All four individuals were found to have significantly lower levels found in the tail region than expected if chromosome X was equally distributed. In the case of chromosome 18 we found no significant evidence of a preferential localisation; analysis revealed chromosome 18 to be equally distributed throughout. Previous reports have demonstrated delayed nuclear decondensation of the apical region of the sperm nucleus after ICSI (Terada et al., 2000). In studies in Rhesus monkeys after ICSI the apical region of the sperm nuclei remains condensed surrounded by the peri-nuclear theca, a unique cytoskeleton structure important in stabilizing sperm structure in mammalian sperm (Sutovsky et al., 2003). This structure begins to dissassemble during incorporation into the egg (hence in natural conception and in IVF), however this structure remains intact in ICSI resulting in atypical decondensation. Terada et al. (2000) hypothesise that the unusual nuclear remodelling in the first cell cycle of ICSI

may prevent the synchronised decondensation of all chromosomes. This in turn may result in increased ICSI derived chromosome anomalies (for chromosomes located within this apical region) by delaying the S-phase entry and hence leading to mitotic errors during the first cleavage. In light of this data several studies have investigated the position of chromosomes 18 and that of the sex chromosomes (due to the increased rate of sex chromosome aneuploidy in ICSI offspring) within sperm nuclei (Luetjens et al., 1999; Sbracia et al., 2002; Terada et al., 2000). The study carried out by Leutjens et al. (1999) reported that chromosome 18 was located in the tail region in 60% of the 190 sperm nuclei analysed, in contrast we found an average of only 33% of chromosome 18 territories within the tail region. Sbracia et al. (2002) reported rates for chromosome 18 of 14.49%, 31.35% and 54.36% in the head, middle and tail region, in contrast we report a more equal distribution throughout all three areas with respective rates of 35%, 32% and 33%. The same study also reported rates of 40.94%, 50.29% and 8.77% for chromosome X and 52.94%, 38.14% and 8.93% for Y chromosome (in head middle and tail regions respectively). In agreement we report similar rates of 45%, 45% and 10% for chromosome X and 44%, 45% and 11% for chromosome Y. The study carried out by Luetjens et al. (1999) reported much higher rates of chromosome X position within the tail region with >40% of 230 sperm nuclei, compared to the results generated in this study with an average of 10% located in the tail region and around 45% located in both the head and middle region. All studies including this one report provide evidence for the preferential location of the sex chromosomes within the head and middle region of the sperm nucleus with significantly lower levels found within the tail region (except for that of the Leutjens study). This is of relevance as the study carried out by Terada et al. (2000), analysed the postion of the sex chromosomes in 12 sperm after ICSI injection into hamster eggs, at 2 and 6 hours. Of these 12 three had a sex chromosome located in

the apical region, in contrast to the other nine analysed, after six hours these sex chromosomes remained condensed. These findings along with the evidence of increased rates of ICSI derived sex chromosomes aneuploidy (Bonduelle et al., 1996; 1999; 2002) have serious implications and suggest that the technique itself may play an important role in the increased rates of aneuploidy seen. Clearly further research should be initiated to determine if this is the case and whether males with compromised semen parameters are at any increased risks. Also these studies have not investigated genome organisation other than in reference to the head, middle or tail region it occupies and make no inference as to whether chromosomes occupy discrete domains and in relation to each other whether specific chromosomes are more peripherally located etc. This is clearly of importance especially given the fact that genome organisation in diseases such as EDMD have been shown to altered (Meaburn and Bridger., 2001). Future studies should be undertaken to determine the "normal situation" in control males with normal semen parameters and those with compromised semen parameters. Investigations should also be investigated in other testicular cells derived from testicular biopsies which will provide us with important information how the genome is organised throughout spermatogenesis.

Chapter 7

General discussion

Chapter 7: General discussion

7.1 Project outcomes relative to specific aims

In general terms, the work in this thesis has been largely successful in the completion of the specific aims set out in section 1.8. Significant steps have been made towards establishing the need for, and implementation of a pre-ICSI aneuploidy screening kit. Clues to the aetiology of the phenomenon of sperm aneuploidy has been provided through the analysis of semen parameters and genome organisation. Furthermore to the best of our knowledge we have provided the first report of a reduction in sperm aneuploidy levels coincident with any medical intervention. Specifically the following has been achieved:

- Statistical analysis of existing questionnaire data, has revealed that sperm aneuploidy is perceived as a serious problem in infertility clinics.

- Statistical analysis of the existing questionnaire data, also established that the majority of individuals surveyed believe there to be merit in pre-screening men for levels of sperm aneuploidy prior to treatment of ICSI.

- Significant steps have been taken towards the development and implementation of a kit that will allow the screening of sperm aneuploidy in males undergoing ICSI.

- Evidence is presented that sperm aneuploidy levels can vary significantly between samples in infertile men.

• Preliminary evidence has been provided that suggests chromosome-specific and parameters-specific mechanisms with regard to sperm aneuploidy.

• Evidence is presented that, in 6 males, sperm aneuploidy levels have reduced significantly coincident with traditional Chinese herbal medicine treatment.

• Evidence has been provided for the anti-oestrogenic and anti-oxidant activities of the individual herbs used in the TCM treatment of these six individuals.

• Evidence, has also been provided for the anti-oestrogenic and anti-oxidant activities of the herb mixtures used in the TCM treatment of two of the six individuals.

• Evidence that chromosomes 18, X and Y occupy discrete domains within human spermatozoa is presented.

• Finally evidence is presented that selected chromosomal domains may occupy different positions in some infertile males.

7.2 Development of a sperm aneuploidy screening kit for clinical use

Within chapter 2, the development of the sperm screening kit is detailed, with the concentration and incubation times established for the decondensation buffers and denaturation. During the development of the technique for the screening kit two probe protocols were tested within the development of the screening kit, at the onset of the study the combinatorial probe approach was utilised, this entailed a four probe approach. In this case chromosome Y was labelled in Spectrum Green, chromosome 21

in Spectrum Orange and chromosome X used a combination of two probes labelled with different fluorochromes to produce a third colour (Spectrum Green and Spectrum Orange to produce a yellow colour), (figures 2.1, 2.2 and 2.3). This approach however, produced several issues with regards to use within a screening kit. The first of which was the fact that it was not reproducible, in that the combination of the two X chromosome probes did not always produce a yellow colour, at times it would be difficult to distinguish it from chromosomes X and 21 as the probe was either more green or red in colour. This probe protocol was hence not suitable for incorporation into such a screening kit. Secondly the fact that it required 4 probes, which would make it more costly, again not ideal for a screening kit. Latterly within this study we switched to a three probe non-combinatorial approach (figure 8.5) using probes labelled in Spectrum Orange (21), Spectrum Green (X) and Spectrum Aqua figure (Y), to eliminate the reproducibility issues found with the first probe protocol. This probe protocol was not used at the outset of the study as the Leica microscope system used did not have the appropriate filter sets for analysis of these fluorochromes. At first we tested the new probe system with a quad band pass (including DAPI, Spectrum Orange, Spectrum Green and Aqua), however we encountered problems identifying the Aqua Y probe from the DAPI counterstain. More recently we have obtained a triple band pass (containing a Spectrum Orange, Spectrum Green and Aqua filters), to eliminate this problem. Since switching to this three-colour probe system we have attracted the interest of a company Stretton Scientific LTD, who are interested in commercialising this kit. Initial studies however still suggest a difficulty in distinguishing the Aqua probe from the DAPI counterstain though this may be the fault of our current filter. At present a number of issues remain that need to be resolved, these include the probe approach used, firstly we need to identify if there is a problem distinguishing the aqua probe from

the DAPI background through the rigorous testing of this protocol on a wide range of samples. Once the new probe protocol has been established and thoroughly tested, we would want to send the protocol and probes out to at least five other laboratories to test the reproducibility and robustness of the kit.

We realise that the implementation of such a test within infertility clinics will not be possible to be undertaken unless it is in conjunction with an automated scoring system (due to the time constraints imposed by the sheer number of cells required to be scored). Within the past year we have tested several automated dot counters (from Applied Imaging and Imaging Associates), both companies have produced systems that are capable of undertaking such analysis reliably. The implementation of an automated scoring system would have many benefits, including: significantly reduced analysis time, analysis that can be carried out 24 hours a day. It will also enable identical scoring criteria to be programmed and set up for each infertility clinic eliminating the potential problems of observer bias that may contribute in part to some of the significant differences in aneuploidy levels found between studies (refer to section 1.5). It will also eliminate any potential problems of not being able to identify the Aqua Y probe from that of the DAPI counterstain. In conjunction with Stretton Scientific LTD, we hope to distribute this screening kit to infertility clinics, enabling some of the questions to be answered from chapter 1. Including the risk of chromosome aneuploidy in children conceived through ICSI, those individuals with high levels of sperm aneuploidy can opt for further follow-up in any conceptions with amniocentesis and the karyotyping of children conceived through ICSI.

7.3 Evidence of a correlation between semen parameters and sperm aneuploidy

As seen in chapter 3 we have provided preliminary evidence of correlations between individual semen parameters and increased disomy of individual chromosome pairs, while statistically significant, warrants further investigation. Closer correlations of disomy rates in men with defects in only one of the three criteria used to measure semen quality will form the basis of future investigations, however this was not possible within this study as no individuals were identified with reduced semen quality in only one parameter. Through these studies, a closer understanding of the mechanistic basis of the relationship between chromosome segregation and infertility will be achieved. Further confirmation of this could also be obtained utilising probes for different autosomes, hence establishing for example if aneuploidy in any other autosome pairs are significantly increased in individuals with reduced sperm count (as established for chromosome 21 disomy but not for the sex chromosomes).

In the light of our results it is possible that a common mechanism leading to oligozoospermia and increased levels of XY disomy involves a perturbation in the mechanisms of synapsis and/or recombination in the XY paring region. These results generated in this study have formed part of a successfully funded Wellcome grant, in which to further test this hypothesis, single sperm PCR (Huang et al., 1995) will be used. In 10 severely oligozoospermic males and 10 controls, primers located within and outside the XY paring region will be used in these studies to determine whether there is evidence to support the absence or reduction of recombination within the X-Y pairing region. A significant difference between oligozoospermic males compared to controls would provide evidence to support our hypothesis. Clearly there is plenty of scope for further investigation based upon these preliminary observations, however one of the

shortcomings of the current study as it stands (presented in chapter 3) there is the lack of a second control group (men of proven fertility). The basis of the study was to compare individuals with reduced semen quality for specific semen parameters, against those that are within the normal range (however, in the majority of cases these individuals had reduced semen quality in the other parameters investigated). Clearly we realise the importance of having a second control group (consisting of individuals of proven fertility) and is something that is lacking from this study, however this was due to difficulties in recruiting control subjects. As part of future studies, such a control group, from which we will obtain at least four repeat samples. At present we have recruited four such individuals, however due to time constraints it was not possible to perform and include the analysis of these individuals.

7.4 Evidence of reduction of sperm aneuploidy levels for chromosomes 21, X and Y in males coincident with TCM treatment and biological activity of TCM prescriptions

We have provided novel evidence of the efficacy of TCM in the treatment of male infertility specifically that of reductions within disomy frequencies for the investigated chromosomes coincident with treatment. Within the past few years there has been an increasing use of TCM (including individuals within the medical profession), to date several studies have been published assessing people's views and attitudes towards TCM. Koh et al. (2003) questioned pharmacists attending the 61st International Congress of the International Pharmaceutical Federation, on their knowledge and attitudes towards complementary alternative medicine (CAM) including that of TCM. In total 84% of the 420 individuals asked revealed that at some point they themselves had used CAM in the treatment of both acute and chronic conditions. Receiving

information on these therapies from a range of sources including: books and magazines, friends and family and the Internet. Of these individuals 79% would recommend CAM treatment to friends and family. This study also indicated that 81% of these individuals felt they had inadequate knowledge to use these treatments in patients, however, of interest is that over 90% felt that the professional curricula should contain more CAM components. Fontanarosa and Lundberg, (1998) reported that the fundamental issue is not Western medicine versus TCM, but medical practice supported by clinical and scientific evidence.

In Western medicine over half of the drugs used in the treatment of illnesses has been derived from plants (New Scientist, 2001; Yuan and Lin, 2000), therefore it is likely that the plant based herbs as used in TCM are likely to contain components that have medicinal benefit. The herbal combinations that are used, in TCM remains an untapped source for the discovery of new drugs. Traditionally Western drug discovery focuses on one plant type at a time in order to identify single active compounds, whereas TCM uses 10-20 different plants within prescriptions, therefore it is likely that in many cases the activity of these plants are based on the interactions of different compounds (Yuan and Lin, 2000).

As reviewed in chapter 4 there have been a number of successful randomised clinical trials that have provided evidence of the efficacy of TCM treatment in a range of diseases including: atopic dermatitis; atopic eczema; bronchial asthma and irritable bowel syndrome. Clearly it is beyond the scope of this study to determine if the effects seen are due to a placebo effect or improvements in lifestyle for example, however we have provided evidence of the biological activities (including that of endocrine and anti-

oxidant activity) of the herbs used in TCM treatment. We have provided strong preliminary evidence to support the potential efficacy of TCM in the treatment of male infertility, clearly much more research is required and warranted. Over the last decade a great deal of pharmacological research has been undertaken to further our understanding and establish reliable composite formulae of TCM natural products (Wang, Z.T et al., 1995). Further research needs to be carried out to determine the principal active components of the investigated herbs; this would be possible through the separation of components through counter-current chromatography (CCC). We have initiated a potential collaboration with Brunel Institute for Bioengineering to use this technique to isolate the individual components of the herbs. These investigations will lead to the isolation and characterisation of the active components of the herbs, which in turn will lead to a better understanding of the chemical properties in the herbs that appear to be effective in the treatment of infertility. The technique of CCC will also establish a generic method for the purification of TCMs, hence providing samples for detailed chemical and biological analysis. It is also clear that the integration of Chinese TCM and western pharmacology constitutes a potentially rich source for drug discovery and development (Gong and Sucher., 1999). The therapeutic efficacy of isolated compounds may be related to the possible interactions of the individual ingredients, or even through the metabolism of these compounds within the body that may be responsible for the resultant activities. Therefore a barrage of tests must be undertaken to investigate the effects of such complex TCM preparations.

Based on our findings of a reduction in sperm aneuploidy coincident with TCM treatment and evidence of the biological activity, we feel there is enough preliminary evidence to justify continuation to a double-blind placebo controlled clinical trial. This

would begin the address the question of whether the evidence provided in this study is as a result of the TCM treatment and will enable the existence of placebo effects and lifestyle changes. A detailed clinical trial will also enable more patient access and produce large amounts of data hence making it possible to determine how these may be acting i.e. the pathways through which these herbs may be acting. It would be possible to produce data on reactive oxidant species (ROS) within sperm (therefore individuals identified with high ROS in sperm, levels could be monitored to determine if this improves with the treatment of anti-oxidant herbs for example). A clinical trial may make it possible to monitor hormone levels coincident with treatment to determine if there are any changes, also possibly measure concentrations of known xenoestrogens e.g. PCB and PE in seminal plasma as in the study carried out by Rozati et al. (2002). This may provide a correlation either providing evidence of a potential link with environmental oestrogens or ruling it out as a possibility. Gathering of detailed patient data such as these are not possible in open-label studies such as these therefore hopefully will determine how these are working if it is through these routes or whether it is through an alternative route.

7.5 Chromosome positioning

The research presented within chapter 6, illustrates that as in other cell types (including lymphoblastoid and fibroblast cells) there is evidence that the investigated chromosomes (18, X and Y) occupy discrete chromosome domains within sperm nuclei. The results presented within this chapter form the basis of preliminary evidence, a pilot study to determine if there is evidence of genome organisation within sperm for chromosomes 18, X and Y. Clearly the evidence provided of genome organisation requires further investigation especially with regards to correlating the data with

aneuploidy frequencies for the investigated chromosomes, it is also clear that more controls and patients need to be recruited. Despite the relative small numbers of individuals investigated the results of this study provide evidence for the genome organisation of chromosomes 18, X and Y in terms of location relative to the centre of the nucleus with chromosome 18 located more peripherally and chromosome X located more towards the interior. We also provided evidence of the preferential location of the sex chromosomes within the head and middle region of sperm nuclei with intact sperm tails, whilst the location of chromosome 18 appeared to be equally distributed throughout the head, middle and tail region. Preliminary evidence was also provided for differences in chromosome location within the nucleus in several of the infertile patients investigated compared to that of the control population. The results presented in chapter 6 suggesting evidence of preferential location within the sperm nucleus in terms of both that of peripheral and interior, and in polarity in terms of head middle and tail, for chromosomes 18, X and Y and differences found between a small number of individuals clearly demonstrate the requirement for further analysis. Future investigations should extend these studies to a larger patient and control cohort, preferably in patients for which significantly increased aneuploid levels have been identified in the sperm for the investigated chromosomes compared to controls. They should also include several other autosomes that are known to be gene rich and gene poor chromosomes (located in the interior of the nucleus and the periphery of nuclei respectively in lymphoblastoid and fibroblast cells investigated). This will determine if chromosome territory position is organised, if there is evidence of organisation, whether it is organised as in the somatic cells previously investigated, or whether genome organisation within germ cells is different. Studies investigating the position of chromosome territories within other testicular cells derived from testicular biopsies will

also generate important information on how the genome is organised throughout the process of spermatogenesis. This is likely to provide evidence as to what stage within spermatogenesis genome organisation may become perturbed, enabling not only spatial errors of genome organisation of infertile males but also the identification of the stage at which these errors occur.

At present our understanding of what structures are responsible for genome organisation in germ cells is limited, however a number of structures of the nuclear envelope and proteins including the nuclear lamina, integral membrane proteins (Alsheimer et al., 1996a) and the nuclear matrix that binds active regions of the nuclear matrix (Vclek et al., 2001) have all been implicated. The nuclear structure of cells undergoing spermatogenesis has been demonstrated to undergo fundamental changes throughout this process. Examples of this is the expression of B3 and C2 lamins in spermatocytes only, and that the constituents and structure of the nuclear matrix changes throughout spermatogenesis, which are likely to be as a result of an adaptation to meiosis-specific nuclear function (Alsheimer et al., 1996b). Alsheimer et al. (1999) determined that lamin C2 is localised at segregated regions of the nuclear envelope in male meiotic cells, with chromosome ends, bound through synaptonemal complexes, associated with these C2 dense regions. Meaburn and Bridger, (2001) report the strict genome organisation found in somatic cells is absent in cells derived from individuals with EDMD, found to have proteins implicated in genome organisation either mutated or lacking. Therefore it would be reasonable to suggest that if nuclear structures and proteins within the cell types undergoing spermatogenesis was altered from that found in the normal situation, may lead to altered genome organisation. This could also result in abberant chromosome segregation which may in turn be responsible for some of the

increases in aneuploidy within the sperm of males with compromised semen parameters. Clearly further studies need to be carried out in normal and severely infertile males with significantly higher levels of chromosome aneuploidy. In order to identify if the preliminary evidence provided here of strict organisation (in terms of peripheral or interior location) is found for all chromosomes and what positions these occupy. Once determined if there is evidence of strict organisation, it will be possible to identify if this is perturbed in males with compromised semen parameters, and enable us to examine the role of these proteins in these patients.

7.6 Self-criticisms of the project

In retrospect there are a number of subjects that could have been dealt with differently, firstly with regards to the screening kit, given the opportunity we should have switched from the combinatorial (four) probe approach to the three colour approach, earlier on in the study, as it was clear, due to the lack of reproducibility of this probe approach that it would not be suitable for use within a screening kit. The reasons for not switching to the three colour approach was due to the fact that the fluoresent microscope did not have the appropriate filters. Switching to the three colour approach earlier would have meant that we could have generated more patient data using this approach and invested more time in resolving the issues that have arisen as a result of this protocol. The development of the screening kit would, hence have been further along through to the testing stages by now.

Secondly, recruitment of patients was also a difficult issue and also for different control groups for the various studies. We have managed to recruit a number of controls for this

study, however for several investigations we could have done with more controls. In particular for the study into correlations between semen parameters and sperm aneuploidy (refer to section 7.3) and also for the studies into genome organisation. The controls used in previous studies could not be used as they were stored in the sperm buffer at 4°C, which may have affected the genome organisation in the sperm nuclei. In this study new control samples had to be used that were fixed and stored at -20°C to prevent this, as a result we were only able to obtain three samples. Given more time it would have been possible to recruit and investigate more patients in each of the studies reported within this thesis particularly for the positioning and TCM aneuploidy studies, however due to time constraints this was not possible.

Thirdly one of the major issues is the lack of karyotype analysis performed. The ideal situation would have been to perform this analysis on all the patients enrolled in the studies. This, however, was not possible due to the consent not given by patients to perform such analyses in the vast majority of individuals with a few rare exceptions. The occurrence of chromosome abnormalities has indeed been reported to be significantly higher within infertile males than that of their normal fertile counterparts (refer to section 1.5). The increased rate of such abnormalities however, is mainly associated with severe oligozoospermic males (those with <5 million/ml) (Patrizio and Broomfield, 1999), within these studies there were no males classified as severely oligozoospermic. Even if karyotype analysis had been performed in each of the patients it is unlikely that any chromosomal aberration identified would have altered any of the conclusions reached in these studies. For example in the TCM studies it is unlikely that even if a karyotype abnormality was identified in one of the patients that it would have altered our conclusions that there is preliminary evidence of the efficacy of TCM in the

treatment of male infertility. Given more time it would have also been possible to carry out Y deletion analysis and analysis of CF mutations (refer to sections 1.3 and 1.2.1.1.1 respectively) however, as with karyotype analysis these existence of these would have been unlikely to affect our conclusions. These karyotype analyses would have enabled us to present a more complete patient assessment and would have ruled out these aberrations as the cause of the infertility. Given the time and opportunity, within these studies these would have been performed.

Fourthly, in retrospect and given more time we should have created detailed lifestyle questionnaires, that should have been completed at the time of each semen assessment (especially for repeat samples). This is of particular importance with regards to the TCM study, and would have enabled us to discount individuals or perhaps provide a possible explanation that may account for the reductions seen in disomy levels if, for example individuals had significantly reduced their dietary intake of cigarettes and alcohol. Future studies will include a well designed lifestyle questionnaire that should be completed by patients throughout treatment. It should be noted that in all cases individuals were required to provide detailed lifestyle questionnaires at the time of the first semen assessment, however, any changes in the lifestyle of these individuals were not monitored. It is unlikely though that changes in dietary factors such as smoking and alcohol would have caused the significant reductions in aneuploidy in these six patients as initial lifestyle questionnaires revealed, that none smoke and all consumed under the recommended allowance of alcohol units per week.

7.7 Concluding remarks

At present there are many questions that arise over the apparent increase in infertility, particularly within Western populations, at present our knowledge and understanding of the mechanisms behind this is limited. Yet we have created radical treatment regimes for infertility (without, in many cases, knowing the cause for this infertility). Indisputably this is a cause for concern, indeed ICSI has raised concerns over the increased genetic risks to unborn children and transmission of infertility. Within these studies we have demonstrated significant differences in aneuploidy levels within the gametes of infertile males, ranging from individuals with levels comaprable to controls and individuals with up to 15 fold increases compared to controls. The differences observed between infertile males clearly have clinical ramifications, with the outcomes of ICSI being monitored carefully especially with regards to the karyotype of offspring and the careful monitoring of the fertility of these individuals. The results presented in this thesis hopefully shed some light onto some of the potential mechanisms that may be involved in infertility, for example the identification of chromosome specific semen parameter correlations and the preliminary investigations into genome organisation. Clearly it is essential for the further understanding of infertility that these studies are continued. Futher research is also warranted in alternative treatments to ICSI, treatments that are less radical and invasive without the potential risks associated such as TCM. We are still a long way from understanding the mechanisms of infertility and only through further research and understanding of these factors will safe and reliable methods be available for the treatment of infertility.

Chapter 8

Materials and methods

Chapter 8: Materials and methods

Within this materials and methods section, unless stated otherwise all chemicals were research grade and were purchased from Sigma-Aldrich Ltd. (Poole, UK). All sterilisation was undertaken by one of two methods, either through filtration (disposable filters with a pore size of 0.2μm Whatman Puradisc) or through the process of autoclaving, heating up to 121°C for 10-15 minutes. From herein sterilisation is specified as being carried out by either filtration or autoclaving.

8.1 Karyotyping

8.1.1 Preparation of human chromosomes from blood

This procedure was carried out in a BSB 3A Gelaire® laminar flow cabinet type. A total of 1ml of human blood was transferred into a sterile plastic tissue culture flask, to this flask 19ml of PB-Karyomax medium (Invitrogen, Paisley, UK) was added. The flask (containing blood and growth medium) was subsequently transferred to a 37°C incubator for 72 hours.

8.1.2 Harvesting of human chromosomes

As before this procedure was carried out in a BSB 3A Gelaire® laminar flow cabinet. After the 72-hour incubation period, 200μl of 50μg/ml colcemid (Invitrogen, Paisley, UK) was added to the flask to arrest the cells in metaphase, the flask was then returned to the 37°C for 40-60 minutes. Meanwhile a 75mM KCl hypotonic solution was prepared and pre-warmed to 37°C.

After the 40-60 minute incubation, the culture was removed from the 37°C incubator gently mixed and equally transferred to two 15ml centrifuge tubes. These tubes were then centrifuged at 1200 rpm for 5-7 minutes, after centrifugation the supernatant was removed and the pellet gently re-suspended (in a drop wise manner) in approximately 10ml of the 75mM KCl hypotonic solution (pre-warmed to 37°C) these centrifuge tubes were then returned to the 37°C incubator for a further 12 minutes. Following this incubation period 5ml of fixative (3:1 methanol: acetic acid) (BDH, Dorset, UK) was added to the centrifuge tube and the tube was inverted to mix. The centrifuge tube was centrifuged as before (1200 rpm for 5-7 minutes), after centrifugation the supernatant was removed and the pellet re-suspended (drop wise) in 5ml of fixative (3:1 methanol: acetic acid). Following the addition of the fixative the centrifuge tube was centrifuged at 1200 rpm for 5-7 minutes, and the process of adding fixative drop wise followed by centrifugation was repeated a further 4-6 times. Subsequently chromosomes are stored at -20°C until required.

8.1.3 Preparation of slides

Centrifuge tubes containing harvested chromosomes are removed from -20°C and centrifuged as before, the supernatant is then removed and the appropriate volume (usually between 0.5-3ml) of fresh fixative is added to the centrifuge tube, to obtain the correct cell density. Using a pipette approximately 15-20µl of the cell suspension was dropped onto a clean glass slide (from a height of between 10-20cms to aid in disrupting cell membranes). On top of this cell suspension two drops of fresh fixative was added to aid in spreading and fixing chromosomes to the slide. The slide was then checked under phase contrast (x10 magnification) to check for the presence of adequate numbers of metaphase spreads and ensuring that metaphases were well spread with little

or no crossovers as possible. Slides were then dehydrated through an ethanol series of (70, 80 and 100%) and placed in a 37°C incubator overnight to age (to harden the chromosomes).

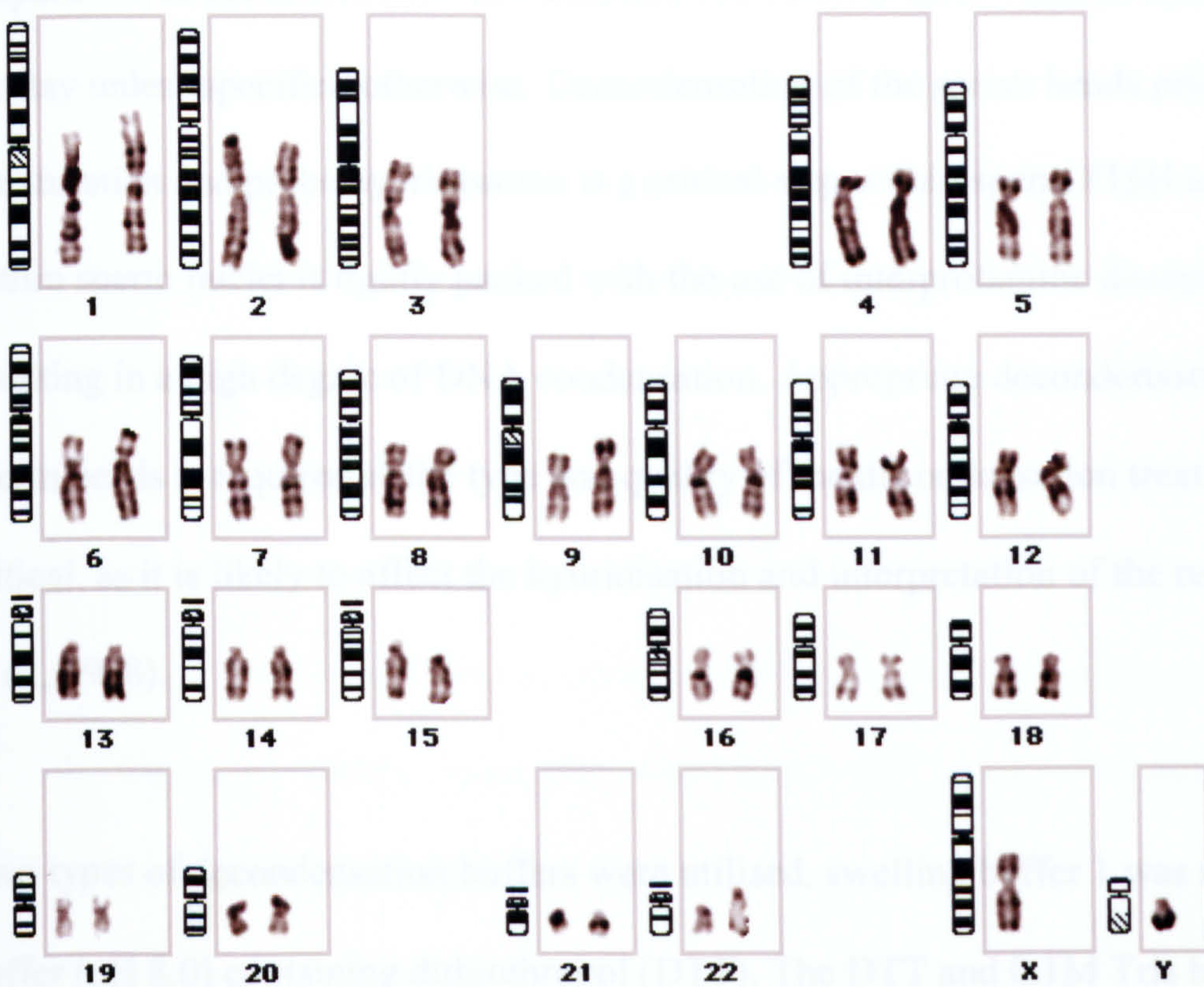
8.1.4 Staining of slides with Vectasheild DAPI

Slides were removed from the 37°C incubator the following day, to each slide 50-100μl of anti-fade Vectasheild mounting medium with DAPI (Vector Laboratories, Peterborough, UK) was added and a coverslip placed on top.

8.1.5 Microscopy and karyotype analysis

Metaphases were analysed using a Leica DM 35 fluorescence microscope and captured using Smart Capture version 3.2 (Vysis Digital Scientific, UK). In total for each patient a minimum of 10 metaphases were karyotyped and the number of chromosomes were counted in at least 30 metaphases (to exclude the likelihood of chromosomal mosaicism). Images to be karyotyped were then exported into Quips CGH Karyotyper (version 3.1.1, Vysis, UK), the image of the DAPI banded chromosomes was then inverted to give a grey scale reverse DAPI banded image (figure 8.1). The metaphase was then karyotyped using the various tools available within the CGH karyotyper package.

Figure 8.1- illustrates the reverse DAPI banded karyotype of a normal 46,XY male



8.2 Methods for determining aneuploidy levels within spermatozoa

8.2.1 Preparation of reagents for sperm FISH analysis:

8.2.1.1 Sperm buffer used for storage of samples:

Preparation of the sperm storage buffer 10mM Tris HCl (BDH, Poole, UK), 10mM NaCl (BDH, Poole, UK) (pH 8.0), was performed as follows: glass bottles were sterilised prior to use by filling with ddH_2O and autoclaving. Once autoclaved the ddH_2O was removed, the sperm buffer was prepared and transferred to the sterilised glass bottles and sterilised as before through autoclaving. Once autoclaved the sperm buffer was stored at room temperature (RT) until use.

8.2.1.2 Decondensation buffers:

Preparation of decondensation and denaturation buffers: these were all carried out on the day unless specified otherwise. Decondensation of the sperm heads prior to denaturation and probe hybridisation is a critical step within sperm FISH as DNA within sperm nuclei is tightly packed with the use of interprotamine disulphide bridges resulting in a high degree of DNA condensation. Appropriate decondensation of the sperm heads is required as the type and quality of the decondensation treatment is critical, as it is likely to affect the hybridisation and interpretation of the results (Rives et al., 1998).

Two types of decondensation buffers were utilised, swelling buffer 1 was a 0.1M Tris buffer (pH 8.0) containing dithiothreitol (DTT). The DTT and 0.1M Tris buffer were made up separately and combined prior to use on the day. A 1M stock of DTT was prepared and transferred into sterile eppendorfs in aliquots of 400μl, and stored at -20°C, the 0.1M Tris buffer (pH 8.0) was prepared in sterile glass bottles and stored at RT. Prior to use the 1M DTT aliquot was removed from -20°C and thawed slowly at RT (in the dark, light sensitive), once thawed this was added to 40ml of 0.1M Tris and placed in the dark until use. Swelling buffer 2 was used in addition to swelling buffer 1 in samples that had reduced/poor hybridisation as we found that it further aided permeabilisation of the sperm head to enable probes to further access the DNA within the sperm head producing more intense hybridisation signals. Swelling buffer 2 comprised of 0.1M Tris buffer (pH 8.0) and lithium salt (LIS). Aliquots of 1M (LIS) was prepared and stored in a sterile universal at 4°C in the dark, on the day of use a 25mM LIS/0.1M Tris buffer (pH 8.0) was prepared and then stored in the dark.

8.2.1.3 Preparation of denaturation solution, probes and post hybridisation washes

A stock of 20x concentrate of SSC was prepared by adding the following components to 1L ddH_2O: 175.3g sodium chloride and 88.2g sodium citrate dihydrate, once made up the 20xSSC was transferred to a sterile glass bottle and the pH was adjusted to 7.0 and stored at RT. A 2xSSC buffer was prepared by diluting the 20xSSC stock solution by 1 in 10 with ddH_2O (pH 7.0).

8.2.1.4 Preparation of denaturation solution:

The denaturation solution (70%formamide/2xSSC) was prepared on the day by taking 35 ml of formamide (BDH, Poole, UK), 10ml of 20xSSC and 20ml of ddH_2O mixed well and the pH was then adjusted to 7.0.

8.2.1.5 Probe preparation:

Three colour FISH was carried out for chromosomes 21, X and Y in each patient using directly labelled commercially available probes (Vysis Inc., Downers Grove, Ilinois, USA).

The probe preparations and denaturation for both protocol 1 and 2 are essentially the same with differences only in the specific probes used. All probe preparation and denaturation was carried out as according to manufacturers guidelines unless stated otherwise. The preparation and denaturation of the probes for both protocols were carried out prior to immediate use and details are included in the sperm FISH protocol. Figure 8.2 illustrates examples of the two probe approaches used.

Figure 8.2- panel A probe protocol 1 and panel B probe protocol 2

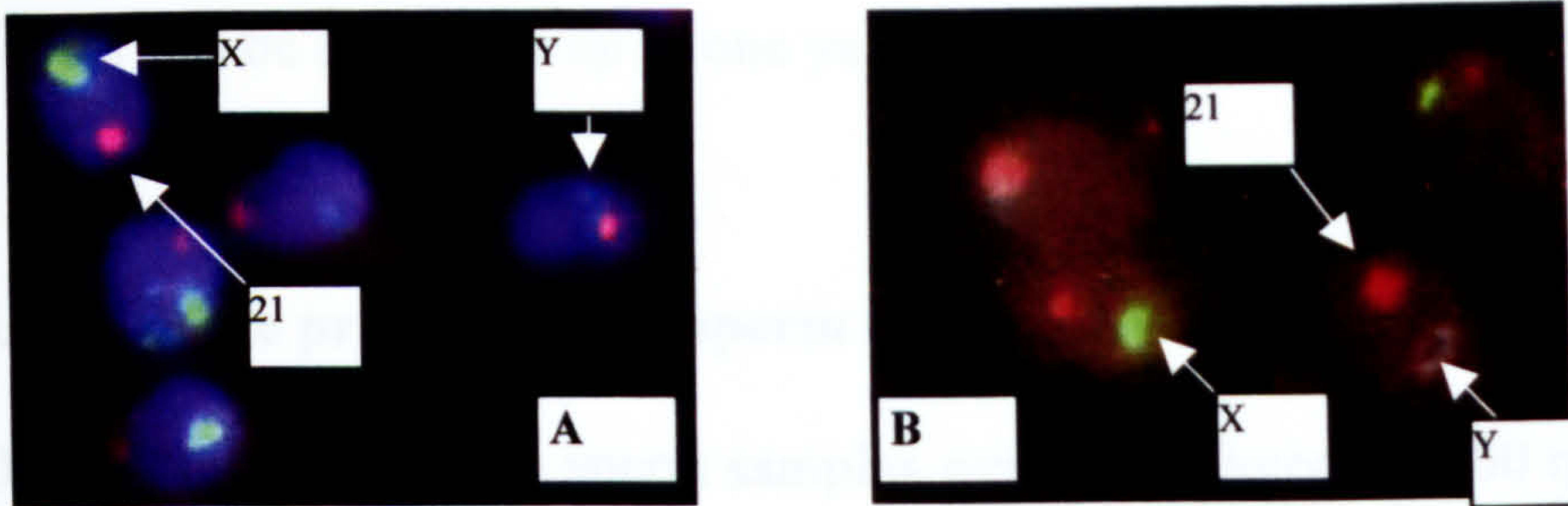


Figure 8.2- Panel A- probe protocol 1- green-Y, red-21 and green and red- X **Panel B-** probe protocol 2- green-X, red-21 and aqua-Y (shown without DAPI counterstain to enable aqua Y to be seen).

8.2.5.6 Post hybridisation washes:

All post hybridisation washes were prepared on the day according to guidelines recommended by the probe manufacturer (Vysis, Downers Grove, Illinois, USA) pre-warmed to 45°C (+ 1°C per slide) and the pH adjusted to 7.0. Three post hybridisation washes were prepared, the first of which was a 50%formamide/2xSSC, the second a 2xSSC and finally a 2xSSC/0.1% tween20 (BDH, Poole, UK).

8.2.3 Sperm FISH protocol

The preparation of semen samples for FISH use was carried out in a Laminar air flow cabinet type II under containment level 2+. Semen samples were immediately washed in 10mM Tris HCl, 10mM NaCl (pH 8.0) (sperm buffer) once received (approximately 1-2 hours after production). Semen samples were transferred to 15ml centrifuge tubes and a total of 10mls of sperm buffer was added to each semen sample. The tubes containing the sample and sperm buffer were then centrifuged at 1200 rpm for 5 minutes, after each centrifugation the pellet was carefully re-suspended in10mls of fresh sperm buffer. The centrifugation step, removal of the supernatant and re-suspension of the pellet in fresh sperm buffer was then repeated twice, this process was undertaken to remove the

seminal fluid and retain the sperm cells only. Samples were subsequently stored at 4°C (slides can be stored for up to one year) until required for FISH analysis.

8.2.3.1 Slide preparation of sperm samples for FISH analysis (Day 1)

Once ready for analysis sperm samples were centrifuged at 1200 rpm for 5 minutes, the supernatant was carefully removed ensuring the pellet was not disturbed. The pellet was then re-suspended in 0.5-1ml of fresh sperm buffer (dependant on the size of the pellet) until a semi-translucent suspension was achieved. Slides were then prepared by spreading 10-20μl of the cell suspension onto a clean glass slide using a plastic coverslip, air-dried and the appropriate cell density required was checked on a phase contrast microscope, ensuring cell density is approximately 70-80%. Slides were then dehydrated and fixed through an ethanol series of 70, 80 and 100% for 5 minutes in each. It is essential to ensure that there were adequate numbers of cells on the slide to score the number of sperm required and that the density of these cells were not too sparse (to be able to score as many as possible within one field of view, reducing scoring time). It was also important to check that the cell density of the slide was not too high or that too many clumps were found (as overlapping sperm heads are not scored). The process of spreading sperm onto slides was repeated until the correct cell density required was achieved, adjusting the volume of the sperm sample added to the slide accordingly. Slides were then stored at RT overnight to age.

8.2.3.3 Decondensation and Denaturation of sperm nuclei (Day 2)

The aged slides, prior to hybridisation were decondensed for 30 minutes in swelling buffer 1. (*N.B. If samples were not adequately decondensed the FISH was repeated with the addition of the second swelling step, however, the majority of cases did not require*

this additional step. In these cases this step comprised of the immediate transfer after incubation in swelling buffer 1 to swelling buffer 2 for a 1 hour incubation). All incubations in the swelling buffer(s) took place at RT in the dark due the light sensitivity of the buffer(s). After decondensation the slides were briefly rinsed (approximately 1 minute) in 2xSSC pH 7.0 and air dried, and subsequently dehydrated in an ethanol series of 70, 80 and 100% (incubated for 5 minutes in each) and air dried. Once air dried slides were denatured for 11 minutes in 70% formamide/2xSSC at 72°C + 1°C per slide (taking into account the drop in temperature the slides will cause to the denaturation solution). After denaturation slides were quenched in ice cold 70% ethanol (-20°C) for 2 minutes, followed by 2 minutes each in 80% and 100% ethanol to dehydrate the slide. Slides were then air dried before the probe was applied.

8.2.3.4 Probe preparation and denaturation (Day 2)

According to manufacturers guidelines, the probes were pre-warmed to 37°C and the following amounts of each of the above probes was aliquoted into an eppendorf for every slide to be hybridised:

Protocol 1: 1.5μl of the LSI 21 probe (Spectrum Orange); 0.5μl CEP Y probe (Spectrum Green); 0.5μl of CEP X probe (Spectrum Orange); 1μl of the CEP X probe (Spectrum Green).

Protocol 2: 1.5μl of the LSI 21 probe (Spectrum Orange); 1μl CEP Y probe (Spectrum Aqua); 1μl of CEP X probe (Spectrum Green).

In both protocols a total of 7μl of the manufacturers hybridisation mix was added to each eppendorf. Once added the probes and hybridisation mix were added they were then vortexed to mix, pulse centrifuged, vortexed again, and subsequently denatured for

5 minutes at 70°C. The total volume of the probe mix (10.5µl) was then added to the air dried slides, and then a 22 x 22mm coverslip was carefully placed on to the slide avoiding air bubbles. The coverslip was then sealed to the slide using rubber solution (Halfords, Redditch UK) in order to prevent evaporation of the probes, and slides were then placed in a humidified chamber overnight at 37°C.

8.2.3.5 Post Hybridisation Washes (Day 3)

Post hybridisation washes were carried out as specified by the manufacturers guidelines, briefly this included washes at 45°C (+ 1°C per slide), 3 x 10 minute washes in 50%formamide/2xSSC, 1 x 10 minute wash in 2xSSC followed by a 1 x 5 minute incubation in 2xSSC/0.1%tween 20. After these washes at 45°C slides were briefly rinsed in ddH_2O and left to air dry in the dark, once dry slides were mounted in anti-fade mountant (Vectasheild) containing a DAPI counterstain (Vector laboratories, Peterborough, UK).

8.2.4 Microscopy and scoring criteria

Slides were observed using the Leica fluorescence microscope with appropriate filter sets (Vysis, Downers Grove, IL, USA), triple band pass filter (DAPI/ Orange and Green), dual band pass filter (Orange/ Green) and single pass filters for (DAPI, Orange and Green). Images were captured using Smart Capture (v3.2).

Approximately 5000 sperm nuclei were scored per patient (or the maximum that could be counted in patients with severe oligozoospermia) triple colour FISH was always undertaken to enable disomic spermatozoa to be distinguished from diploid ones. For scoring slides a stringent criteria was applied: Sperm nuclei were only scored if

spermatozoa were intact, displaying a similar degree of decondensation and clear hybridisation signals; disrupted or overlapping spermatozoa were excluded from analysis. Spermatozoa were regarded as abnormal if two copies of the same chromosome (disomic) were found within the sperm nuclei. Sperm cells were scored as disomic if two signals recorded for the same chromosome were clear, similar in size and intensity, and separated from each other by at least one signal domain and clearly positioned within the sperm nuclei, (therefore excluding divided (split) signals being wrongly scored as disomies). Spermatozoa exhibiting two signals (as above) for each chromosome 21 and the sex chromosomes were counted as diploid. Cells that presented with an absence of either a sex chromosome or chromosome 21 were not counted as nullisomic as it is not possible to distinguish whether this is true nullisomy or failure of the probe to hybridise, therefore nullisomic spermatozoa were not included in analyses (Griffin et al., 1995; 1996). Examples of normal and aneuploid spermatozoa for chromosomes X, Y and 21 (using protocol 1 combination probe approach) are presented in figure 8.3.

Figure 8.3- examples of normal and abnormal spermatozoa using the FISH probe protocol 1

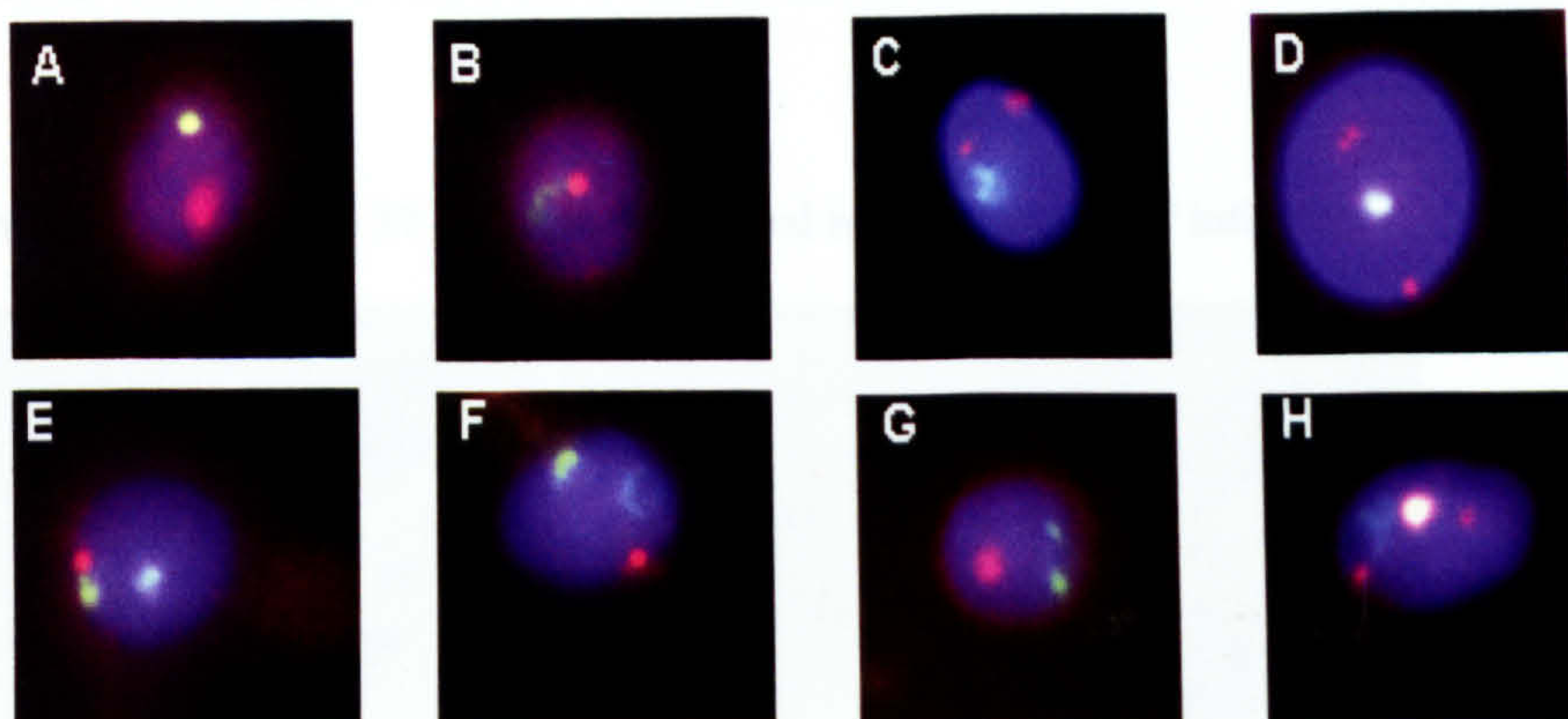


Figure 8.3 chromosomes X (labelled in yellow, produced by a combination of red and green), Y (labelled in green) and 21 (labelled in red). Image A and B = normal X and Y bearing sperm (respectively) for the chromosomes investigated with the presence of one sex chromosome and one chromosome 21. Images C - H are all examples of aneuploid sperm, images C and D = Y and X bearing sperm with disomy 21; images E - G are all examples of sex chromosome disomies (E = disomy X, F = XY disomy, G = YY disomy) and image H = disomic for chromosome 21 and also contains 2 sex chromosomes (X and Y), hence is likely to be a diploid spermatozoa.

8.3 Preparation of herbal extracts:

We obtained 37 plant based herbs (figure 8.4 and table 8.1) used in the treatment of male infertility by traditional Chinese herbal medicine. The recommended preparation of the herbal prescriptions was to add water (an exact amount is not specified), ensuring that all the herbs were covered (with at least a 1cm excess coverage). This infusion was then left overnight and boiled the following day for 30 minutes. The herbal infusion is then left to cool down and is then taken as a tea. The standard preparation of each of the herbs for all assays assessing herbal activities followed the same protocol given to patients. The herbs were prepared for each assay as follows: pyrex boiling tubes were rinsed with 100% ethanol followed by ddH_2O and sterilised by autoclaving. Once sterilised 0.15g of each herb to be investigated was weighed out and transferred to these boiling tubes, following this 1ml of ddH_2O was added to the boiling tube (more than enough to cover the herbs). The boiling tube was then covered with parafilm and

aluminium foil and were left to infuse over night at RT. Following overnight incubation the herbs were then boiled for 30 minutes at 100°C.

Figure 8.4- showing the 37 different herbs used in the treatment of infertility

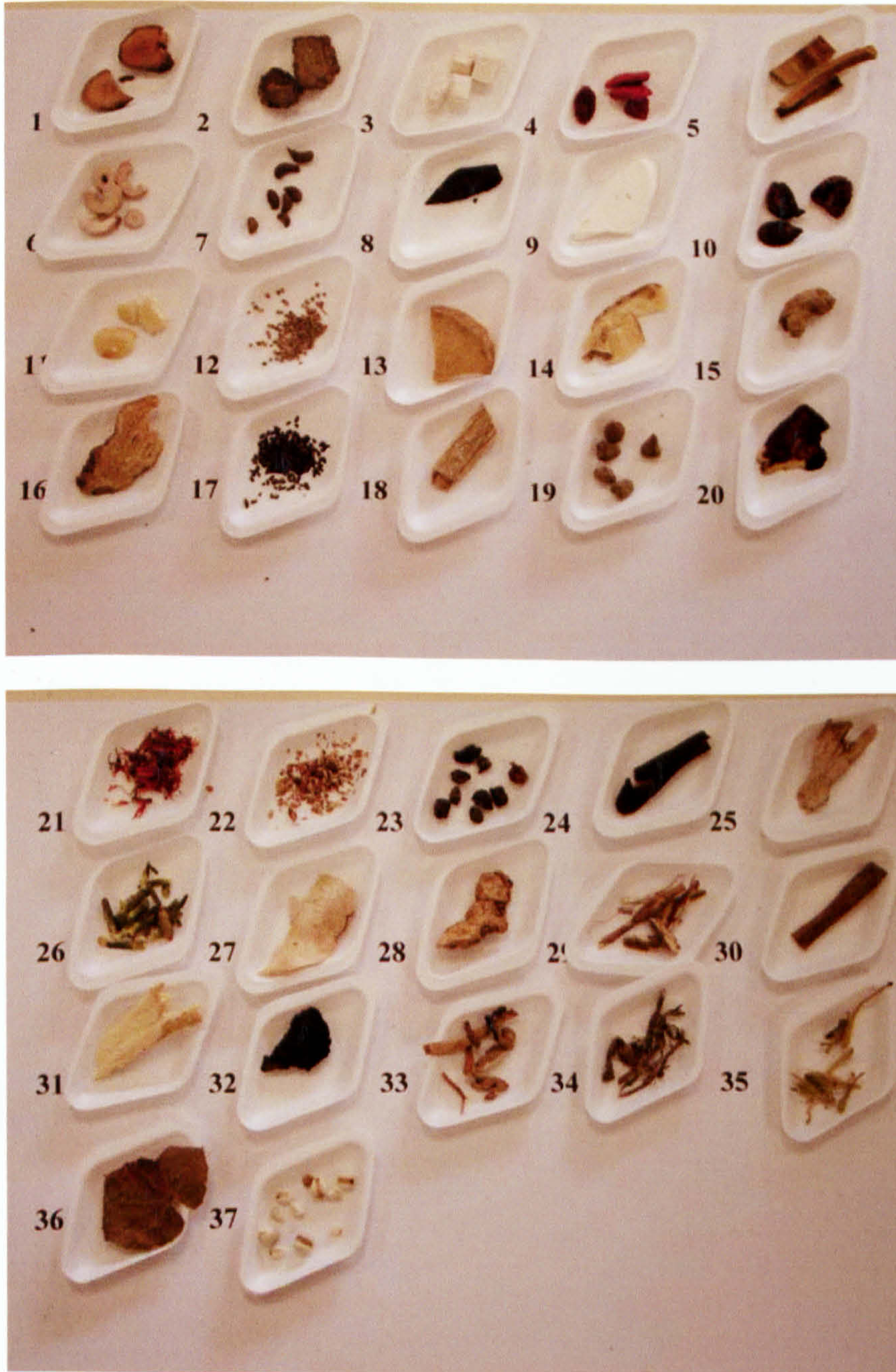


Table 8.1- Pin-Yin, latin and common names of each of the 37 individual herbs where possible

Herb No.	Pin Yin Name	Latin Name	Common Name
Herb 1	Chi Shao	***Radix paeoniae rubrae***	Red peony root
Herb 2	Chuan Niu Xi	***Radix cyathula officinalis***	Cyathula root
Herb 3	Fu Ling	***Sclerotium poriae cocos***	Poria
Herb 4	Gou Qi Zi	***Fructus lycii chinensis***	Lycium fruit
Herb 5	Huang Bi	***Cortex phellodendri***	Phellodrendron bark
Herb 6	Mu Dan Pi	***Cortex moudan radicis***	Moutan
Herb 7	Nu Zhen Zi	***Fructus ligustri lucidi***	Ligustrum seed / glossy privet fruit
Herb 8	Shu Du		
Herb 9	Shan Yao	***Rhizoma dioscoreae***	Chinese yam
Herb 10	Shan Zhu Yu	***Fructus corni officinalis***	Cornus fruit / dogwood fruit
Herb 11	Tao Ren	***Semen persicae***	Persica
Herb 12	Tu Si Zi	***Semen cuscutae***	Dadder seed/ cuscuta seed
Herb 13	Ze Xie	***Rhizoma alismatis plantago-aquaticae***	Alismatis rhizome/ water plantain tuber
Herb 14	Zhi Mu	***Rhizoma anemarrhenae asphodeloidis***	Anemarrhena
Herb 15	Ba Ji Tian	***Radix moridae officinalis***	Morinda root
Herb 16	Bai Zhu	***Rhizoma atractylodis macrocephalae***	Alba atractylodes
Herb 17	Che Qian Zi	***Semen plantaginis***	Plantago seed
Herb 18	Dang Shen	***Radix codonopsis pilosulae***	Codonopsis
Herb 19	Fu Pen Zi	***Fructus rubi***	Rubus
Herb 20	Gan Cao	***Radix glycyrrhizae uralensis***	Liquorice root
Herb 21	Hong Hua	***Flos carthami tinctorii***	Safflower flower
Herb 22	Wang Bu Liu Xing Zi	***Semen varricariae pyramidata medic***	Vaccaria seed
Herb 23	Wu Wei Zi	***Fructus schisandrae chinensis***	Schisandra fruit
Herb 24	Zhi Ke	***Aurantii fructus***	Aurantium fruit
Herb 25	Shi Chang Pu	***Acori graminei rhizoma***	Acorus rhizome
Herb 26	Lian Zi Xin	***Nelumbinis embryo***	Lotus embryo
Herb 27	Bi Xie	***Rhizoma dioscoreae septemlobae***	Hypoglauca yam
Herb 28	Cang Zhu	***Rhizoma atractylodis***	attractylodes
Herb 29	Chai Hu	***Radix bupleuri chinensis***	Bupleurum
Herb 30	Sheng Ma	***Rhizoma cimicifugae***	Bugbane rhizome
Herb 31	Huang Qi	***Radix astragali seuhedysari***	Astragalus root
Herb 32	Sheng Di Huang	***Radix rehmanniae glutinosae***	Fresh rehmannia
Herb 33	Chen Pi	***Pericarpium citri reticulata blanco***	Citrus peel
Herb 34	Han Lian Cao		Eclipta
Herb 35	Jin Yin Hua	***Flos lonicerae japonicae***	Lonicera flower
Herb 36	Yin Yang Huo	***Herba epimedii***	Epimedium
Herb 37	Yi Yi Ren	***Semen coicis***	Coix seed / jobs tear seed

8.4 Methods for determining endocrine activities including: oestrogenic, androgenic, anti-oestrogenic and anti-androgenic

The purpose of this assay was to test plant extracts (boiled in water) (refer to section 8.3 for preparation) for oestrogenic, androgenic, anti-oestrogenic and anti-androgenic activities.

8.4.1 Preparation of and storage of medium components:

Prior to use all glassware was rinsed twice with methanol (BDH, UK), followed by a final rinse in ethanol (Hayman, Essex, UK).

8.4.1.1 Preparation of sterile minimal medium:

The minimal medium was prepared by the addition of the following chemicals to 1L of double-distilled water: KH_2PO_4 13.61g, $(NH_4)_2SO_4$ 1.98g, KOH pellets 4.2g, $MgSO_4$ 0.2g, $Fe_2(SO_4)_3$ solution (20mg/ml ddH_2O) 1ml, L-leucine 50mg, L-histidine 50mg, adenine 50mg, L-argenine-HCl 20mg, L-methionine 20mg, L-tyrosine 30mg, L-isoleucine 30mg, L-lycine-HCl 30mg, L-phenylalanine 25mg, L-glutamic acid 100mg, L-valine 150mg, L-serine 375mg. Once all components were added the medium was placed on a heated stirrer in order to ensure that all components had dissolved and that the medium was well mixed. Subsequently 45ml aliquots of the medium were measured out and placed into glass bottles, these were then autoclaved and stored at RT.

8.4.1.2 Preparation of components for addition to minimal medium

Copper (II) sulphate solution (20mM) was prepared, and filter sterilised, into sterile glass bottles and stored at RT.

L-aspartic acid stock solution was prepared by adding 4mg/ml (ddH_2O), this was then transferred to sterile glass bottles in aliquots of 20ml and sterilised by autoclaving and stored at RT.

L-threonine stock solution was prepared by adding 24mg/ml (ddH_2O), this was then transferred to sterile glass bottles in aliquots of 5ml and sterilised by autoclaving and stored at 4°C.

The vitamin solution was prepared by the addition of the following components to 180ml of ddH_2O: panthothenic acid (8mg), pyridoxine (8mg), thiamine (8mg), inositol (40mg) and biotin solution (2mg/100ml ddH_2O) (20ml). The vitamin solution once prepared was sterilised by filtration and transferred to sterile glass bottles in 10ml aliquots and stored at 4°C.

A 20% glucose solution was prepared by making a 20%w/v solution of D-(+)-glucose, which was transferred to sterile glass bottles in 20ml aliquots, sterilised by autoclaving and stored at RT

A 10mg/ml stock solution of CPRG was prepared and sterilised through filtration, aliquots of 2ml were transferred to sterile glass jars and stored at 4°C.

8.4.1.3 Growth Medium preparation

This segment of the assay was undertaken in a Laminar air flow cabinet type II (Flow laboratories Gelaire, class 100, Irvine). The growth medium was prepared by adding the following to 45ml of minimal medium: 5ml of glucose (20%w/v), 1.25ml of L-alanine,

0.5ml vitamins, 0.4ml L-threonine, 125µl of $CuSO_4$. For the preparation of growth medium for the oestrogen or androgen yeast assay 125µl of the human oestrogen receptor (HER) yeast stock or the 125µl of the human androgen receptor (HAR) was added respectively. The culture flask was then swirled to mix and placed on an incubated shaker (250rpm) at 28°C overnight for 24 hours.

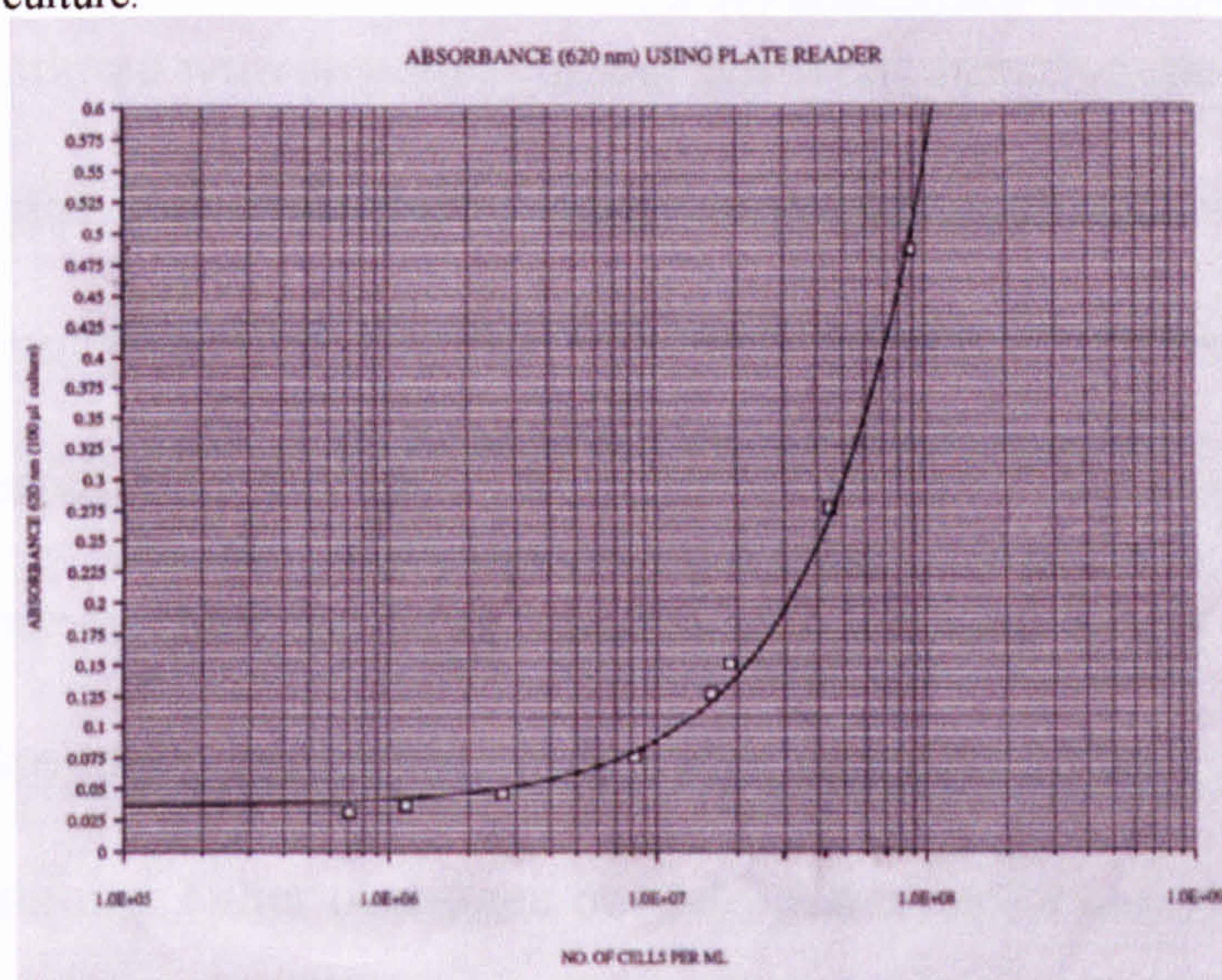
8.4.1.4 Preparation of recombinant yeast assay media

As with the preparation of the growth media this part of the assay was undertaken in a Laminar air flow cabinet type II. The assay medium was prepared by adding the following to 45ml of minimal medium: 5ml of glucose (20%w/v), 1.25ml of L-alanine, 0.5ml of CPRG, 0.5ml vitamins, 0.4ml L-threonine, 125µl of $CuSO_4$.

Once the assay medium was prepared it was necessary to add the appropriate amount of yeast from the cultures derived from the cultures grown overnight. The assay requires an amount of 4×10^7 cells per 50ml. The amount of yeast to add was determined by taking two 100µl aliquots from each growth culture, this was then transferred onto a 96 well microtitre plate (Linbro, ICI Biomedicals, Ohio, USA). The turbidity of each of these aliquots was determined by the use of a spectrophotometer plate reader (Molecular Devices, California, USA) at the wavelength of 620nm. These absorbance's were noted and utilised to estimate the number of cells per ml by reading off from a yeast growth curve (figure 8.5). Using the following equation it was then possible to determine the amount of the growth culture that was required to be added to the assay medium to give the required 4×10^7 cells per 50ml.

$$\text{Volume of the growth culture} = \frac{4 \times 10^7}{\text{No. of cells calculated from growth curve}}$$

Figure 8.5 illustrates the yeast growth curve used to calculate the number of cells per ml present in the overnight (growth) culture.



Once calculated the appropriate aliquots were removed from the growth culture and added to the assay medium.

8.4.2 Recombinant yeast screen assay procedure

This part of the assay was undertaken in a Laminar air flow cabinet type II. For each assay carried out a 96 well microtitre plate containing positive and negative controls, including: a standard curve (positive control), a row of ethanol blanks and a row of the yeast medium alone (both negative controls to check for contaminants. In the case of the oestrogen screen the standard utilised was 17β-oestrodiol (covering the range of 1.3-2724ng/l), and for the androgen screen dihydro-testoterone (DHT) (covering the range of 7-14520ng/l) was used. Each of the standards were serially diluted in absolute ethanol in a 96 well microtitre plate, for each dilution a total of 10μl was added to the assay plate using a multichannel pipette. Two sample plates were prepared to cover a

larger concentration range. The first of these was prepared in the same way as the standard curve with the sample extract being serially diluted in absolute ethanol covering a range of $5x10^{-3}$-10µl, with 10µl being transferred to the assay plate, these plates will be referred to as the dilution plate from herein. For the second plate samples were not serially diluted with absolute ethanol but were directly pipetted (spotted) onto the 96 well microtitre plate, with 1.25µl added to the first well, this amount was then doubled to 2.5µl and so on until the final volume of 40µl was reached in well 6, these plates will be referred to as spot plates from herein. Once all standards, ethanol blanks, yeast medium alone and samples were pipetted onto the assay plates these were left in the Laminar air flow cabinet type II to evaporate (until dry). Subsequently 200µl of the assay media, containing either oestrogen or androgen receptor carrying yeast (dependant on the screen being carried out) was pipetted into each of the wells on the assay plate using a multichannel pipette. Plates were subsequently sealed with autoclave tape (to prevent evaporation), and were shaken for 2-3 minutes on a microtitre shaker (Lab Plant, Huddersfield, UK), once shaken plates were placed into an incubator overnight at 32°C.

Day 2

All assay plates were removed from the 32°C incubator and shaken on the microtitre shaker as before, oestrogen screen plates were subsequently returned to the 32°C incubator, androgen screen plates were then placed in an incubator at 28°C.

Day 3

As before the assay plates were removed from the incubators and shaken on the microtitre plate shaker. The plates for both screens were then left out overnight at RT,

to prevent the yeast cells from growing too rapidly, thus ensuring that the chromogenic substate (CPRG) change did not develop too rapidly.

Day 4

Assay plates were shaken on the microtitre plate shaker as before. The assay plates were then analysed using the spectrophotometer plate reader. All assay plates were measured at two wavelengths, the first of which was 540nm (to measure colour change) and the second wavelength of 620nm to measure the turbidity of the medium (providing an estimate of yeast growth rate).

In order for the accurate measurements for colour change, the amount of turbidity has to be corrected for, this is done by the following equation:

Corrected value = chem.. abs. (540nm)- [chem. Abs. (620nm)-blank abs. (620nm)].

The plates were read on a spectrophotometer plate reader, the software used to analyse the data was that of Softmax pro, this calculation has been entered into the software and hence automatically takes into account the yeast turbidity. The values calculated could then be plotted showing the endocrine response of the investigated herbal extracts.

8.5 Antagonist assays (anti-oestrogen and anti-androgen yeast based assays).

8.5. 1 Assay procedure:

Growth medium for the yeast cells were prepared as stated before section 8.4.1.3

8.5.1.1 Preparation of assay medium (Day 1):

The assay medium was prepared as stated previously in section 8.4.1.4, however prior to the medium being transferred to the assay plates the agonist was added. In the anti-oestrogen assay 17β-oestrodiol was added to the medium to produce a final concentration of $2.5x10^{-10}$, to the anti-androgen assay DHT was added to produce a final concentration of $2.5x10^{-9}$. To the medium the appropriate amount of yeast cells was added to the culture (described in section 8.4.1.4) and the chromogenic substrate CPRG was added. Once prepared the assay plates were prepared with standards, samples and controls.

8.5.1.2 Preparation of standards and samples:

The assay was undertaken in a Laminar air flow cabinet type II, samples were prepared as stated before in section 8.4.2, in brief samples were either serially diluted (dilution plates) or aliquoted directly onto assay plates, (spot plates) covering a range of 1.25μl to 40μl. As with previously described oestrogen and androgen yeast based assays a standard curve along with several negative controls are prepared (including an ethanol blank and yeast medium alone). In the case of the anti-oestrogen screen the standard used was hydroxy-tamoxifen (OHT) (covering the range of 8.4 to 19375 ng/l), the standard used for the anti-androgen screen was that of flutamide (covering the range of 7-13810 ng/L) as before these were all serially diluted and 10μl transferred to the assay plates. As before all samples and standards were left to evaporate to dryness, once complete 200μl of the assay medium was transferred to each of the wells on the assay plates. Once complete the lids to the assay plates were placed on and were sealed with autoclave tape, assay plates were then vigorously shaken on a microtitre plate shaker for 2 minutes and transferred to a 32°C incubator overnight.

Day 2:

All assay plates were removed form the 32°C incubator and vigorously shaken on the microtitre plate shaker for 2 minutes, ensuring that the yeast cells are thoroughly mixed and dispersed throughout the wells. Anti-oestrogen screen plates were then placed back in the 32°C incubator, while anti-androgen screen plates were placed in a 28°C incubator overnight.

Day 3:

As before all plates were removed from the incubators (28°C and 32°C) and vigorously shaken on the microtitre plate shaker for 2 minutes, once shaken assay plates were left at RT overnight.

Day 4:

All assay plates were vigorously shaken on the microtitre plate shaker for 2 minutes, assay plates were then analysed using a spectrophotometer plate reader at two wavelengths. The first of which was 540nm (to measure colour change) and the second at 620nm (measuring the turbidity of the medium and hence providing an estimate of the growth rate of the yeast cells).

As before for accurate analysis of the rate of colour change the amount of turbidity had to be corrected for, the following equation took this into account:

Corrected value = chem.. abs. (540nm)- [chem. Abs. (620nm)-blank abs. (620nm)].

8.6 FRAP assay to determine anti-oxidant activity

For this section of work it was necessary to have an assay system that would enable us to test plant extracts (boiled in water) (see section 8.3 for preparation) for antioxidant activity.

8.6.1 Preparation of the FRAP assay components:

The FRAP assay requires three components (A, B and C) to make the working reagent, these are made up as follows:

Reagent A- is a 300mM acetate buffer pH 3.6, and is prepared as follows: (3.1g sodium acetate, 16 ml of glacial acetic acid (BDH, Poole, UK) made up to 1L with ddH_2O, once made up this reagent is stored at RT.

Reagent B- is made up on the day and consists of 10mM TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40mM HCl (3.1mg/ml).

Reagent C- is also made up on the day and consists of 20mM $FeCl_36H_2O$, in ddH_2O (5.4mg/ml).

Reagent A, B and C are then mixed in a 10:1:1 ratio respectively to produce the working FRAP reagent.

8.6.2 FRAP assay protocol:

For this assay all the individual and herb mixes were prepared as stated in section 8.3, a standard of 1mM Ascorbic acid was also prepared (0.176mg/ml) in ddH_2O, as in section 8.4 the anti-oxidant potential of herbs and standards were tested at a variety of concentrations, (1.25, 2.5, 5, 10, 20 and 40μl), with boiled ddH_2O used as a negative control.

Each of the herb and standard concentrations were added to 1ml of the working FRAP reagent at time intervals of 1 minute, each sample was then incubated in the dark at RT for 10 minutes. Following the 10 minute incubation the absorption was measured on a spectrophotometer at a wavelength of 593nm, that was first blanked against a sample containing 1ml of FRAP and 40µl of ddH_2O, following this the wavelength of each of the herbs, standards and negative controls were measured and recorded after the 10 minute incubation.

8.7 Chromosome positioning in human sperm nuclei

8.7.1 Preparation of sperm nuclei

This was carried out as in section 8.2.3, briefly semen samples were washed three times in 10mM Tris HCl, 10mM NaCl (pH 8.0) by centrifugation (1200 rpm for 5 minutes) after each centrifugation the pellet was carefully resuspended. After the final centrifugation the majority of the supernatant was then removed (to give the correct cell density for FISH). Slides were then prepared by spreading 10-20µl of the cell suspension onto a clean glass slide and air-dried. Slides were then fixed for two hours in 3:1 methanol:acetic acid (BDH, Poole, UK) in order to flatten the sperm nuclei, once fixed the slides were air dried and then dehydrated through an ethanol series 70, 80 and 100% for 5 minutes in each. Slides were then stored at –20°C until further required.

8.7.2 Preparation of denaturation solution, probes and post hybridisation washes:

Please refer to section 8.2.1.2 for details on the preparations of decondensation buffers and denaturation solutions.

8.7.2.1 Probe preparation:

Commercially available directly labelled whole chromosome paints (WCP probes) specific for chromosomes X, Y and 18 were utilised in this assay. Paints were obtained from Q-Biogene (Livingston, UK). Probes and all post hybridisation washes were carried out according to manufacturers guidelines.

8.7.2.2 Post hybridisation washes:

All post hybridisation washes were prepared on the day according to guidelines recommended by the probe manufacturer (Q-Biogene) and the pH adjusted to 7.0. These probes required 2 post hybridisation buffers to be prepared, and included: 50%formamide/2xSSC, and 0.1xSSC. For both washes 150mls of each was prepared (enough for three washes in each), the 50%formamide/2xSSC was prepared as described in section 8.2.5.6, and the 0.1xSSC was prepared from the 20xSSC stock (refer to section 8.2.1.3 for stock preparation).

8.7.3 Chromosome positioning sperm FISH protocol:

8.7.3.1 Slide preparation (Day 1)

For chromosome positioning slides have previously been prepared and stored at -20°C, prior to use these were removed from and left at RT overnight to age ready for use.

8.7.3.2 Sperm nuclei decondensation and denaturation (Day 2)

Once aged the slides were decondensed and denatured as in section 8.2.3.3 (sperm FISH segment). However, in the chromosome positioning assay the second swelling buffer (LIS) was omitted as this is used to salt out DNA in DNA halo preparations and hence is likely to effect DNA organisation within the sperm nuclei.

8.7.3.3 Probe preparation (Day 2)

FISH was carried out using directly labelled commercially available whole chromosome paints (WCP) for chromosomes X, Y and 18, unlike the Vysis probes the Q-Biogene probes come already mixed with the hybridisation buffer, therefore an aliquot of the total amount needed is taken directly from the tube. For each slide a total of 4.5μl of the probe (and hybridisation mix) was transferred into a sterile eppendorf and were placed at 37°C for 5 minutes, denatured at 72°C for 5 minutes followed by being incubated at 37°C for 30-60 minutes to preanneal. Once the slide had been decondensed, denatured, dehydrated through an ethanol series 70, 80 and 100%, air dried and the probe preparation finished 4.5μl of the probe was applied directly to the slide and a 13x13mm coverslip placed on top. As in section 8.2.3.4 the coverslips were then sealed with rubber cement and placed in a humidified chamber overnight at 37°C.

8.7.3.4 Post Hybridisation Washes (Day 3)

The two post hybridisation washes were pre-warmed in water baths to the following temperatures: 50%formamide/2xSSC at 45°C (+ 1°C per slide) and 0.1xSSC at 60°C (+ 1°C per slide). Once these washes reached the required temperatures, the slides were removed from the (overnight) humidified chamber and the rubber cement was carefully removed with forceps taking care not to disturb the coverslip. The slide was then briefly placed in 2xSSC to float the coverslip off, the slides were immediately placed in the first of three 5 minute 50%formamide/2xSSC washes (45°C + 1°C per slide). Once this wash was complete the slides were then immediately transferred to 50%formamide/2xSSC washes for 5 minutes for a further two times. Once the third wash was complete the slides were directly transferred to the first of three 5 minute washes in 0.1xSSC (60°C + 1°C per slide), this process was then repeated for the final

two washes. Once the final wash was complete slides were then briefly rinsed in ddH_2O and left to air dry in the dark before being counterstained with anti-fade mountant containing DAPI (Vector Laboratories).

8.7.4 Microscopy and analysis of chromosome position in sperm nuclei

Slides were observed using the same fluorescent microscope and image capture programme as reported in section 8.3. For positioning analysis a total of 50 sperm nuclei were captured for each slide, spermatozoa considered had to follow strict criteria. Spermatozoa had to exhibit a similar degree of decondensation, only clear hybridisation signals located clearly and completely within the sperm nuclei were considered, any disrupted or overlapping spermatozoa were excluded from analysis. Two types of analysis were made with regards to chromosome territory position relative to the centre and chromosome territory position relative to the polarity of the sperm nucleus (i.e. in relation to head and tail).

8.7.4.1 Chromosome positioning analysis within sperm nuclei:

8.7.4.1.1 Collection of data with regards to chromosome territory position relative to the centre of the sperm nuclei

In order to undertake the analysis of these 50 nuclei images were captured in smart capture and exported as TIFF files and imported into Adobe Photoshop v5.2. Using Adobe Photoshop the sperm nuclei was enlarged to three times its original size (300%), and the longest point of the sperm nuclei (from edge to edge) was measured by hand on the screen using a ruler. The position of the chromosome territory was then measured by measuring the distance from the centre of the chromosome territory to the nearest edge of the sperm nuclei, and also the length of the chromosome territory as stated

previously (Bridger, personal communication), see figure 8.6 for diagrammatic explanation. These measurements were directly recorded onto an excel spreadsheet.

Figure 8.6- diagrammatic explanation of the protocol used to measure the location of chromosome territories with reference to the centre of the nuclei

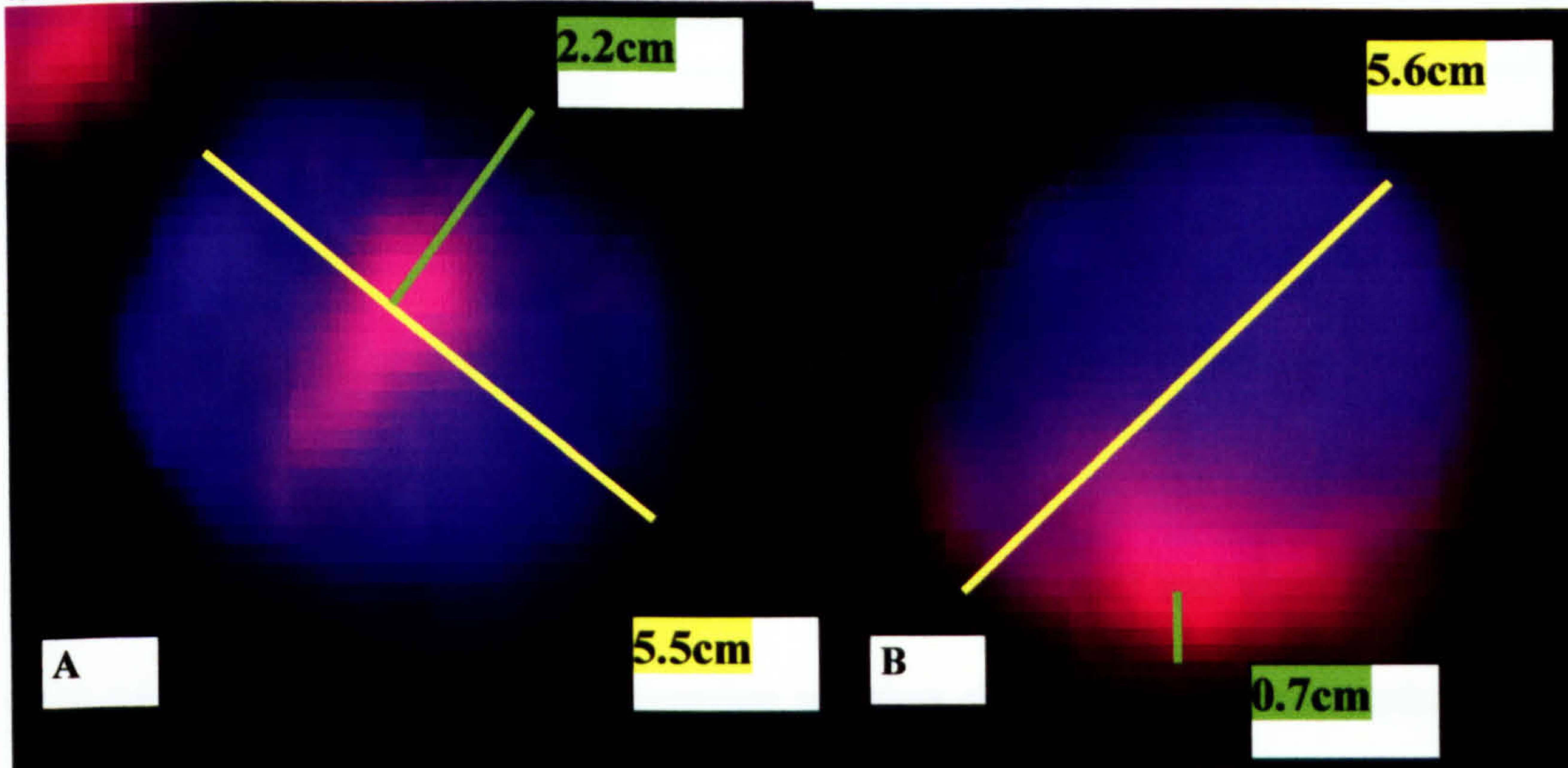


Figure 8.6- two sperm nuclei (magnified 3 times the original size are shown), a whole chromosome paint probe for chromosome X has been hybridised (red). The two panels A and B show chromosome X to be centrally located (A) and peripherally located (B). The first step of the analysis is to measure the longest point of the sperm nuclei, as illustrated with the yellow line this is then recorded (i.e 5.5cm in A), the next step is to then measure the distance from the centre of the chromosome territory to the nearest edge, as illustrated with the green line, thus providing the measurement for the location (i.e. more interior or peripheral).

This was repeated for 50 nuclei per chromosome per patient, each chromosome and patient was also repeated (therefore 100 nuclei in total were analysed per patient per chromosome). At the same magnification an image of a graticule (figure 8.7) with 10μm sections was captured, this image was also exported as a Tiff file and imported into Adobe Photoshop, the image as with the sperm nuclei was magnified to 300% and using a ruler the length of a 10μm section was measured in centimetres, based on this 6cm was equivalent to10μm. This enabled the measurements calculated for sperm length and centre of the chromosome territory to the nearest edge of the sperm nuclei to be converted into microns.

Figure 8.7- Picture of the graticule with 10μm markings.

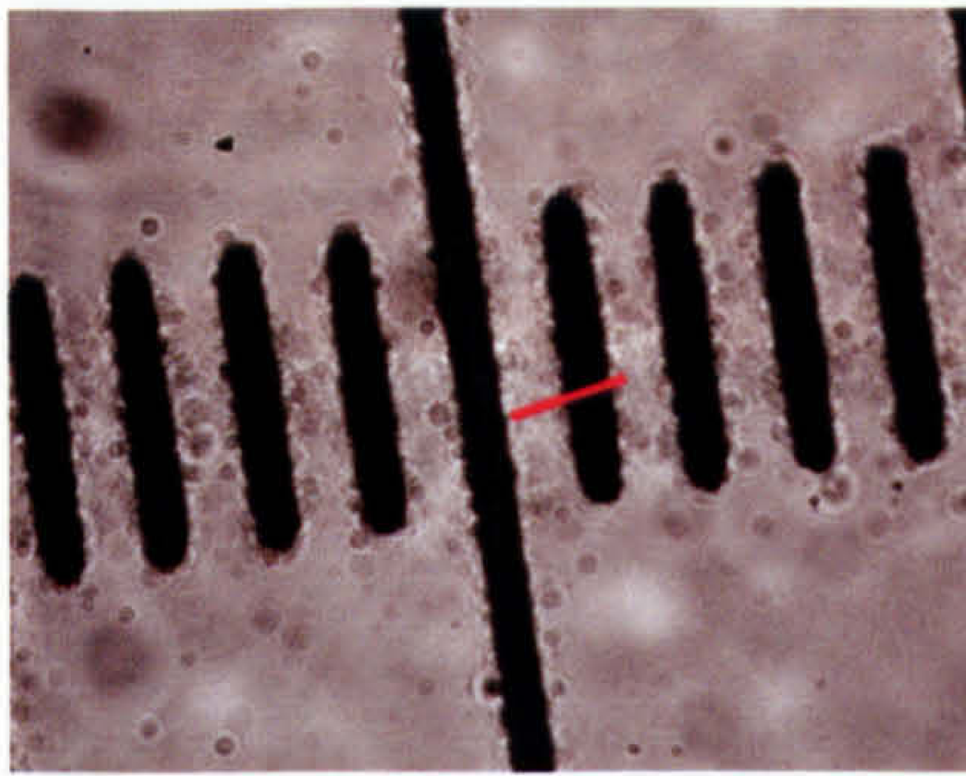
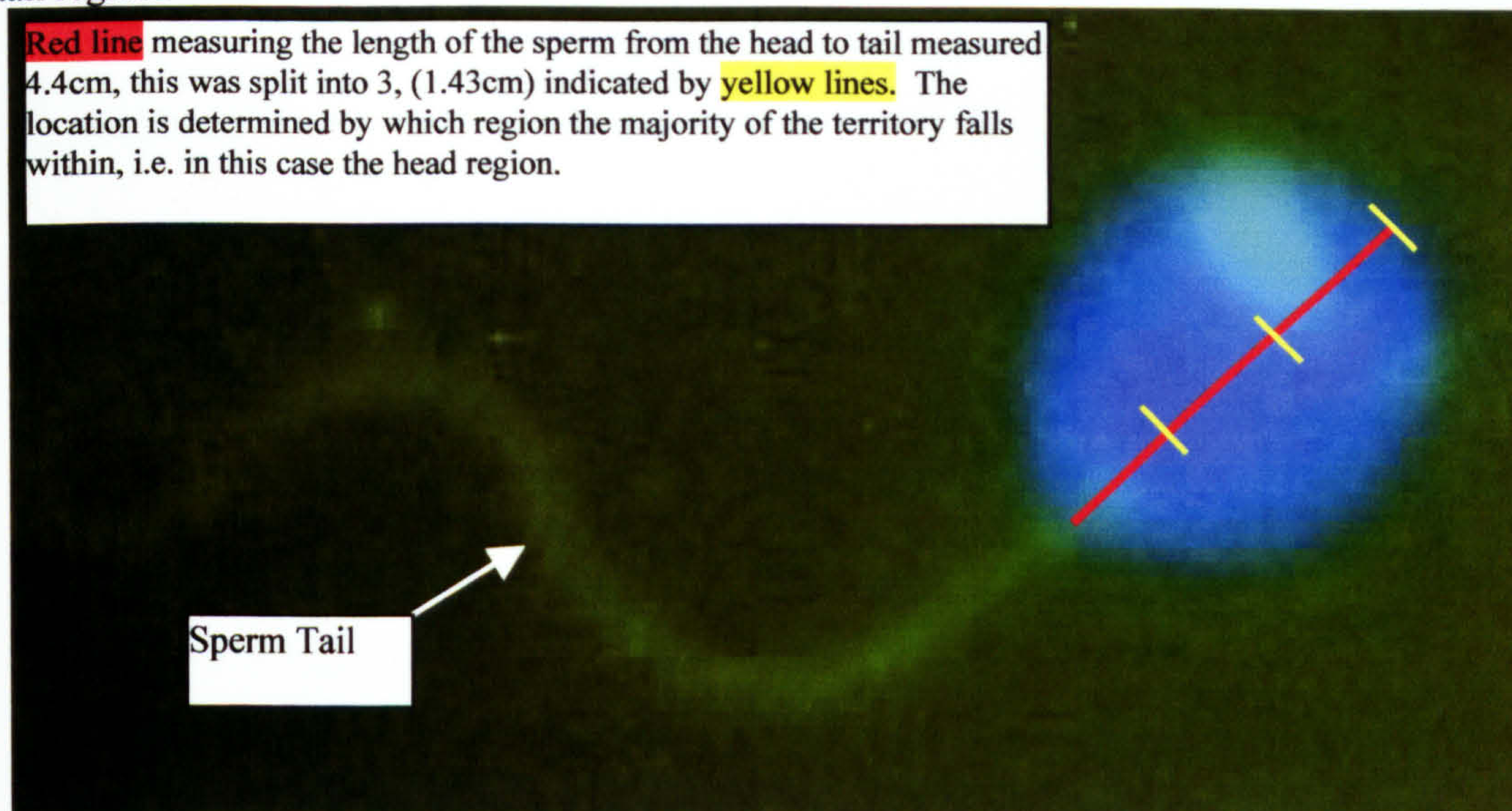


Figure 8.7- image (taken at the same magnification of the sperm nuclei) of the graticule measuring 10μm, as with the sperm nuclei this was imported into Adobe Photoshop, and was enlarged to the same magnitude as the sperm nuclei. A 10μm section (as indicated by the red line), was then measured =6cm, enabling the measurements (in cms) to be converted into microns.

8.7.4.1.2 Collection of data with regards to chromosome territory position relative to the polarity of the sperm nuclei

The position of the sperm tail was also noted if still present and visible after the FISH procedure. This enabled us to determine whether chromosomes preferentially locate within head, middle or tail region of the sperm nuclei. The sperm head nuclei were divided into three equal parts along the longest length of the sperm (figure 8.8). The location of the chromosome territory was determined by which of the three regions the majority of the chromosome territory localised to, i.e. head middle or tail region (figure 8.8) (Bridger, personal communication).

Figure 8.8- illustrating how chromosome territories were determined to be located in the head middle or tail regions



8.7.4.2 Analysis of the data generated by chromosome positioning study

The position of the chromosome territory was established (relative to the centre of the sperm nuclei as described in section 8.7.4.1.1). All measurements were converted to microns, and the data was normalised to take into account the varying sizes of sperm nuclei. This was carried out by dividing the number of microns from the centre of the chromosome territory to the nearest edge of the sperm nuclei by total length of the sperm nuclei at the longest point (also in microns) (Bridger, personal communication. The value obtained from this calculation is the value used to determine the location of the chromosome territory (relative to the centre of the sperm nuclei).

Analysis of the location of the chromosome territory in respect to polarity was made possible with respect to head, middle or tail position, whenever the tail was still present after the FISH procedure (refer to section 8.7.4.1.2). In these cases a note was made for each sperm as to whether the majority of the chromosome territory was located in either the head, middle or tail region.

Chapter 9

Appendices

Appendix A:

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DEBATE—continued

Safety issues in assisted reproduction technology

Should men undergoing ICSI be screened for chromosome abnormalities in their sperm?

D.K.Griffin[1,3], P.Hyland[1], H.G.Tempest[1] and S.T.Homa[2]

[1]Cell and Chromosome Biology Group, Department of Biological Sciences, Brunel University, Uxbridge, Middlesex UB8 3PH and [2]10, Harley Street, London W1G 9PF, UK

[3]To whom correspondence should be addressed. E-mail: darren.griffin@brunel.ac.uk

The incidence of aneuploidy in gametes of men undergoing ICSI has raised the prospect of there being risks associated with ICSI and the question of whether or not to screen men for sperm aneuploidy before treatment. We report results of a questionnaire undertaken to address how IVF staff perceive this problem, whether ICSI men are already being screened for sperm aneuploidy and the extent to which IVF specialists feel that there is merit in such a test. The results suggest that this is seen as a problem but most feel the risks outweigh the benefits. Most claimed their clinics do not screen sperm for aneuploidy but feel that there is merit in doing so. There are considerable benefits to screening i.e. couples would get additional information about the genetic repercussions of ICSI and could make informed decisions before treatment; screening would also facilitate the design of a large research study to give clearer answers on the safety of ICSI. However, we acknowledge counter arguments i.e. families would not necessarily benefit as most would have the ICSI procedure regardless of screen results; sex chromosome trisomies clinically are not severe enough to worry about in this context and there are other potential risks of ICSI that screening would not address.

Key words: aneuploidy/ICSI/OAT/opinions/sperm

Introduction

The proportion of aneuploid sperm (i.e. with extra or missing chromosomes) in males with severe defects in the conventional parameters of semen quality, has been the topic of numerous studies in recent years (Lahdetie *et al.*, 1997; Calogero *et al.*, 2001; Hansen *et al.*, 2002; Healy and Saunders., 2002). Severe oligozoospermia has been associated with aneuploidy levels of up to 70% (Pang *et al.*, 1999; Pfeffer *et al.*, 1999; Ohashi *et al.*, 2001), i.e. extrapolations from sperm fluorescent in-situ hybridization (FISH) studies involving a few chromosomes at a time have indicated that the most severely affected males may have up to 70% of sperm that have at least one extra or one missing chromosome (Pang *et al.*, 1999; Pfeffer *et al.*, 1999). The vast majority of studies have provided evidence for a highly significant relationship between decreased semen quality parameters and increased sperm aneuploidy (Bernardini., 1997; Lahdetie *et al.*, 1997; Li and Hoshiai, 1998; Martin, 1998; McInnes *et al.*, 1998; Aran *et al.*, 1999; Colombero *et al.*, 1999; Nishikawa *et al.*, 2000; Shi and Martin, 2000; Ushijimal *et al.*, 2000 ; Vegetti *et al.*, 2000). In other words, we are aware of ~50 studies that address this issue, and the vast majority suggest that men with severe reductions in the usual criteria used to measure sperm have significantly elevated levels of sperm disomy. In our own experience, we have found up to a 15-fold increase in sperm aneuploidy for the chromosomes thought to be most prone to non-disjunction i.e. chromosomes X, Y and 21 (H.G.Tempest, unpublished data) in men with sub-optimal semen parameters. However, the magnitude of the increase varies from laboratory to laboratory and from patient to patient, indeed we are aware of about four studies, including Guttenbach *et al.*, that show no relationship at all (Guttenbach *et al.*, 1997). The apparent discrepancy between reports may be explained by technical reasons (e.g. laboratory specific differences in stringency of scoring criteria) or there may be genuine differences depending on the population of males studied. Specifically, intrinsic (e.g. DNA polymorphisms) or extrinsic (e.g. environmental pollutants) factors may influence sperm aneuploidy depending on the patient cohort studied. Differences in aneuploidy among populations are not widely reported, however both intrinsic and extrinsic factors have been linked to errors in chromosome segregation. For example, cigarette smoke, alcohol and chemotherapy regimes can all cause increased sperm aneuploidy (Robbins *et al.*, 1997; Rubes *et al.*, 1998; Shi *et al.*, 2001) while factors such as age have been clearly associated with increased aneuploidy in both males and females (Griffin *et al.*, 1995; Hassold *et al.*, 1996). Moreover, a recent report has identified a genetic polymorphism as a significant risk factor for Down's syndrome (Hobbs *et al.*, 2000).

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Men with severe oligozoospermia are usually treated by ICSI. Therefore this raises the question of whether, in cases where men have significantly elevated levels of sperm aneuploidy, there is an increased risk of producing aneuploid offspring. This is of especial concern for trisomies of the sex chromosomes that arise 50% of the time in the sperm. Studies that have been performed examining the chromosomal constitution of ICSI conceptions suggest that there is some cause for concern. Bonduelle *et al.* reported that 9/1084 ICSI conceptuses respectively had sex chromosome abnormalities (Bonduelle *et al.*, 1999a); this is 5–10 times the published population frequency (Hassold *et al.*, 1996). More recently Bonduelle *et al.* compared outcomes of ICSI versus neonate pregnancies and found that 6/1000 had sex chromosome abnormalities in the ICSI group compared with 2/1000 in the control group—a small yet statistically significant increase (Bonduelle *et al.*, 2002).

Given the level of interest in the literature it seems reasonable to suggest that the increase in sperm aneuploidy associated with male infertility is of significant concern for infertility clinics and families embarking upon ICSI treatment. The question then arises 'should the male partner be screened for sperm aneuploidy prior to ICSI?'—at least for the sex chromosomes. In our opinion, before this question can be discussed fully, a number of questions remain that, to the best of our knowledge, have hitherto been poorly investigated; these include the extent to which: (i) sperm aneuploidy for ICSI males is perceived as a problem in infertility clinics; (ii) men are already screened for sperm aneuploidy in IVF clinics; and (iii) specialists in infertility clinics feel that there is merit in screening ICSI males for sperm aneuploidy by molecular cytogenetic techniques.

In the first part of this debate we report and discuss the results of a questionnaire-based study in which specialists in infertility clinics were surveyed with these questions in mind. They suggest that there is sufficient interest in this issue to warrant investment in molecular cytogenetic technology in infertility clinics to screen males for levels of aneuploidy as an adjunct to conventional semen analysis. In the second part we place these results in the wider context of a debate on the pros and cons of implementing a sperm aneuploidy screening service for ICSI couples.

Materials and methods

A one-page questionnaire was sent to scientists, clinicians, nurses and counsellors to all infertility clinics operating in the UK (Figure 1). In total 590 questionnaires were sent; the questionnaire was made deliberately short (consisting of only 5 questions) in order to maximize the return. For this reason, a self-addressed envelope was also included. In order to avoid leading questions, participants were asked about a number of genetic factors associated with male infertility e.g. constitutional chromosome abnormalities and Y-chromosome deletions. The brief covering letter accompanying the questionnaire explained the purpose of the study. The questionnaire was anonymous.

Statistical analysis

In order to establish whether the responses were statistically significant, standard χ^2-analysis was used.

Results

Of the 590 questionnaires sent, 190 (32.2%) were completed and returned; 78 (13.2%) were returned as "no longer known at this address". In order to maximize return, the respondents were not asked to give their name. It was therefore difficult to determine accurately what proportion of UK clinics were represented; however analysis of the postmark on the return envelopes suggested that 55–70% of all UK clinics were represented by at least one respondent. Figure 1 also shows the number of answers obtained for each question in this study.

All individuals questioned were invited to state their position in the infertility clinic; however the majority declined to do so; thus the analysis is based on the cohort in general rather than opinions among individual career groups within IVF clinics. However, of the 49 that did state their occupation, 21 gave their job description as 'consultant' suggesting that the 'decision makers' of the IVF clinics were represented significantly in the study.

Approximately 82% (156) of respondents worked at clinics where ICSI was routinely performed. Of these, 126 (80.7%) indicated that their clinics routinely performed karyotype analysis on patients undergoing ICSI, 65 (41.7%) performed Y-chromosome deletion analysis and 18 (11.5%) of the participants worked in clinics where chromosome analysis of sperm before treatment was performed.

Approximately half (49.5%) of the respondents felt there was an increased risk of a child being born with congenital abnormalities when conceived by ICSI versus standard IVF treatment. This was significantly more than the 54 (28.4%) ($P < 0.005$ by χ^2-test) who felt there was no increased risk of congenital abnormalities in babies conceived by ICSI versus standard IVF treatment [42 respondents (22.1%) were unsure].

Of the participants who felt that there was an increased risk of congenital abnormalities associated with ICSI, 61, by far the largest majority (64.9%), felt this risk was due to chromosomal abnormalities in the sperm used for the procedure. Of the remainder, 11 (11.7%) felt it was due to the actual ICSI procedure itself and 14 (14.9%) were unsure of the cause. Around 8 (8.5%) respondents felt other factors were responsible for the increase and, when invited to comment on what they thought they were, responses included "chromosomal abnormalities in the egg used for the procedure", "artificial selection of sperm" and "individual circumstances" Thus a significant majority ($P < 0.0005$) cited chromosome abnormalities in the sperm as the major risk factor (in their opinion) for ICSI treatment.

When asked to choose a statement that best described their current opinion about ICSI, 122, the significant majority (64.2%) ($P < 0.0005$), felt there were minimal risks with ICSI but that the risks were outweighed by the benefits. Of the remainder, 35 (18.4%) required further information before forming an opinion; 17 (8.9%) felt there were no additional risks with ICSI over standard IVF treatment; 10 (5.3%) felt that there were considerable risks with ICSI but that it was still a worthwhile treatment overall. Significantly, none of the respondents felt the risks of ICSI outweighed the benefits and they would therefore recommend it to patients. Of the 6 (3.2%)

Sperm chromosome screening before ICSI

Please state your current job title: ____________________

QUESTIONNAIRE:

1. Does your clinic currently perform intracytoplasmic sperm injection (ICSI)?

Yes 156 No 34

1a. If Yes, does your clinic counsel and arrange for patients to have:

Karyotype analysis?	Yes	126	No	28	Unsure	2
Y chromosome deletion analysis?	Yes	65	No	55	Unsure	6
Chromosome analysis of sperm before treatment?	Yes	18	No	128	Unsure	10

2. In your opinion, is there an increased risk of congenital abnormalities in babies conceived by ICSI versus standard in vitro fertilisation (IVF) treatment?

Yes 94 No 54 Unsure 42

2a. If Yes, what do you feel causes this increase?

Chromosomal abnormalities in sperm used for procedure	61	ICSI procedure itself	11
Unsure	14	Other (please specify)	8

3. Which of the following statements best applies to your opinion about ICSI?

No additional risks with ICSI over standard IVF	17	Minimal risks with ICSI but outweighed by benefits	122
Considerable risks with ICSI but still worthwhile overall	10	Risks outweigh benefits would not recommend to patients	0
Further information required before forming opinion	35	Other (please specify)	6

4. Are patients in your clinic currently counselled about potential risks of ICSI?

Yes 184 No 6 Not applicable 0

5. Do you think there is merit in pre-screening sperm of ICSI males for chromosomal abnormalities prior to treatment?

Yes 97 No 43 Unsure 50

Thank you for your help in completing this questionnaire. If you have any further comments please feel free to write them on the reverse of this sheet. Please return completed questionnaire in the stamped envelope provided.

Figure 1. Questionnaire sent to clinics and number of responses for each question.

that held opinions described as 'other' responses included "safety depends on indication for ICSI" and "individual circumstances must be taken into account".

Our results demonstrate that counselling about the risks of ICSI is widespread in IVF clinics as 184 (96.8%) indicated their patients were counselled and just 6 (3.2%) suggested they were not.

Finally, we were interested to note that 97, a significant majority (51.1%), felt that there was merit in pre-screening the sperm for chromosomal abnormalities prior to ICSI. This compared with 43 (22.6%) who felt there was no merit in so doing and 50 (26.3%) who were unsure. In other words over twice as many IVF specialists are supportive of our hypothesis that such a screen should be implemented in addition to

assessing regular semen parameters, compared with those who were not. This is highly significant ($P < 0.0005$) by χ^2-analysis.

Discussion

Questionnaire results

The non-Mendelian transmission of aneuploidy to the offspring of couples undergoing ICSI is a serious medical issue and one of considerable debate among the scientific community (Lahdetie *et al.*, 1997; Calogero *et al.*, 2001; Hansen *et al.*, 2002; Healy and Saunders, 2002). The results of the questionnaire part of this study seem to suggest that there is enough concern about sperm aneuploidy in infertility clinics to warrant inclusion of a screen for sperm aneuploidy prior to ICSI. It seems that, for the most part, these opinions are made on the basis that the majority of IVF specialists questioned believe that there is an increased risk associated with ICSI (most likely to do with increased sperm aneuploidy) compared with standard IVF, but that the risks outweigh the benefits. They also indicate that screening for sperm aneuploidy is not a practice that is widespread with only 18 (11.4%) of the respondents suggesting that this occurs in their clinics compared with 126 (80.7%) who said that constitutional karyotyping was commonplace. Of these 18, nine were almost certainly from the same clinic as they were returned in the same envelope with a 'with compliments' slip attached. Further questioning of a senior member of that particular laboratory (results not shown) revealed that information gleaned from screening sperm for aneuploidy beforehand was used for research purposes only and information was not yet passed on to patients. Informal discussions with colleagues suggest that this is also the case in other clinics that examine sperm for aneuploidy. Thus, although 18 respondents said that their clinic counselled and arranged for patients to have sperm chromosome analysis, it seems certain that some (if not most) are counselled about the risks of ICSI and then given the opportunity to take part in an anonymous research programme. A further possibility is that some respondents did not understand the term chromosome analysis of sperm in this context. At this time therefore we are not aware of any clinic in the UK which screens for sperm aneuploidy and then uses that information as a means for genetic counselling of patients. It is clear that about twice as many respondents of this questionnaire think it is a good idea compared with those who do not. Clearly however, while very important, the opinions of IVF specialists are only one part of the decision-making process on this issue and the hard scientific facts should be considered in context.

Pros and cons of screening ICSI males for sperm aneuploidy

Screening for sex chromosome aneuploidy in sperm before ICSI might allow genetic counsellors to allay the fears of those families who are at low risk (i.e. those that have low levels of sperm aneuploidy). 'Normal' levels of sex chromosome aneuploidy are thought to be around 0.3% i.e. 2–4 sperm with an extra or missing sex chromosome in every 1000 (Griffin *et al.*, 1995). Men about to embark upon ICSI with similar levels should be, theoretically, at no increased risk of producing offspring with sex chromosome aneuploidy. In some men however, sex chromosome aneuploidy in sperm may be 10–30 times that of normal levels (Pang *et al.*, 1999). In this scenario, based on existing data regarding the maternal versus paternal contribution to sex chromosome aneuploidy (Hassold *et al.*, 1996), reasonably accurate estimates of potential risk of transmission can be made by genetic counsellors. Equipped with this information families can then make informed decisions about whether or not to continue with ICSI and, if they do, whether to have prenatal diagnosis of any subsequent pregnancy. The question of where to draw the line between 'high risk' and 'low risk' is, however likely to be a contentious one. An interesting analogy is that of the triple screen test for pregnant women. On the whole, families who are given a risk of around 1 in 250 as a result of the triple screen are offered amniocentesis (Wellesley *et al.*, 2002). The figure of 1 in 250 has been chosen on the balance of several lines of evidence as the most appropriate threshold point. Perhaps therefore a threshold based on similar levels of risk should be established for ICSI men with elevated levels of sperm aneuploidy.

An additional benefit of providing an assessment of sperm aneuploidy to a large number patients is that it would be the first stage in which a very large number of ICSI males could be screened as part of a research study. Pregnancies could be followed up subsequently in order to establish more accurate estimates of the risk of transmitting trisomy through ICSI. The purpose of this study would be, ultimately, to generate enough data to provide even more accurate assessments of the risk of transmitting trisomy for ICSI couples. In particular, the issue of whether ICSI perpetuates the risk of trisomy 21 could be addressed. A further advantage of such a technology being widespread is that the regular screening of sex chromosome aneuploidy might provide a means by which the efficacy of any new chemotherapeutic treatment regimes might be monitored. This would be particularly applicable if any pharmacogenomic approaches that addressed the problems of chromosome segregation directly were developed. Should such approaches prove effective, they might then provide means of treating male infertility that are less labour intensive and do not have the risks associated with producing aneuploid offspring as has been suggested for ICSI.

It is often argued that there is an apparent discrepancy between the high levels of sperm aneuploidy in ICSI males and the relatively reassuring figures for ICSI outcome. However, closer analysis of the data might suggest that this is not necessarily the case. Klinefelter syndrome (47,XXY) arises as a result of XY sperm disomy ~50% of the time whereas a 47,XYY karyotype is 100% paternally derived (Hassold *et al.*, 1996). Thus, a significant increase in sperm aneuploidy should, in theory, lead to an increased risk of these and other disorders in ICSI offspring. Recent work provides significant evidence that there is a several fold increase in sex chromosome abnormalities associated with ICSI treatment (Bonduelle, 1999a,b, 2002) however, to the best of our knowledge these men were not screened for the incidence of sex chromosome abnormalities in their sperm beforehand. Had this been the case, then a more accurate correlation between the incidence of sex chromosome aneuploidy in sperm and the karyotype of the child might have been possible and we suggest that then there would have been a very close correlation. Indeed to the best of our knowledge, there have been no large controlled studies that have screened sperm aneuploidy before ICSI, then monitored pregnancies throughout and then estimated the levels of paternally derived aneuploidy in the subsequent offspring. Clearly such a project would be an extremely difficult undertaking but it would help us resolve more fully the

question of the relationship between sex chromosome sperm aneuploidy and ICSI outcome. In this case, we might expect a very close correlation between the level of YY disomy and XYY children (since all XYY conceptuses are paternally derived). We might expect a slightly lower but nonetheless significant correlation between Klinefelter syndrome and XY sperm disomy since ~50% of these errors ordinarily arise in the sperm. However we would expect only a weak correlation between XX disomy and XXX conceptuses as the majority of these normally arise as a non-disjunction event in the ovary (Hassold *et al.*, 1996). In order to screen for sex chromosome abnormalities in sperm, it is necessary to look at at least one autosome to distinguish disomic from diploid sperm: i.e. a sperm with two sex chromosomes and one autosome is assumed to be disomic whereas a sperm with two sex chromosomes and two autosomes is assumed to be diploid (Griffin *et al.*, 1995). In this case it seems reasonable to suggest that the autosome screened should be chromosome 21. While Down's syndrome (trisomy 21) is predominantly (~90%) maternally derived (Hassold *et al.*, 1996), a tenfold increase in the incidence of disomy 21 in the sperm could potentially double overall risk of trisomy 21 in the offspring. We are not aware of any studies demonstrating an increased risk of Down's syndrome associated with ICSI. However any increase would be unlikely to have been seen using the methodologies of current studies (Bonduelle *et al.*, 1999a,b, 2002). This is for two reasons: (i) because of the relatively small number of live births studied (around 1000 each, in test and control groups); and (ii) because the parental origin of the aberrant chromosome was not determined. As a comparison, had the same approach been used to address the question of whether Down's syndrome (incidence 1 in 700 live births) was associated with maternal age, it is questionable whether a statistically significant difference would have been seen between the two groups. Paternally-derived Down's syndrome accounts for about 1 in 7000–14 000 live births; while this cannot be thought of as common, its incidence is similar to that of myotonic dystrophy and Edward's Syndrome. It is therefore reasonable to suggest that any significant increase in this disorder associated with the use of ICSI treatment might be of clinical concern. It is questionable however whether clinicians and patients would necessarily distinguish between the maternally- and paternally-derived forms given that the clinical features are identical. Nevertheless given that screening sperm for sex chromosome abnormalities requires the examination of at least one autosome, we propose that this autosome should be chromosome 21. In fact, the majority of aneuploid conceptuses (including presumably those that arise as a result of injection of an aneuploid sperm) are thought not to reach the stage of clinical recognition (Hassold *et al.*, 1996). Of those in which a pregnancy is demonstrabley established, the majority result in spontaneous abortions. In fact only cases of sex chromosome aneuploidy or trisomy 21 can usually result in live births (with very rare exceptions e.g. trisomies 18 and 13). While spontaneous abortions can be very stressful for families (particularly if there have already been several pregnancy losses), it is, in our experience, the prospect of having aneuploid offspring (even with mild clinical features) that is of most concern to ICSI families. Thus, we propose that, at the moment, if sperm aneuploidy screening of ICSI men is to go ahead, the sex chromosomes and chromosome 21 should be screened alone. To conclude, the increased risk of transmitting trisomy through ICSI is therefore quite well established for the sex chromosomes but not for chromosome 21. There is, theoretically, an increased risk of transmitting paternally derived trisomy 21 although this has yet to be determined on a large cohort of ICSI outcomes. Moreover, an increase in sperm aneuploidy for the 'non-21' autosomes is of less concern in terms of screening in ICSI males but could, theoretically, lead to increased incidence of spontaneous abortion (Hassold *et al.*, 1996) thus further limiting the chances of a live birth.

If a screen for sperm aneuploidy were implemented, appropriately trained individuals could estimate the potential of conceiving aneuploid offspring by performing FISH and counting the proportion of aneuploid sperm in the male partner. The test could be done at the same time as the semen assessment and, probably, could be organized in order not to delay the progress of the ICSI cycle. This procedure is nevertheless costly in that it involves development or procurement of high quality fluorescent probes, staff training, the cost of each individual test, the purchase of a high specification epifluorescence microscope and the costs involved in establishing the technique as routine. A number of clinical laboratories, however, now already have epifluorescence microscopes and the cost of the test, if borne by the patient, would be minor in comparison with the total cost of the ICSI procedure. A second drawback of implementing the procedure is that scoring about 5000 sperm per patient is very time consuming. This problem may ultimately be circumvented by automated approaches for the scoring of sperm aneuploidy that should be less labour intensive; these are under development in a number of laboratories. Thus, before such a service is offered, it needs to be established that these and other drawbacks are outweighed by the benefits.

What is not clear (and, possibly, is unlikely to be so until such a service is implemented) is how patients would respond given the information that they were at high risk of transmitting aneuploidy to their offspring. Giltay *et al.* (1999) reported that a significant majority [42 out of 75 (56%) Dutch couples] continued with ICSI even though they were carrying constitutional chromosome abnormalities and were counselled extensively that they were at a significant risk of having an affected child (Giltay *et al.*, 1999). It could be argued however that the patients in that study benefited from the information that they were at risk of an affected child so that they could come to an informed decision, indeed nearly half of these opted for amniocentesis given this information. If this were the case, then it would be a strong argument in favour of screening sperm for aneuploidy levels prior to ICSI. An alternative interpretation however is that the results suggest that, even given information about elevated sperm aneuploidy, families would, on the whole, go ahead with ICSI anyway and thus screening for sperm aneuploidy would be a fruitless exercise. We have difficulty with this second viewpoint for the following reasons: closer analysis of the data by Giltay *et al.* (1999) reveals that 31% of the patients in the study declined ICSI given the information about their genetic risk. It is, in fact highly unlikely for a sub-fertile man to decline ICSI under normal circumstances (S.T.Homa, personal communication). This leads us to conclude that a significant proportion may take the decision to refrain from ICSI if they were identified as high risk whereas more still would opt for prenatal diagnosis with a view to either terminating or preparing for an affected child. In either case, therefore, the information is highly valuable to the patients and their reproductive planning.

A further issue is that factors other than elevated sperm aneuploidy may lead to an increased risk of trisomic offspring as a result of ICSI. These include the disruption of the meiotic spindle during the injection process (e.g. Pang *et al.*, 1999) and inadequate decondensation of the sex chromosomes in the sub-acrosomal region (Sbracia *et al.*, 2002). If either of these hypotheses prove to at least contribute to the observed increase in sex chromosome abnormalities in ICSI children then this might lessen the need to screen ICSI males for aneuploidy. However, given that sex chromosome aneuploidy levels are elevated in ICSI males and elevated levels of sex chromosome abnormalities have been reported in ICSI children it seems unlikely that there would not be at least some relationship between the two findings.

A final consideration is whether sex chromosome abnormalities are clinically severe enough to be of sufficient concern for ICSI clinics and families. The majority of sex chromosome abnormalities are only diagnosed if they are picked up prenatally or when affected individuals are having reproductive problems. Indeed, most are not detected at all and fewer couples are opting for a therapeutic abortion when a sex chromosome abnormality is diagnosed (Abramsky *et al.*, 2001). In the context of this study then it raises the question of whether these relatively mild symptoms during childhood warrant investing in an extensive screening programme for sperm aneuploidy. On one side of the argument, if couples would mostly continue with ICSI anyway and the disorder would be unlikely to be detected unless it was looked for specifically, then there is little point in implementing the screen. On the other, the reproductive and secondary sexual development problems associated with sex chromosome abnormalities vary from individual to individual and can be quite severe. We should therefore inform couples (to the best of the genetic counsellor's knowledge) of the risk of having an affected child, particularly if they are willing to pay for the test. Moreover, although the risk of paternally-derived trisomy 21 is only theoretical at this stage, it may be argued that it is of sufficient concern to warrant further investigation. This could be achieved by screening a large number of ICSI males for disomy 21 levels in sperm and monitoring obstetric outcome by karyotyping.

In conclusion, screening for sperm aneuploidy is not commonly practised in IVF clinics. There are pros and cons of implementing such a screen, and, in our experience, about twice as many IVF specialists favour the idea compared with those who do not. The benefits of screening at least for chromosomes X, Y and 21 include the added information it gives to families about their reproductive health and the fact that it can be implemented without delaying ICSI. With this knowledge, the patients can then make informed decisions regarding their treatment, and whether they wish to opt for prenatal diagnosis and/or plan for a potentially affected child. There is a further benefit that it would allow a larger research programme that would ultimately give clearer answers on the safety of ICSI e.g. with regard to the risk of transmitting Down's syndrome. Indeed, it could be argued that, if karyotyping is recommended for these men (in which an abnormality is found in about 12% of ICSI men: Nakamura *et al.*, 2001) then sperm aneuploidy certainly should be implemented as abnormal levels are likely to be found in far more infertile men. On the other hand the following can be argued in response: (i) families would not benefit from such a screen as the majority would go ahead with ICSI regardless of the information given; (ii) sex chromosome abnormalities are clinically not severe enough to worry about in this context and (iii) even if we do screen for sperm aneuploidy it would not take into account other associated risks of the ICSI procedure. In our opinion the first point is questionable given a closer analysis of the data (Giltay *et al.*, 1999). The second point is central to the debate and is likely to be resolved more clearly when the question of the risk of transmitting Down's syndrome is more clearly established. The third point is theoretical at this stage and it seems likely that there is at least some significant relationship between levels of sperm aneuploidy and incidence of trisomic offspring in ICSI couples. The only certainty is that the debate will continue for some time before the test becomes widespread.

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Appendix B:

Online Table: Mouse mutations causing reproductive defects. Only single mutant defects are described. Fertility defects of unknown gene origin are not described. M, male; F, female; Hetero, heterozygote phenotype taken from Matzuk and Lamb, (2002).

Mutant gene	Male/ Female	Reproductive Phenotype	Fertility Status
Acrosin (Acr)	M	Sperm are capable of binding and penetrating the zona pellucida	Delayed fertility
Activin receptor-type IIA (Acvr2)	B	Antral follicle block in females; small testes, delayed fertility in males	Infertility F Subfertility M
Activin/inhibin βB subunit (Inhbb)	F	Delivery and nursing defects	Subfertility
Acyl-CoA synthetase 4 (ACS4; Facl4)	FH	Enlarged uteri with prostaglandin accumulation	Subfertility
Adamts1 (a disintegrinlike and metalloprotease with thrombospondin type 1 motif, 1)	F	Cystic formations in uteri; defects in preovulatory follicle development	Subfertility
Adamts2 (procollagen N-proteinase)	M	Defects in spermatogenesis; marked decrease in sperm within testes tubules	Infertility
ADP-ribosylation factor-like 4 (Arl4)	M	Significantly reduced testicular weights and sperm counts	Normal fertility
Alpha 1 microglobulin/bikunin (Ambp; Urinary trypsin inhibitor)	F	Defects in ovulation and cumulusoocytes complex (COC) integrity	Subfertility
Angiotensin-converting enzyme (Ace)	M	Compromised ability of sperm to fertilize ova	Subfertility
Androgen receptor (Ar; tfm or Testicular feminization)	M	Feminized external genitalia; hypogonadal; cryptorchidism with a block in spermatogenesis	Infertility
Anti-Müllerian hormone (Amh)	B	Uteri development in males causes obstruction and secondary infertility; females exhibit early delpetion of primordial follicles	Secondary Infertility
AMH receptor (Amhr2)	M	Uteri development in males causes obstruction and secondary infertility	Secondary Infertility
Apaf1 (Apoptotic protease activating factor 1)	M	Spermatogonial degeneration Variable lethality;	Infertility
Apolipoprotein B (Apob)	MH	Decreased sperm count, motility, survival time, and ability to fertilize ova	Infertility
Aryl-hydrocarbon receptor (Ahr)	F	Early development of primordial follicles; decreased numbers of antral follicles	Subfertility
Ataxia telangiectasia (Atm)	B	Germ cells degenerate; disruptions evident in meiosis I	Infertility
ATP-binding cassette transporter 1 (Abca1)	F	Placental malformations leading to impaired embryo growth, embryo loss and neonatal death	Subfertility
Basigin (Bsg)	B	Defects in fertilization and implantation (F); block in spermatogenesis at metaphase I (M)	Partial lethality; Infertility
Bax (Bcl2-associated X protein)	B	Premeiotic arrest of spermatogenesis; increased oocytes and primordial follicles postnatally	Infertility M
Bcl2 (B-cell leukemia/ lymphoma 2)	F	Fewer oocytes/primordial follicles in the post-natal ovary	Subfertility
Bcl6	M	Apoptosis in metaphase I spermatocytes	Subfertility
BclX (Bcl2l) hypomorph	B	PGCs are lost by E15.5	Infertility
Bclw (Bcl2l2, Bcl2-like 2)	B	Late meiotic arrest with loss of germ cells (M) and reduced PGC survival (F)	Infertility M Subfertility F
Bone morphogenetic protein 4 (Bmp4)	B	Absent primordial germ cell (PGC) population; defect in PGC development	Lethal
Bmp8a	M	Degeneration of germ cells and epididymis	Progressive Infertility
Bmp8b	B	Reduced or absent PGCs (developmental defect); Postnatal male germ cell proliferation/differentiation defect and spermatocyte apoptosis	Subfertility/ Infertility
Bmp15	F	Defects in cumulus-oocyte complex (COC) formation and ovulation	Subfertility
BMP receptor, type IB (Bmpr1b)	F	Defects in estrous cyclicity, cumulus expansion, and endometrial gland development	Subfertility
Calmegin (Clgn)	M	Defect in sperm-zona pellucida binding	Infertility
Camk4(calcium/calmodulindependent protein kinase IV)	M	Impaired chromatin packaging during spermiogensis	Infertility
cAMP-responsive element	M	Defective spermiogenesis with aberrant post-meiotic gene	Infertility

modulator (Crem)		expression	
cAMP-specific phosphodiesterase type 4 (Pde4d)	F	Diminished sensitivity of the granulosa cells to gonadotropins	Subfertility
Casein kinase II 1 (Csnk2a2)	M	Globozoospermia (no acrosomal cap)	Infertility
Caspase-2 (Casp2)	F	Decreased apoptosis of female germ cells	Increased Fertility
CatSper (putative sperm cation channel)	M	Defects in motility and fertilization	Infertility
CD9 antigen (Cd9)	F	Sperm-egg binding defect	Subfertility
Cell division cycle 25 homolog B (Cdc25b) (Cdc25b phosphatase)	F	Oocytes are arrested in meiotic prophase, with defects in maturation promoting factor activity	Infertility
Centromere protein B (Cenpb)	B	Males are hypogonadal and have low sperm counts; females have straindependent uterine epithelium defects	Subfertility F
C/EPBβ (CCAAT/enhancerbindin g protein β)	F	Reduced ovulation and block in CL differentiation	Infertility
Claudin 11 (Cldn11; Osp-11)	M	No tight junctions between Sertoli cells	Infertility
Colony stimulating factor (macrophage) (Csf1)	B	Males have reduced testosterone; females have implantation and lactation defects	Subfertility
Colony stimulating factor (granulocytemacrophage) (Csf2)	B	Mean litter size decrease with disproportionate loss of males pups (F); maternal effects most pronounced in intercrosses with knockout males	Intercrossing Subfertility
Connexin 37 (Gja4; Cx37)	F	Defects in late folliculogenesis and oocyte meiosis	Infertility
Connexin 43 (Gja1; Cx43)	B	Small ovaries and testes; decreased numbers of germ cells from E11.5	Neonatal lethality
Cpeb (cytoplasmic polyadenylation element binding protein)	B	Disrupted germ cell differentiation and meiosis I synaptonemal complex formation	Infertility
Cut-like 1(Cutl1; CDP/Cux) truncation mutant	M	Severely reduced male fertility	Subfertility
Cyclin A1 (Ccna1)	M	Block in spermatogenesis before the first meiotic division	Infertility
Cyclin D2 (Ccnd2)	B	Failure of granulosa cell proliferation (F); males fertile with decreased testis size	Infertility F
Cyclin dependent kinase 4 (Cdk4)	F	Defects in the hypothalamic-pituitarygonadal axis	Infertility
Cyclooxygenase 2 Ptgs2)	F	Defects in ovulation and implantation	Most Infertile
Cyp11a (Cytochrome 450, 11a, cholesterol side chain cleavage)	B	Males feminized with female external genitalia, underdeveloped sex organs; gonads degenerate	Lethal
Cyp19 (Cytochrome P450, 19, aromatase)	B	Early spermatogonial arrest, Leydig cell hyperplasia, and defects in sexual behavior (M); folliculogenesis block and ovulation defects	Progressive Infertility M; Infertility F
Cyp40 (P450 25-hydroxyvitamin D-1(.hydroxylase)	F	Uterine hypoplasia and absence of CL	Infertility
Cyritestin (Adam3)	M	Altered sperm protein expression and adhesion defects during fertilization	Infertility
Dax1 (Nr0b1)	M	Progressive degeneration of the germinal epithelium	Infertility
Dazl (Deleted in azoospermia-like)	B	Reduced germ cells; differentiation failure and degeneration of germ cells	Infertility
Desert hedgehog (Dhh)	M	Complete absence of mature sperm; defects in Sertoli-to-Leydig cell signaling	Infertility
Dmc1h (Disrupted meiotic cDNA 1 homolog)	B	Defects in chromosome synapsis in meiosis; female germ cells degenerate during embryogenesis	Infertility
Dnmt1o (DNA methyltransferase)	F	Embryos of knockout females die during gestation due to imprinting defects; maternal effect gene	Subfertility
DNA polymerase λ	M	Immotile spermatozoa	Lethality; Infertility
Doublesex and mab-3 related transcription factor 1 (Dmrt1)	M	Defects in post-natal testes differentiation; disorganized seminiferous tubules and absence of germ cells	Infertility
Dynein heavy chain 7 (Dnahc1)	M	Defects in sperm flagellar motility	Infertility
Early growth response 1 (Egr1; NGFI-A) targeted lacZ insertion	B	Lack of LH (M); downregulation of LHR, not remedied with gonadotropin treatment (F)	Infertility
Early growth response 1 (Egr1) targeted neo insertion	F	LH insufficiency; loss of estrous cyclicity, no CL; rescued by treatment with gonadotropins	Infertility
Early growth response 4 (Egr4)	M	Germ cells undergo apoptosis during pachytene stage	Infertility
ELKL motif kinase (Emk)	B	β-gal gene trap insertion creates a null allele; homozygotes intercrossed are not fertile	Intercrossing Infertility
Empty spiracles homolog 2 (Emx2)	B	Defective development of gonads and urogenital tracts	Lethal
Estrogen receptor α (ERα)(Esr1)	B	Females have hemorrhagic ovarian cysts and uterine defects,	Infertility

		decreased lordosis response; males develop disruptions of the seminiferous epithelium due to abnormal epididymal function, no ejaculations	
Estrogen receptor β (ERβ)(Esr2)	B	Females are subfertile; males are fertile, but develop prostate hyperplasia	Subfertility
Fanconi anemia complementation group A (Fanca)	B	Hypogonadism, reduced fertility, more dramatic and progressive in females	Subfertility
Fanconi anemia complementation group C (Fancc)	B	Hypogonadism, compromised gametogenesis	Subfertility
Fanconi anemia complementation group G (Fancg)	B	Hypogonadism, compromised gametogenesis	Subfertility
Fertilin β (Adam2)	M	Altered sperm protein expression and adhesion defects during fertilization	Infertility
Fibroblast growth factor 9 (Fgf9)	M	XY male-to-female sex reversal; phenotype ranges from testicular hypoplasia to complete sex reversal	Lethal
Figla or FIGα (Factor in the germline α)	F	No primordial follicles develop at birth and oocytes die	Infertility
Fragile-X mental retardation syndrome 1 homolog (Fmr1)	M	Macroorchidism	
FSH hormone β-subunit (Fshb)	B	Female pre-antral block in folliculogenesis; males decreased testis size	Infertility F
FSH receptor (Fshr)	B	Female pre-antral block in folliculogenesis; males decreased testis size	Infertility F
Fus1 (translocated in liposarcoma; TLS)	M	Defects in spermatocyte chromosome pairing	Infertility
β1,4-Galactosyltransferase	B	Male infertility; defects in sperm-egg interaction; females exhibit defects in delivery and lactation	Variable lethality
γ-Glutamyl transpeptidase (Ggtp)	B	Both males and females are hypogonadal and infertile; phenotype corrected by feeding mice Nacetylcysteine	Infertility
Gdi1 (guanosine diphosphate dissociation ihibitor 1; Rho GDIα)	B	Impaired spermatogenesis, vaculolar degeneration in males; postimplantation pregnancy defects in females	Infertility
Glial cell line-derived neurotrophic factor (Gdnf)	MH	Depletion of stem cell reserves; spermatogonia differentiate	Fertile
Glycoprotein hormone α-subunit (Cga)	B	Hypogonadal due to FSH and LH deficiency	Infertility
Gpr106 (G proteincoupled receptor 106)	M	Crsp males homozygous for transgene integration exhibit a high intraabdominal position of the testes, complete sterility	Infertility
Growth differentiation factor-7 (Gdf7)	M	Defects in seminal vesicle development	Infertility
Growth differentiation factor-9 (Gdf9)	F	Folliculogenesis arrest at the one-layer follicle stage	Infertility
Growth hormone receptor (Ghr)	F	Delayed puberty and prolonged pregnancy	
H19	FH	Loss of maternal allele in developing embryos causes somatic overgrowth due to loss of IGF2 imprinting	
Heat shock protein 70-2 (Hsp70-2)	M	Meiosis defects and germ cell apoptosis	Infertility
Heatshock transcription factor 1 (Hsf1)	F	Maternal effect gene; pre- and postimplantation defects	Infertility
Hepatocyte nuclear factor (HNF-1α)(transcription factor 1; Tcf1)	B	Infantile uterus; normal ovarian histology (F); vestigial vas deferens, seminal vesicles and prostate, impaired spermatogenesis, no mating behavior (M)	Infertility
High mobility group box 2 (Hmgb2)	M	Sertoli and germ cell degeneration and immotile spermatozoa	Subfertility
Histone H2A family, member X (H2afx)	M	Pachytene stage arrest in spermatogenesis; defects in chromosome segregation and MLH1 foci formation	Infertile
Histone 3.3A gene (H3f3a) Insertional mutation	M	β-gal gene trap insertion creates a hypomorphic allele; homozygous males have reduced copulatory activity and fewer matings result in pregnancy	Subfertility
Homeobox A10 (Hoxa10)	B	Variable infertility; males have cryptorchidism and females have frequent embryo loss prior to implantation	Progressive, infertility M; subfertility F
Homeobox A11 (Hoxa11)	B	Females have uterine defects; males have malformed vas deferens and undescended testes	Infertility
Hrb (HIV-1 Rev binding protein) (RAB/Rip)	M	Round-headed spermatozoa lack an acrosome (globozoospermia)	Infertility
Inhibin α (Inha)	B	Granulosa/Sertoli tumors, gonadotropin hormone-dependent	Infertility F Secondary infertility M
Inositol polyphosphate- 5-	M	Sperm have reduced motility and reduced ability to fertilize	Infertility

phosphatase (Inpp5b)		eggs; defects in fertilin β processing	
Insulin-like growth factor 1 (Igf1)	B	Hypogonadal and infertile; disrupted spermatogenesis and vestigial ductal system, defects in mating behavior (M); impaired antral follicle formation (F)	Infertility
Insulin-like growth factor 2 receptor (Igf2r); T-associated maternal effect (Tme) mutation	FH	Mutation of maternal allele in pups causes developmental defects and embryonic/perinatal death	Lethality; maternal effect
Insulin-like hormone 3 (Insl3)	B	Bilateral cryptorchidism results in abnormal spermatogenesis in males; female subfertility associated with irregular estrous cycles	Subfertility
Insulin receptor substrate 2 (Irs2)	F	Small, anovulatory ovaries with reduced numbers of follicles	Infertility
Interleukin 11 (Il11)	F	Compromised implantation and decidualization	Infertility
JunD (Jund1)	M	Anomalous hormone levels and sperm structural defects	Infertility
Kit ligand (Kitl)	B	steel defect mutation causes defect in PGC migration/survival; panda mutation causes blocks in folliculogenesis in females	Infertility
Kit receptor (Kit)	B	White spotting null mutation causes PGC defects	Infertility
Leptin (Lep; ob/ob) mutant	B	Obese and infertile with hypogonadotropic hypogonadism	Infertility
Leptin receptor (Lepr; db/db) mutant	B	Obese and infertile with hypogonadotropic hypogonadism	Infertility
Leukemia inhibitory factor (Lif)	F	Failed implantation	Infertility
Limk2 (LIM motif containing protein kinase 2)	M	Degeneration of spermatogenic cells in the seminiferous tubules; increased apoptosis	
Lipase, hormone sensitive (HSL) (Lipe)	M	Multiple abnormalities in spermatogenesis	Infertility
Luteinizing Hormone Receptor (Lhcgr)	B	Underdeveloped sex organs and infertility in both males and females; spermatogenesis arrested at round spermatid stage; preantral folliculogenesis block	Infertility
Man2a2 (α-mannosidase IIx)	M	Defect in adherence of spermatogenic cells to Sertoli cells; germ cells prematurely released from the testis	Mostly infertile
Mater (maternal antigen that embryos require)	F	Development beyond the two-cell stage is blocked; Maternal effect gene	Infertility
Mlh1 (MutL homologue 1)	B	Meiotic arrest and genomic instability	Infertility
Mos (Moloney sarcoma oncogene)	F	Parthenogenetic activation, cysts and teratomas	Subfertility
Msh4 (MutS homologue 4)	B	Prophase I meiotic defects apparent at the zygotene/pachytene stage; germ cells lost within a few days post-partum	Infertility
Msh5 (MutS homologue 5)	B	Zygotene/pachytene meiotic defects with aberrant chromosome synapsis and apoptosis	Infertility
Microtubule-associated protein (Mtap7)(EMAP-115) insertional mutation	M	Abnormal microtubules in germ cells and Sertoli cells	Infertility
Morc (microrchidia) insertional mutation	M	Early arrest in meiosis and germ cell apoptosis	Infertility
Mybl1 (A-myb) myeloblastosis oncogene-like 1	M	Germ cell meiotic arrest at the pachytene stage	Infertility
Na(+)-K(+)-2Cl(-) cotransporter (NKCC1) solute carrier family 12, member 2 (Slc12a2)	M	Low spermatid counts and compromised sperm transport	Infertility
Neuronal Helix-Loop- Helix 2 (Nhlh2)	B	Males are infertile and hypogonadal; females are fertile when reared with males	Infertility
Neuronal insulin receptor (NIR)	B	Hypothalamic hypogonadism; impaired spermatogenesis and follicle maturation	Infertility
Nitric oxide synthase 3, endothelial cell (Nos3; eNos)	F	Compromised ovulation, delayed meiotic progression from metaphase I	Subfertility
Nuclear receptor coactivator (Ncoa1); steroid receptor coactivator-1 (SRC1)	B	Decreased responsiveness to steroid hormones in uterus, mammary glands (F), testes and prostate (M)	Fertile
Nuclear receptor corepressor RIP40 (Nrip1)	F	Ovulation defect; ovaries accumulate luteinized, unruptured follicles	Infertility
Nuclear receptor subfamily 5, group A, member 1 (Nr5a1); Steroidogenic factor-1 (SF-1)	B	Gonadal agenesis in both sexes	Lethal
Otx1(orthodenticle homolog 1)	B	Prepubescent dwarfism and hypogonadism; progressive recovery of follicular development and sperm development and fertility	Delayed fertility
Ovo	M	Reduced fertility and underdeveloped genitalia	Subfertility
P2X1 receptor (P2rx1)	M	Oligospermia and defective vas deferens contraction	Infertility
p18Ink4c (Cdkn2c)	M	Leydig cell hyperplasia and reduced testosterone production	Fertile

p19Ink4d (Cdkn2d)	M	Testicular atrophy and germ cell apoptosis	Fertile
p27Kip1 (Cdkn1b)	B	CL differentiation failure and granulosa cell hyperplasia (F); males fertile with testicular hyperplasia	Infertility F
p57Kip2 (Cdkn1c)	B	Surviving mice show sexual immaturity	Mostly lethal
PAC1; adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1)	F	Prolonged and irregular diestrous phase	Subfertility
PC4 (testicular germ cell protease) (Pcsk4)	M	Sperm have impaired fertilization ability	Infertility
Pentraxin 3 (Ptx3)	F	Defects in cumulus-oocyte complex (COC) integrity and ovulation	Subfertility
Phosphatidylinositol 3'- kinase (Pi3k)	M	Defects in proliferation and increased apoptosis of spermatogonia	Infertility
Phosphatidylinositol glycan, class A (Piga)	CM	Abnormal testes, epididymis and seminal vesicles	Variable Infertility; no allele transmission
Pit1 (pituitary specific transcription factor 1)	B	Snell dwarf mice have multiple anterior pituitary hormone deficiencies and hypogonadism	Infertility
Polyomavirus enhancer activator 3 (Pea3)	M	Normal mating behavior, but males do not set plugs or release sperm	Infertility
Postmeiotic segregation increased 2 (Pms2)	B	Abnormal chromosome synapsis in meiosis (M); female knockout zygotes have microsatellite instability in both maternal and paternal genomes; Maternal effect gene	Infertility M
Progesterone receptor (Pgr)	F	Defects in ovulation, implantation, sexual behavior, and mammary gland development	Infertility
Prolactin (Prl)	F	Females are infertile with irregular estrus cycles	Infertility
Prolactin receptor (Prlr)	B	Compromised ovulation, fertilization and preimplantation development in knockouts (F); defects in maternal behavior in knockouts and heterozygotes (F); variable infertility and subfertility in males	Infertility F; Subfertility M
Prop1 (paired like homeodomain factor 1; prophet of Pit1)	B	Ames dwarf mice have multiple anterior pituitary hormone deficiencies and hypogonadism	Infertility
Prostaglandin E2 EP2 receptor (Ptger2)	F	Decreased fertilization and defects in cumulus expansion	Subfertility
Prostaglandin F receptor (Ptgfr)	F	Females do not undergo parturition; failed luteolysis	Infertility
Protamine 1 (Prm1)	CM	Protamine haploinsufficiency; abnormal spermatogenesis	Infertility
Protamine 2 (Prm2)	CM	Protamine haploinsufficiency; abnormal spermatogenesis	Infertility
Protease inhibitor protease nexin-1 (PN- 1) knockout (Serpine2)	M	Abnormal seminal vesicle morphology and altered semen protein composition	Subfertility
Protein kinase A, catalytic subunit α (Prkaca)	M	Most mice die; few viable mice have sperm motility defects	Mostly lethal
Protein phosphatase 1 catalytic subunit γ (Ppp1cc)	M	Defects in spermiogenesis	Infertility
Protein phosphatase 1 regulatory subunit 1B (Ppp1r1b)(DARPP-32)	F	Knockouts exhibited defects in progesterone facilitated sexual receptivity	Not reported
Puromycin-sensitive aminopeptidase (Psa)	F	Lack of CL formation and prolactin production cause early pregnancy loss	Infertility
Retinoic Acid Receptor alpha (Rara)	M	Complete arrest and degeneration or germ cell depletion	Infertility
Retinoic acid receptor γ (Rarg)	M	Squamous metaplasia of the seminal vesicles and prostate	Infertility
Retinoid X receptors (Rxrb)	M	Germ cell maturation defects and tubular degeneration	Infertility
Ros1 (c-ros) protoncogene)	M	Sperm motility defects	Infertility
Scavenger receptor, class B1 (Srb1)	F	Defects in oocyte maturation and early embryo development due to abnormal lipoprotein metabolism	Infertility
Serpina5 (Serine proteinase inhibitor A 5; Protein C inhibitor)	M	Sertoli cell destruction	Infertility
SH2-B	B	Males have small testes and reduced sperm count; females have small, anovulatory ovaries with reduced numbers of developing follicles	Subfertility M Infertility F
Smad1 (MAD homolog 1; Madh1)	B	Developing embryos lose PGCs	Lethal
Smad5 (MAD homolog 5; Madh5)	B	Developing embryos lose PGCs	Lethal
Sp4 trans-acting transcription factor (Sp4)	M	Defects in reproductive behavior	Infertility
Spam1 (sperm adhesion molecule) mutations	F	Sperm defects in hyaluronic-acid binding	Subfertility
Sperm-1	M	Defect in haploid sperm function	Subfertility

Sperm mitochondrionassociated cysteine-rich protein (SMCP)	M	Defects in sperm motility and migration into the oviduct; defects in fertilization	Subfertility and Infertility
Spermatid perinuclear RNA-binding protein (Spnr) insertional mutation	M	Defects in seminiferous epithelium and spermatogenesis	Subfertility
SPO11 homolog (Spo11)	B	Defects in meiosis; oocytes lost soon after birth	Infertility
Steroid 5α-reductase type 1 (Srd5a1)	F	Defects in parturition	Infertility
Steroidogenic acute regulatory protein (Star)	B	Males have female external genitalia; both sexes die of adrenocortical insufficiency	Lethal
Styx (phosphoserine/ threonine/tyrosine interaction protein)	M	Defects in round and elongating spermatid development	Infertility
Superoxide dismutase 1 (Sod1)	F	Folliculogenesis defect; failure to maintain pregnancy	Subfertility
Sycp3 (synaptonemal complex protein 3)	B	Defects in chromosome synapsis during meiosis; germ cell apoptosis in males; embryonic loss in females due to aneuploidy	Infertility M Subfertility F
Taf4b (TAF4B RNA polymerase II, TATA box binding proteinassociated factor; TAFII105)	F	Defects in follicular development, oocyte maturation/fertilization	Infertility
TATA-binding proteinlike protein (Tlp; TRF2)	M	Post-meiotic spermiogenesis block (defective acrosome formation in early stage spermatids)	Infertility
Telomerase reverse transcriptase (Tert)	B	Progressive infertility in both sexes; females have few oocytes and uterine abnormalities	Progressive Infertility
Theg (kisimo) (Transgene integration)	M	Abnormal elongated spermatids; asthenospermia	Infertility
Thyroid stimulating hormone β (Tshb; hyt/hyt) mutant	F	Hypothyroid; females show continuous dioestrus, and poor response to gonadotropin-induced superovulation	Infertility
Tial1 (cytotoxic granule-associated RNA binding proteinlike 1)	B	PGCs lost by E13.5	Infertility
Tnp1 (transition protein 1)	M	Abnormal chromosome condensation, sperm motility	Subfertility
Tnp2 (transition protein 2)	M	Abnormal chromosome condensation	Subfertility
Tumor necrosis factor type I receptor (Tnfrsf1a)	F	Enhanced prepubertal response to gonadotropins; early ovarian senescence	Subfertility
Ube2b (E2B ubiquitinconjugating enzyme; HR6B)	M	Alterations in sperm chromatin structure, an incomplete meiotic arrest, abnormal sperm morphology	Infertility
Ubiquitin-like DNA repair gene HR23B (Rad23b)	M	Most knockouts die during development or shortly after birth; surviving mice have multiple abnormalities and male sterility	Variable lethality; Infertility
Ubiquitin protein ligase E3A (Ube3a; E6-AP ubiquitin protein ligase)	B	Testicular hypoplasia, defects in spermatogenesis and prostate gland development (M); ovarian hypoplasia, defects in ovulation and uterine development (F)	Subfertility
Ubiquitin protein ligase seven in absentia 1A (Siah1a)	M	Block in spermatogenesis and germ cell apoptosis; failure to complete transition to telophase of meiosis I	Partially lethal; Infertility
VASA homolog (Ddx4; DEAD box polypeptide 4)	M	Defective proliferation/differentiation of PGCs	Infertility
Vitamin D receptor (Vdr) knockout	B	Defects in estrogen biosynthesis in males and females; elevated serum gonadotropins	Infertility
Voltage-dependent Anion Channel Type 3 (Vdac3)	M	Immotile sperm; axonemal defects with sperm maturation	Infertility
Wilms tumor homolog (Wt1)	B	Gonadal agenesis	Lethal
Wip1 (p53-induced phosphatase)	M	Runting and testicular atrophy	Subfertility
Wingless-related MMTV integration site 4 (Wnt4)	F	Ovaries depleted of oocytes; Müllerian ducts do not form	Infertility
Wnt7a	B	Females show abnormal development of oviducts and uterus; males do not have Müllerian duct regression	Infertility
Zfx (Zinc finger protein X-linked)	B	Reduced germ cell numbers; males have reduced sperm, but are fertile; females subfertile	Subfertility F
Zona pellucida protein 1 (Zp1)	F	Defects in fertilization	Subfertility
Zp2	F	Fragile oocytes with defects in developmental competence	Infertility
Zp3	F	Fragile oocytes	Infertility

Appendix C:

The following tables include the semen assessments for each sample from each of the six patients (NLCL, IDSS, PHEH, ALTAL, JFEF and SGSW) undergoing TCM treatment.

Semen assessment results for seven consecutive samples from patient NLCL undergoing TCM treatment

Patient: NLCL	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
Date	8/12/99	19/1/00	8/3/00	16/5/00	8/8/00	7/11/00	3/7/01
Appearance	N	N	N	N	N	N	N
Volume (ml)	2.8	2.5	2.0	2.3	3.5	3.0	2.1
Viscosity	SV	N	N	N	N	N	SV
Liquefaction	C	C	C	C	C	C	P
PH	8.0	8.0	8.0	8.0	8.0	8.0	8.4
Agglutination	NS	NS	NS	NS	NS	NS	NS
M.A.R test	BD	<10%	<10%	<10%	<10%	<10%	<10%
Sperm concentration/ml	$69x10^6$	$10x10^6$	$9.5x10^6$	$63x10^6$	$32x10^6$	$57x10^6$	$24x10^6$
Total no. sperm		26	19	145	112	170	50
Motility %	10%	26%	11%	17%	41%	37%	58%
Progression (overall)	G2-slow	G1-2	G2	G2	G2	G2	G1-2
1- rapid, linear (good)	0%	46%	15%	28%	28%	6%	30%
2- slow linear or non-linear (weak)	56%	22%	30%	44%	44%	52%	42%
3- very limited forward progression	32%	22%	40%	12%	16%	22%	16%
4- locally sluggish	12%	10%	15%	12%	12%	18%	8%
5- locally quickly shaking	0%	0%	0%	4%	0%	4%	4%
Abnormal forms %	91%	87%	97%	97%	96%	96%	87%
Head defects - amorphous	14%	4%	0%	2%	3%	2%	10%
- tapered	11%	1%	3%	3%	6%	3%	1%
- pyriform	2%	10%	8%	5%	6%	9%	5%
- large	4%	4%	3%	3%	2%	2%	10%
- round	10%	19%	26%	17%	29%	30%	20%
- other	1%	18%	26%	19%	8%	11%	7%
Tail defects - coiled	35%	13%	5%	30%	23%	23%	22%
- double	0%	0%	2%	0%	0%	1%	1%
Mid-piece defects	14%	18%	24%	19%	19%	15%	11%
Comments			*	*	*	*	*

N= normal, C= complete, SV= slightly viscous, BD= beyond detection, G= gelatinous, NS= non-specific, P=partial

Semen assessment results for six consecutive samples received from patient IDSS undergoing TCM treatment

Patient: IDSS	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Date	8/12/99	19/1/00	23/2/00	29/3/00	2/5/00	13/6/00
Appearance	N	N	N	N	N	N
Volume (ml)	3.8	4.0	4.5	2.75	4.5	4.2
Viscosity	N	N	N	N	N	N
Liquefaction	C	C	C	C	C	C
PH	8.0	8.0	8.0	8.0	8.0	8.0
Agglutination	NS	NS	NS	NS	NS	NS
M.A.R test	BD	<10%	<10%	<10%	<10%	<10%
Sperm concentration/ml	$17x10^6$	$12x10^6$	$21x10^6$	$80x10^6$	$13x10^6$	$7.9x10^6$
Total no. sperm		48	95	220	60	33
Motility %	18%	11%	30%	8%	26%	42%
Progression (overall)	G3- v ltd	G2-3	G2	G3	G3	G1-2
1- rapid, linear (good)	0%	12%	8%	3%	0%	26%
2- slow linear or non-linear (weak)	28%	38%	56%	23%	27%	38%
3- very limited forward progression	60%	29%	26%	30%	43%	22%
4- locally sluggish	12%	21%	10%	44%	30%	14%
5- locally quickly shaking	0%	0%	0%	0%	0%	0%
Abnormal forms %	96%	97%	96%	98%	97%	98%
Head defects – amorphous	20%	8%	6%	2%	2%	4%
- tapered	8%	5%	2%	2%	4%	0%
- pyriform	4%	0%	4%	5%	3%	1%
- large	4%	4%	8%	11%	6%	7%
- round	28%	42%	19%	24%	16%	34%
- other	8%	28%	29%	23%	26%	28%
Tail defects - coiled	0%	2%	4%	3%	7%	1%
- double	0%	4%	0%	16%	0%	8%
Mid-piece defects	24%	4%	24%	14%	34%	15%
Comments						

N= normal, C= complete, SV= slightly viscous, BD= beyond detection, G= gelatinous, NS= non-specific

Semen assessment results for five consecutive samples received from patient PHEH undergoing TCM treatment

Patient: PHEH	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Date	12/1/00	16/2/00	22/3/00	16/5/00	30/6/00
Appearance	N	N	N	N	N
Volume (ml)	4.6	4.0	4.5	3.2	3.2
Viscosity	N	N	SV	N	SV
Liquefaction	C	C	C	C	C
PH	8.0	8.0	8.0	8.5	8.0
Agglutination	+	NS	+	NS	+
M.A.R test	BD	<10%	<10%	<10%	<10%
Sperm concentration/ml	$12x10^6$	$7.5x10^6$	$37x10^6$	$16x10^6$	$30x10^6$
Total no. sperm $x10^6$	55	30	167	51	96
Motility %	36%	53%	27%	45%	40%
Progression (overall)	G2	G2	G1-2	G1-2	G3
1- rapid, linear (good)	28%	20%	32%	16%	6%
2- slow linear or non-linear (weak)	34%	37%	36%	36%	26%
3- very limited forward progression	14%	33%	12%	10%	32%
4- locally sluggish	20%	10%	16%	20%	28%
5- locally quickly shaking	4%	0%	4%	18%	8%
Abnormal forms %	97%	99%	97%	98%	100%
Head defects - amorphous	3%	6%	0%	1%	7%
- tapered	1%	3%	0%	3%	1%
- pyriform	5%	0%	0%	1%	2%
- large	2%	3%	5%	4%	15%
- round	10%	36%	19%	14%	30%
- other	64%	38%	64%	59%	19%
Tail defects - coiled	2%	5%	1%	0%	0%
- double		2%	3%	9%	9%
Mid-piece defects	10%	6%	5%	7%	18%
Comments					

N= normal, C= complete, SV= slightly viscous, BD= beyond detection, G= gelatinous, NS= non-specific

Semen assessment results for four consecutive samples received from patient ALTAL undergoing TCM treatment

Patient: ALTAL	Sample 1	Sample 2	Sample 3	Sample 4
Date	15/12/99	19/1/00	16/2/00	22/3/00
Appearance	N	N	N	N
Volume (ml)	1.8	3.0	1.8	1.5
Viscosity	N	N	N	N
Liquefaction	C	C	C	C
PH	8.0	7.5	8.0	8.0
Agglutination	NS	NS	NS	NS
M.A.R test	>99%	>99%	>99%	>99%
Sperm concentration/ml	61×10^6	37×10^6	22×10^6	63×10^6
Total no. sperm $\times 10^6$	110	113	40	95
Motility %	24%	7%	45%	13%
Progression (overall)	G3	G3	G3-4	G3-4
1- rapid, linear (good)	0%	0%	0%	1%
2- slow linear or non-linear (weak)	4%	17%	4%	3%
3- very limited forward progression	56%	53%	64%	61%
4- locally sluggish	40%	30%	32%	35%
5- locally quickly shaking	0%	0%	0%	0%
Abnormal forms %	95%	95%	99%	94%
Head defects – amorphous	20%	11%	6%	4%
- tapered	7%	6%	5%	4%
- pyriform	17%	19%	5%	0%
- large	5%	6%	1%	2%
- round	20%	6%	17%	14%
- other	8%	19%	8%	16%
Tail defects - coiled	5%	11%	2%	18%
- double	0%	4%	2%	2%
Mid-piece defects	13%	13%	53%	34%
Comments				

N= normal, C= complete, SV= slightly viscous, BD= beyond detection, G= gelatinous NS= non-specific

Semen assessment results for three consecutive samples received from patient JFEF undergoing TCM treatment

Patient: JFEF	Sample 1	Sample 2	Sample 3
Date	5/4/00	1/8/00	26/9/00
Appearance	G	N	G
Volume (ml)	4.25	3.2	5.8
Viscosity	N	N	N
Liquefaction	C	C	C
PH	8.0	8.0	8.0
Agglutination	+	Slight	Slight
M.A.R test	50%	80%	20% IgG 10% IgA
Sperm concentration/ml	$25x10^6$	$8x10^6$	$22x10^6$
Total no. sperm $x10^6$	106	26	128
Motility %	48%	38%	41%
Progression (overall)	G4	G2-3	G2
1- rapid, linear (good)	0%	10%	22%
2- slow linear or non-linear (weak)	5%	36%	44%
3- very limited forward progression	35%	23%	20%
4- locally sluggish	60%	31%	12%
5- locally quickly shaking	0%	0%	4%
Abnormal forms %	99%	97%	98%
Head defects – amorphous	5%	17%	9%
- tapered	11%	13%	2%
- pyriform	4%	4%	7%
- large	2%	4%	2%
- round	33%	29%	29%
- other	24%	10%	11%
Tail defects - coiled	11%	6%	13%
- double	2%	1%	5%
Mid-piece defects	8%	13%	20%
Comments			

N= normal, C= complete, SV= slightly viscous, BD= beyond detection, G= gelatinous NS= non-specific

Semen assessment results for three consecutive samples received from patient SGSW undergoing TCM treatment

Patient: SGSW	Sample 1	Sample 2	Sample 3
Date	16/2/00	22/3/00	18/7/00
Appearance	N	N	N
Volume (ml)	3.2	4.0	3.1
Viscosity	Viscous	Viscous	Viscous
Liquefaction	C	IC	IC
PH	8.0	8.0	8.0
Agglutination	NS	NS	NS
M.A.R test	BD	<10%	<10%
Sperm concentration/ml	$18x10^6$	$49x10^6$	$44x10^6$
Total no. sperm $x10^6$	58	196	136
Motility %	53%	44%	56%
Progression (overall)	G4	G3	G1-2
1- rapid, linear (good)	0%	0%	42%
2- slow linear or non-linear (weak)	17%	31%	40%
3- very limited forward progression	24%	31%	10%
4- locally sluggish	59%	31%	4%
5- locally quickly shaking	0%	7%	4%
Abnormal forms %	92%	96%	92%
Head defects - amorphous	4%	20%	9%
- tapered	9%	16%	13%
- pyriform	8%	12%	8%
- large	1%	8%	1%
- round	13%	8%	9%
- other	20%	20%	15%
Tail defects - coiled	2%	4%	9%
- double	9%	0%	0%
Mid-piece defects	26%	12%	28%
Comments			

N= normal, C= complete, IC= incomplete, BD= beyond detection, G= gelatinous NS= non-specific

Appendix D: Publications and manuscripts arising from this thesis

Griffin, D.K., Hyland, P., Tempest, HG. and Homa, S.T. (2002). Should men undergoing ICSI be screened for chromosome abnormalities in their sperm? Human Reproduction 18:229-235.

Shah, K., Sivapalan, G., Gibbons, N., Tempest, HG. and Griffin, D.K. (2003). The genetic basis of infertility. Reproduction., 126:13-25

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Tempest, HG., Christopikou, D., Zhai, XP., Homa, S. and Griffin, D.K. (2001). Looking to the East: Evidence that traditional Chinese medicine can significantly reduce high rates of sperm aneuploidy in infertile males. Chromosome Research., 9(1):P146.

List of publications for submission

Tempest, H.G., Christopikou, D., Dalakiouridou, M., Zhai, X.P., Homa, S. and Griffin, D.K. (2003). The association between male infertility and sperm aneuploidy: Evidence for mechanisms specific to individual semen parameters and individual chromosome pairs. Submitted to Human Reproduction (Invited for re-submission).

Tempest, H.G. and Griffin, D.K. The relationship between male infertility and increased levels of sperm disomy. Submitted to Cytogenetic and Genome Research.

Tempest, HG., Bridger, J.M., Salapidou, G., Foster, H. and Griffin, D.K. Genome organisation in human sperm. Invited and in preparation for Cytogenetic and Genome Research.

Tempest, H.G., Christopikou, D., Dalakiouridou, M., Zhai, X.P., Ramsey, J., Homa, S. and Griffin, D.K. Molecular cytogenetic evidence for the efficacy of Chinese herbal medicine in the treatment of male infertility: A role for anti-oestrogens and anti-oxidants? Manuscript in preparation

Chapter 10

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Chapter 10: References

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