AN INVESTIGATION OF DNA INTEGRITY BIOMARKERS IN GAMETOGENESIS
AND PRE-IMPLANTATION EMBRYO DEVELOPMENT TO PREDICT
REPRODUCTIVE POTENTIAL

by

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‘I, Amanthi Balasuriya, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.’
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ABSTRACT
Unsuccessful fertilization, aberrant embryo development, implantation failure and recurrent miscarriages can occur despite any obvious reasons during assisted reproduction. DNA fragmentation and aneuploidy in gametes have been implicated as a possible cause for infertility, but the use of DNA fragmentation tests is controversial. The SCD-FISH test claims to analyse DNA fragmentation and aneuploidy in the same cell. In this thesis SCD-FISH was compared to single FISH and SCD, which showed that SCD-FISH was an unreliable method to study these parameters. Sperm preparation techniques in assisted reproduction technologies (ART) potentially generate exogenous stresses that cause additional DNA damage. This study subjected mature sperm to environmental insults that normally occur during ART, and highlighted the significant increase in sperm DNA fragmentation due to heat, freezing and oxidative stress. Since it is not possible to investigate the level of DNA damage in the egg or sperm, and still maintain its viability for use in fertilization, DNA damage in cumulus and granulosa cells were studied. There was no relationship between DNA fragmentation in these cells and fertilization or pregnancy outcome. DNA fragmentation was significantly higher in cumulus than granulosa cells. Allegedly minimising DNA damage, GM-CSF is a component added to IVF culture media. Its effect on murine embryo DNA fragmentation was studied and it was found to have no significant effect. The exposure of human embryonic stem cells to pre-tested toxins and its impact on DNA fragmentation and aneuploidy, and the correlation between these two parameters were also investigated. These studies emphasise the belief that the introduction of DNA fragmentation assays to the clinical arena is premature as its role is unsubstantiated, the lack of a transparent relationship between DNA fragmentation and pregnancy outcome and the importance of minimising damage to sperm and embryos due to external stresses during laboratory research and ART. Overall, the aim of this thesis was to examine the hypotheses that DNA fragmentation, aneuploidy and phosphatidylserine translocation are potential biomarkers of reproductive potential that exist in variable degrees of at different stages of gametogenesis and preimplantation embryo development, and are susceptible to environmental stresses.
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Published papers

- ‘Processes involved in assisted reproduction technologies significantly increase sperm DNA fragmentation and phosphatidylserine translocation’ Balasuriya, A, Serhal, P., Doshi, A., Harper, JC. in Andrologia (December, 2012)


Abstract Presentations - Oral and Poster


- ‘Hydrogen peroxide, heat and cryopreservation significantly increase sperm DNA fragmentation and phosphatidylserine translocation’ Balasuriya, A., Serhal, P., Doshi, A., Harper, JC. Poster presentation at the Institute for Women’s Health 7th Annual Meeting (May, 2012)

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- ‘Human embryonic stem cells as a model to study apoptosis and aneuploidy in response to toxins in the preimplantation embryo’ Balasuriya, A., Koutsouraki E., De Sousa P., Harper JC. Oral presentation at the 3rd Institute for Women’s Health Annual Postgraduate Student Day (January, 2011)


- ‘DNA Fragmentation and Aneuploidy’ Balasuriya, A., Smith, B., Serhal, P., Doshi, A., Harper, JC. Poster presentation at the 2nd Institute for Women’s Health Annual Postgraduate Student Day

ABBREVIATIONS

8-OHd 8-oxo-2'-deoxyguanosine
AMH Anti-Müllerian hormone
Annexin V-FITC Annexin V- fluorescein isothiocyanate
AO Acridine orange
ART Assisted reproductive technology
ATP adenosine triphosphate
BAX Bcl-2–associated X protein
BCL2 B-cell lymphoma 2
BSA Bovine serum albumin
CC Cumulus cells
Cd Cadmium
CGH Comparative genomic hybridization
COC Cumulus-oocyte-complex
COH Controlled ovarian hyperstimulation
Comet (also known as the) Single Cell Gel Electrophoresis assay
CRGH Centre for Reproductive and Genetic Health
CSF Colony-stimulating factor
DAPI 4’,6-diamidino-2-phenylindole
DBD-FISH DNA breakage detection-FISH
DDR DNA damage response
DFI DNA fragmentation index
DGC Density gradient centrifugation
DNMT DNA methyltransferase
DS Down syndrome
DSB Double-stranded breaks
DTT Dithiothreitol
E2 Estradiol
EDTA Ethylenediaminetetraacetic acid
EGF Epidermal growth factor
ESC Embryonic stem cell
ESNATS Embryonic stem cell-based novel alternative testing strategies
FF Follicular fluid
FFA Follicular free fatty acid
FGF Fibroblast growth factor
FISH Fluorescence in situ hybridization
FSH Follicle-stimulating hormone
GC Granulosa cells
GM-CSF Granulocyte macrophage colony-stimulating factor
GnRH Gonadotropin-releasing hormone
H2O2 Hydrogen peroxide
HB-EGF Heparin-binding EGF-like growth factor
Hcg Human chorionic gonadotropin
HCl Hydrochloric acid
hESc Human embryonic stem cell
ICM Inner cell mass
ICSI Intracytoplasmic sperm injection
IFNt Interferon-t
IGF Insulin-like growth factors
IMSI Intra-cytoplasmic morphologically selected sperm injection
1. INTRODUCTION
1.1 The need for biomarkers in reproductive medicine

A biomarker is “A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group, 2001). In reproductive medicine, biomarkers may be used to predict outcome, discriminate between subgroups with different etiologies of disease and identify subgroups receptive to treatment (Palmer and Barnhart, 2013). Used for early diagnosis of a disease, the identification of individuals for disease prevention or as a possible drug target (Bonassi et al., 2001; McMichael & Hall, 1997), biomarkers may also be used to predict or detect recurrence or progression of a disease. However, biomarkers are insignificant if they only detect diseases at a late stage that can be identified by examination and other clinical tests. A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Woodcock, 2010). Biomarker evaluations can include morphology, imaging technologies, genetic markers or molecular entities. Molecular biomarkers should be ubiquitous, comprise an assay that can produce rapid results, and relate to a disease process for which non-invasive or early detection has clinical benefit (Palmer and Barnhart, 2013). The search for such markers has intensified in many areas of therapy. As the number of candidate markers in reproductive medicine increase, extensive testing, validation and adjustments need to be implemented prior to establishing a biomarker’s clinical efficacy in order to exploit their potential (Palmer and Barnhart, 2013).

1.2 Unexplained male infertility and semen analysis

The World Health Organization describes infertility as a ‘a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse’ (Zegers-Hochschild et al., 2009). Amongst the various reasons a couple may experience difficulty in achieving a successful pregnancy, male-factor infertility is a significant cause for referral (Aitken et al., 2009) and accounts for approximately 50% of all cases attending infertility clinics (Morris, 2002; Evers, 2002; Devroey et al., 2009; Devroey, 2009). Afflicting approximately 15% of men of reproductive age, defective sperm function is the single major reason behind human infertility (Hull et al., 1985; McLachlan & de Kretser, 2001).

A complex disease with a significant genetic basis, several genetic causes of spermatogenic impairment, including chromosomal aberrations and single gene mutations, are responsible for male infertility (Morris et al., 2002; Sakkas et al., 2003). Men are classified as having ‘idiopathic’ or ‘unexplained’ male infertility when they are infertile despite having normal semen analysis, normal
history and physical examination, and when female factor infertility has also been ruled out (Hamada et al., 2011). On average, the incidence of male infertility is 15%, although reports range from 6%-37% (Templeton and Penney, 1982; Collins and Crosignani, 1992; Moghissi and Wallach, 1983). Possible causes that may explain the difficulty to conceive in idiopathic male infertility include the presence of antisperm antibodies, sperm DNA damage, elevated levels of reactive oxygen species, and sperm dysfunction (reviewed by Hamada et al., 2012). Another possible factor is unexplained female infertility. The advent of ICSI (intracytoplasmic sperm injection), however, has managed to circumvent the issue of male infertility (Palermo et al., 1992), even when the spermatozoon is severely compromised in terms of motility, morphology, maturity or DNA damage (Aitken, 2006). The achievement of a successful pregnancy under such sub-optimal conditions is a credit to the inbuilt robustness of the fertilization process and the effectiveness of the oocyte repair mechanism. Despite these factors being represented in the successful clinical outcomes following ICSI, a number of questions including the nature of DNA damage, how it occurs, it’s biological and clinical significance, detection, and management have to be answered. A successful pregnancy achieved using dysfunctional sperm may carry the same risk of transmitting the same infertility traits to the male offspring (Hamada et al., 2012).

Semen analysis remains the backbone of conventional male factor infertility assessment, in addition to detailed medical history and physical examination (Esteves et al., 2011). It depends on the gross visualization of sperm concentration, motility and morphology as measured by light microscopy (Tomlinson et al., 2001; Morris et al., 2002). However, the criteria for normal semen parameters vary depending on the edition of the World Health Organization (WHO) laboratory manual for the examination and processing of human semen used (Esteves et al., 2012). The WHO established new reference values for human semen parameters in 2010, noticeably lower than those previously reported (World Health Organization: WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. 2010.). Implementation of the new WHO manual reference values in clinical practice may re-classify many infertile couples. Those previously classified as having male-factor infertility with semen parameters higher than the new limits but lower than the old reference values will now be diagnosed as having unexplained or female-factor infertility (Esteves et al., 2012).

Some laboratories have additional tests that include estimations of vitality, anti-sperm antibodies, contaminant cells, and total motile counts prior to and after assisted conception (Lewis, 2007). These criteria for normality, however, offer limited prognostic values in predicting pregnancy outcome (Irvine et al., 1994). Although tests for morphology with more stringent criteria have been developed to enhance existing tests (Kruger et al., 1988), they are yet to succeed in detecting subtle
sperm defects, and approximately 15% of patients with male factor infertility exhibit normal spermiograms (Agarwal and Allamaneni, 2005). Ironically, the implementation of suitable sperm function tests has been deferred due to the success of intracytoplasmic sperm injection for men with male infertility. Reports that ICSI was successful even in the absence of normal semen parameters resulted in its immense influence in ART clinics (Nagy et al., 1995; Svalander et al., 1996). Since ICSI has resulted in ‘take-home baby’ rates that are as good or better than those following IVF, there has been little incentive for ART centres to advance the research and development of sperm selection tests (Lewis and Simon, 2010). However, conventional semen analysis is now thought to be of limited prognostic value in determining a couple’s fertility status (reviewed by Lewis, 2007). Instead, sperm DNA testing has been welcomed as a potentially more promising test to diagnose male infertility and predict the outcome of ART (Aitken and de Iuliss, 2007; Evenson et al., 2007; Barratt et al., 2010), although this is controversial.

The observation that men with severe semen defects display a higher level of chromatin damage (Sun et al., 1997) has led to studies that have associated damaged paternal DNA to detrimental effects of embryo development, and ill effects on the offspring and subsequent generations (Fernandez-Gonzalez et al., 2008). Although controversial, it has been suggested that ‘normal’ semen samples can consist of significant DNA damage and those with apparently poor sperm quality can carry minimal DNA fragmentation; sperm chromatin and DNA defects can be indicative of male sub-fertility (Evenson et al., 1999). A foremost cause of pregnancy loss and mental retardation in humans (Griffin, 1996; Hassold and Hunt, 2001), increased frequencies of aneuploidies have also been shown to draw parallel with increasing severity of infertility (Martin et al., 2003; Tempest and Griffin, 2004; Faure et al., 2007; Collodel et al., 2007).

1.3 DNA fragmentation and aneuploidy in spermatozoa

1.3a Sperm DNA fragmentation assays

Over the last twenty years, a number of DNA fragmentation assays have been introduced for both clinical and research purposes. These tests include Comet, TUNEL, DBD-FISH, SCSA and SCD, amongst others. When analysing cells for DNA fragmentation, it is important to first determine whether the type of breaks produced is single- or double-stranded. Detecting these breaks may also require an initial step of denaturation using an acid or alkaline pH, as in the case of the SCSA, SCD or Comet (with acid/alkaline pH). It is important to keep in mind that when DNA damage is detected under acid or alkaline conditions as opposed to neutral pH, it is in fact acid/alkali labile DNA sites that are under consideration. The TUNEL and Comet (at neutral pH) do not require an initial step of
denaturation, and measure both single- and double-stranded breaks directly. Considering the intracellular pH of the oocyte is approximately 7.0, single-stranded DNA breaks and acid/alkali labile sites are unlikely to have a major effect on male pronucleus development. This is because DNA strands do not dissociate at neutral pH and single-stranded damage is easier to repair than double-stranded DNA breaks.

Thus, the tests used to assess DNA fragmentation can be viewed as either direct or indirect. Despite the method of assessment, all assays attempt to quantify the total amount of DNA fragmentation, regardless of the genomic location. Although breaks occurring in some areas of the genome, affecting specific genes, may be more detrimental than if the damage occurred in ‘silent’ regions, no assay can analyse this factor yet. It is therefore still difficult to distinguish between clinically significant and insignificant fragmentation.

The alkaline Comet assay has been used to study DNA fragmentation in several cell types and has been modified to use on sperm since the extent of DNA packaging in sperm cells prevent DNA strands by migrating during electrophoresis in conventional alkaline assay protocols (Hughes et al., 1996, 1997, 1998 and 1999; Donnelly et al., 1999; Lewis and Agbaje, 2008) (Figure 1.3a). The cells are lysed, electrophoresed, and stained with a fluorescent DNA-binding dye. Supercoils are released in the presence of DNA strand breaks, and the fragments migrate towards the anode (Haines et al., 1998). Spermatozoa with high DNA damage display fluorescence of high intensity (Hughes et al., 1996) and increased length (Singh and Stephens, 1998). This test measures both single and double DNA strand breaks using alkaline (Hughes et al., 1996; Donnelly et al., 2001) or neutral (Morris et al., 2002) pH conditions. The former (pH 10) recognizes both single- and double-stranded breaks by denaturing the sperm DNA. The neutral Comet assay (pH 9) is better equipped to identify DNA damage associated with infertility, because the conditions of the assay do not denature DNA. It only measures double-strand and closely-associated single strand breaks, but not those associated with alkali labile sites (Singh and Stephens, 1998).

In their modified protocol, the lysis and decondensation steps involved immersion (within agarose gels) in freshly prepared cold lysis solution for 1 h at 4°C (Lewis and Agbaje, 2008). This step was followed by incubation with dithiothreitol for 30 min at 4°C, and then lithium diiodosalicyclate for 90 min at 20°C to decrease the disulphide bonds. The process is carried out under yellow light to avoid further induced DNA damage (Donnelly et al., 1999). This modified protocol was found to be an apparently reliable and reproducible test to assess DNA damage in sperm, with a baseline level of damage in normozoospermic samples at ~10–15% (Donnelly et al., 1999).
The Comet test is the only technique that allows the quantitative measurement of DNA damage in individual cells, and is particularly useful in heterogeneous cell populations like sperm (Lewis and Simon, 2010). Although the Comet assay apparently allows FISH analysis (Santos et al., 1997), Comets with fragmented DNA appear dispersed and discrimination of signals proved to be difficult (Muriel et al., 2007). It requires fewer cells for analysis than other tests and may therefore be suitable in the assessment of testicular and oligozoospermic sperm samples where cell numbers are scant (Lewis and Simon, 2010). Unlike the SCSA and TUNEL assay which detect breaks in chromatin associated with histones, the Comet assay detects chromatin breaks associated with both histones and protamines (Lewis and Simon, 2010). It is claimed to be more sensitive than the other existing DNA damage tests (Klaude et al., 1996; Irvine et al., 2000; Barratt et al., 2010).

Figure 1.3a: The sperm Comet test; the ‘tail’ of the sperm Comet is the damaged DNA. The image on the left shows sperm cells with little damage in their DNA, whilst the image in the center shows sperm with damaged DNA. The image on the right shows a single sperm cell with DNA damage. Reprinted from the Lewis Fertility Testing website, Copyright©2011, with kind permission from Lewis Fertility Testing.

The sperm DNA fragmentation assay most studied from a clinical point of view is the SCSA test. This indirect assay exploits the susceptibility of somewhat damaged DNA to reach different degrees of denaturation under acidic conditions, depending on the level of the DNA breaks. The degree of damage is quantified by flow cytometry and expressed as a DNA fragmentation index (DFI) (Figure 1.3b). The SCSA is a rapid, flow cytometry-based measurement of DNA fragmentation in individual spermatozoa. Random selection by non-biased machine criteria makes it the most statistically robust and objective assessment of sperm DNA quality (Evenson et al., 2002). SCSA is based on the principle that nuclear DNA is more resistant to acid-induced denaturation in fertile compared to infertile men (Evenson et al., 1985). It exploits the metachromatic properties of acridine orange (AO), which only attaches to the ends of broken DNA, thus differentiating between fragmented (red fluorescence, single stranded) and native (green fluorescence, double stranded) DNA (Darzynkiewicz and Kapuscinski, 1990). Aliquots of raw semen samples used for ART are flash frozen using liquid nitrogen and the raw semen sample is thawed, and diluted to a concentration of 1-2 million cells/ml. Stress is applied in the form of a low pH buffer to denature DNA and the cells are stained with
acridine orange dye. A flow cytometer is then used to measure the fluorescence of 5000-10,000 cells per sample in <5 minutes (Evenson et al., 2002). This provides objective, machine-derived criteria that avoid human bias, a high level of repeatability (0.98-0.99). The percentage of green versus orange–labelled sperm quantifies the extent of DNA denaturation, and software allows the creation of a cytogram (graphic plot) of the percent of damaged sperm, providing the DNA fragmentation index (DFI). A DFI of >30% has been established for significant lack of fertility potential, 15%-30% reasonable, and <15% for high fertility status (Evenson et al., 2007).

Figure 1.3b: Typical SCSA cytograms obtained from a patient with a normal DFI (top image) and a patient with a high DFI (bottom image) (Evenson et al., 1999). The cytograms and histogram show the source of each component of SCSA data. (Left cytogram) X axis, fragmented DNA (red fluorescence); Y axis, native DNA stainability (green fluorescence). The reader can visualize where each constituent part (non-detectable, moderate, high) of fragmented DNA is located and how the percentage of high DNA stainability (HDS) is calculated. (Center cytogram) X axis, DFI (a ratio of red to total fluorescence); Y axis, total DNA stainability (total fluorescence; ie, red + green). (Right histogram) DNA fragmentation index with each component (non-detectable, moderate, and high) clearly marked. Reprinted from the Journal of Andrology, Vol. 23, Issue 1, January/February 2002, Pages 25-43, Evenson P et al., Sperm Chromatin Structure Assay: Its Clinical Use for Detecting Sperm DNA Fragmentation in Male Infertility and Comparisons With Other Techniques, Copyright©2002, with kind permission from John Wiley & Sons Ltd.
The recently developed SCD procedure involves the immersion of sperm in an agarose matrix on a slide, denaturation of fragmented DNA strands by treating with an acid solution, and then cells are lysed to remove the membranes and proteins. The latter step produces nucleoids that comprise a core and peripheral halo due to the release of DNA loops, signifying the absence of DNA fragmentation. The DNA breakage fluorescent *in situ* hybridization (DBD-FISH) process suggests that sperm nuclei with DNA fragmentation either produce very small halos of dispersion of DNA loops, or none at all (Fernandez and Gosalvez, 2002). Fernandez *et al.*, (2005) claim that the SCD test is the only assay that allows the analysis of DNA fragmentation and aneuploidy simultaneously, on the same sperm cell (SCD-FISH), and recently modified it to promote the Halosperm® Kit (Halosperm® kit; INDAS Laboratories, Madrid, Spain) (Figure 1.3c).

![Figure 1.3c: Sperm DNA fragmentation assessed by Halosperm®. Reprinted from the Halotech® DNA SL website, Copyright©2011, with permission from Halotech® DNA.](image)

When using the SCD test, the DNA fragments are said to remain close together. Furthermore, it was claimed that the Halosperm® kit facilitated the preservation of flagellum and nuclear chromatin, of which the latter is more resistant to the denaturation and washing steps employed in FISH. These features can be advantageous to study the aneuploidy frequency in sperm cells with fragmented DNA, compared to those without (Muriel *et al.*, 2007) (Figure 1.3d).
Figure 1.3d: Sequential FISH on SCD-processed spermatozoa (SCD-FISH); X = green, Y = red, 18 = blue. Cores and halos = grey-blue. Sperm cell no. 1) halo present, no DNA fragmentation; XY disomy (18,X,Y); 2) no halo, DNA fragmentation, normal chromosome complement (18, Y); 3) & 4) halo present, no DNA fragmentation, normal chromosome complement (18, Y), 5) no halo, DNA fragmentation, diploidy (18, 18, Y, Y). Reprinted from Journal of Andrology, Vol. 28, Issue 1, January/February 2007, Pages 38-49, Muriel L et al., Increased Aneuploidy Rate in Sperm With Fragmented DNA as Determined by the Sperm Chromatin Dispersion (SCD) Test and FISH Analysis, Copyright©2007, with kind permission from John Wiley & Sons Ltd.

The DeadEnd™ Fluorometric TUNEL System was designed to detect and quantify the proportion of apoptotic cells within a cell population by measuring nuclear DNA fragmentation, an important biochemical hallmark of apoptosis in many cell types. It provides a simple, accurate and rapid detection of DNA fragmentation in situ at the single-cell level or in cell suspensions, and can be used on several cell types. This assay measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at single- and double-stranded DNA breaks using the template-independent enzyme Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT). rTdT forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay (Gavriel et al., 1992). The fluorescein-12-dUTP-labeled DNA can then either be visualized directly by fluorescence microscopy or quantitated by flow cytometry (Figure 1.3e).
The efficacy of these assays depends on various factors, and their applicability with the patient pathway should also be determined because they can be used as replacement, triage or add-on. Thus far, most DNA damage assays have not been evaluated critically, and they should be (reviewed by Barratt, 2010).

1.3b Basis of DNA damage in spermatozoa

Many theories exist as to the source of DNA damage, but a consensus is yet to be reached (reviewed by Sakkas and Alvarez, 2010). Comprising multifactorial etiology, sperm DNA damage may be due to intrinsic or extrinsic factors. Intrinsic factors include deficiencies in recombination during spermatogenesis (Bennetts et al., 2008; Windt et al., 1994), abnormal spermatid maturation (Mengual et al., 2003), protamine deficiency (García-Peiró et al., 2011; Simon et al., 2011; Oliva, 2006; Carrell and Liu, 2001), oxidative stress (Mahfouz et al., 2010; Agarwal et al., 2008; Aitken et al., 2012; Aitken and Curry, 2011; De Iulissi et al., 2009; Badouard et al., 2008; Saleh et al., 2003), un repaired DNA breaks during chromatin remodelling (Erenpreiss et al., 2006) and abortive apoptosis during spermatogenesis (Sakkas et al., 2004; Zini and Libman, 2006). Extrinsic factors include testicular hyperthermia (Ahmad et al., 2012), lapse of time following ejaculation (Gosalvez et al., 2011), collection methods, extenders and post-ejaculation treatments (Jiménez-Rabadán et al., 2012), sperm preparation techniques for ART (de Lamirande et al., 2012), storage temperature and cryopreservation (Jackson et al., 2010; Imrat et al., 2012; Gosalvez et al., 2011), mechanical...
conditions (sex-sorting) (Gosalvez et al., 2011), post-testicular oxidative stress (Gil-Guzman et al., 2001; Greco et al., 2005), varicocele (Zini and Dohle, 2011), bacterial infections (Gallegos et al., 2008), age (Bello et al., 2009; Vagnini et al., 2007), abstinence (De Jonge et al., 2004), temperature of testis (Paul et al., 2008), reaction to clinical procedures, medicines or vaccines (Morris, 2002; Tanrikut et al., 2010; Gosalvez et al., 2008), and exposure to environmental chemicals (Specht et al., 2012; Rubes et al., 2007; Evenson and Wixon, 2005). The three major mechanisms accepted involve chromatin remodelling by topoisomerase, oxidative stress and abortive apoptosis. Apoptosis eliminates excessive and defective cells to control somatic and germ-cell populations (Ricci et al., 2002). This theory is re-enforced by the approximation that a single spermatogonia in the human testes produces approximately 100 spermatids and not the theoretical value of 4096 (Shen et al., 2002).

Several types of DNA damage occur in mammalian germ cells, most of which originate in the male gamete (reviewed by González-Marín et al., 2012). Characterized by single- (SSB) and double-stranded breaks (DSB), DNA fragmentation is commonly observed in the ejaculates of subfertile men. In general, SSBs are considered to have a better prognosis, and is easier to repair than DSBs. Single-strand breaks can occur due to unrepaired DNA nicks generated during chromatin remodelling (Marcon and Boisonneault, 2004; Lewis and Aitken, 2005), and oxygen radical-induced damage. The hydroxyl radical and ionizing radiation initially produce 8-OH-guanine and 8-oxo-2′-deoxyguanosine (8-OHdG) generating single stranded DNA breaks. If sperm caspases and endonucleases are also activated, the formation of hydroxyl radicals can inadvertently induce double-stranded DNA fragmentation (Badouard et al., 2008). Extremely harmful lesions, DNA double-strand breaks can result in genomic instability if not properly repaired. The extent of double-stranded DNA damage induced by oxygen radicals during sperm transport through the epididymis is determined by the levels of oxygen radicals produced by immature sperm, epididymal epithelial cells, activated leukocytes, and the levels of antioxidant enzymes present in the lumen of the epididymis (reviewed by Sakkas and Alvarez, 2010; Britan et al., 2006). Genome integrity is controlled by a complex cellular network, the DNA damage response (DDR), which mobilizes proteins in response to genotoxic stress (Polo and Jackson, 2011). The persistence of unrepaired DSBs can induce cellular apoptosis or senescence in an attempt to prevent the accumulation of potentially tumorigenic mutations. In the event all damage responses fail, de novo mutations can appear (Wang et al., 2012).
1.3c Sperm DNA damage and infertility

Clinical studies show that sperm DNA integrity is compromised amongst infertile men compared to fertile cohorts (Sergerie et al., 2005; Lewis and Aitken, 2005; Agarwal and Said, 2003), but studies of the relationship between sperm DNA fragmentation and ART outcomes are inconclusive (Spano et al., 2005). Published reports describe conflicting results about the relationship between sperm DNA fragmentation and IVF or ICSI outcome, fertilization rate and embryo cleavage (reviewed by Tamburrino et al., 2012). Generally, sperm DNA fragmentation does not appear to correlate well with fertilization rate when assessed by SCSA or TUNEL (Yamauchi et al., 2011; Zini et al., 2008). Whilst some studies describe the influence of sperm DNA fragmentation on embryo cleavage, blastocyst development and clinical pregnancy, others refute it (reviewed by Tamburrino et al., 2012).

The few available studies show that sperm DNA fragmentation prolongs the time to pregnancy and is associated with lower rates of natural pregnancies when using the SCSA test (Giwercman et al., 2010; Evenson and Wixon, 2008; Spano et al., 2000; Evenson et al., 1999) and when measuring the levels of 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) and deoxyguanosines (dG) in nuclear sperm using high-performance liquid chromatography with electrochemical and UV detection (Loft et al., 2003; Loft and Poulsen, 1997). Sperm DNA damage has also been linked to significantly decreased pregnancy rates following intra-uterine insemination (IUI) when analysed using the SCSA test (Evenson and Wixon, 2008; Bungum et al., 2007), SCD test (Muriel et al., 2006) and the TUNEL assay (Duran et al., 2002). Although sperm DNA fragmentation has been found to correlate with the outcome of IUI, it remains inconsistent with the outcome of second-level ART, causing doubts about the clinical utility of sperm DNA fragmentation tests for couple undergoing ART treatment (Zini et al., 2008; Collins et al., 2008).

A systematic review and meta-analysis of valid published studies that used the SCSA test (Boe-Hansen et al., 2006; Bungum et al., 2007 and 2008; Lin et al., 2008), TUNEL (Benchab et al., 2007; Borini et al., 2006; Frydman et al., 2008; Henkel et al., 2003; Huang et al., 2005), chromomycin AR (CMA3) (Tarozzi et al., 2009) and chromatin compaction (CC) (Filatov et al., 1999) conducted by Zini (2011) concluded that sperm DNA and chromatin damage had a modest influence on pregnancy rates following IVF. This observation was supported by a more limited meta-analysis conducted by Collins et al., (2008), where the studies reviewed employed the TUNEL and SCSA assays. Another found that sperm DNA fragmentation, when evaluated by the TUNEL assay, was not related to fertilization rates in IVF cycles (r=0.08) when using the female patient’s own oocytes or donor oocyte (Esbert et al., 2011). Furthermore, DNA fragmentation was similar in pregnant and non-pregnant
women. However, they observed a higher miscarriage rate in the presence of higher DNA fragmentation despite the origin of the oocyte (Esbert et al., 2011).

The systematic review and meta-analysis of several ICSI studies indicate that sperm DNA damage has no significant effect on ICSI pregnancy (Zini, 2011; Collins et al., 2008) when assessed by SCSA (Boe-Hansen et al., 2006; Bungum et al., 2007; Check et al., 2005; Gandini et al., 2004; Lin et al., 2008; Micinski et al., 2009; Zini et al., 2005), TUNEL (Benchab et al., 2007; Borini et al., 2006; Henkel et al., 2003; Huang et al., 2005), CMA3 (Tarozzi et al., 2009) and analine blue staining to determine chromatin decondensation (Hammadeh et al., 1996). The results of a meta-analysis on mixed IVF and IVF-ICSI studies showed that the rate of pregnancy correlated with sperm DNA fragmentation when analysed by the SCSA test (Larson-Cook et al., 2003; Payne et al., 2005; Virro et al., 2004; Speyer et al., 2010), TUNEL (Seli et al., 2004) and the oxiDNA assay (Meseguer et al., 2008). In summary, the impact of sperm DNA fragmentation on ART outcomes was found to decrease from IUI to IVF, and was least useful in ICSI (Collins et al., 2008; Zini and Sigman, 2009).

Studies have also indicated an important association between sperm DNA fragmentation and the rate of pregnancy loss after IVF and ICSI (reviewed by Zini et al., 2008; Lin et al., 2008; Frydman et al., 2008; Bungum et al., 2007; Benchab et al., 2007; Borini et al., 2006; Zini et al., 2005; Check et al., 2005). In vivo studies using the SCSA test demonstrated a negative correlation between pregnancy rate and high sperm DNA fragmentation (Evenson and Wixon, 2008; Spano et al., 2000; Giwercman et al., 2010) in men with normal semen parameters. A study by Bungum et al., (2007) described significantly lower rates of clinical pregnancy when sperm DNA fragmentation exceeded 30% measured by the SCSA test. These data suggest that pregnancy loss after IVF or ICSI may be a result of impaired embryo/blastocyst development related to sperm DNA damage (Seli et al., 2004; Zini et al., 2005). However, inconsistent relationships have also been observed between sperm DNA fragmentation and pregnancy loss (Lin et al., 2008; Kennedy et al., 2011; Zini et al., 2008; Carrell et al., 2003).

The clinical usefulness of a sperm DNA fragmentation test remains controversial as it depends on an odds ratio based on threshold values that are highly variable (Collins et al., 2008). These include the type of assay, sperm preparation and scientific choice (Lewis and Simon, 2010). When using the TUNEL and SCSA assays in IVF, the combined odds ratio was 1.57 (95% confidence interval 1.18, 2.07; p<0.05). Simon et al., (2010) reported two thresholds to calculate the odds ratios for IVF when using the Comet assay; 56% for native, unprocessed sperm and 48% for the DGC sperm. They suggest that the relatively high values proposed are due to the apparent sensitivity of the Comet assay, where all double and single strand breaks and alkali labile sites are exposed following lysis and
decondensation (Lewis and Simon, 2010). In their study, the odds ratio for clinical pregnancy following IVF were 7.79 (1.74–48.6) in native semen and 6.58 (1.46–41.11) in sperm after DCG. The odds ratio for clinical pregnancy following ICSI was not significant at 2.27 (0.94–5.5) in native semen and 1.61 (0.67–3.89) in the DGC sperm (Simon et al., 2010). This observation was similar to the combined odds ratio of 1.14 reported by Zini and Sigman (2009).

A threshold value of 30% was drawn from a study by Evenson et al., (1999), where 165 apparently fertile couples were examined using the SCSA test. Couples with >30% sperm DNA damage failed to achieve a clinical pregnancy, so this value was considered to be the threshold above which DNA fragmentation was not compatible with fertility. However, the cut-off value for the SCSA test ranges from 20% (Boe-Hansen et al., 2006), 27% (Larson et al., 2000; Larson-Cook et al., 2003) to 30% (Evenson et al., 1999; Virro et al., 2004; Payne et al., 2005; Zini et al., 2005). It is unclear whether these values are appropriate for consideration in the case of infertile couples undergoing IVF or ICSI, and may vary further for sperm from native, unprocessed sperm and semen prepared for ART using density gradient centrifugation (Lewis and Simon, 2010). Previous studies have stated that a DFI >27% is associated with pregnancy failure in ART, but subsequent studies refute this claim. However, it must be noted that the SCSA has been scrutinized more than other tests and future studies may reveal limitations to assays such as TUNEL and the SCD/ Halosperm® (reviewed by Sakkas and Alvarez, 2010).

The threshold values for the TUNEL assay were shown to have even more variation in terms of clinical relevance. They ranged from 4% (Host et al., 2000; Huang et al., 2005), 10% (Borini et al., 2006), 15% (Benchaab et al., 2007), 20% (Benchaab et al., 1993; Seli et al., 2004) to 35% (Frydman et al., 2008). This discrepancy is reflective of the different laboratory protocols for the TUNEL assay (Mitchell et al., 2011).

Based on a study by Simon et al., (2013), sperm DNA fragmentation is thought to have a negative impact on live-birth rates following IVF. Using the Comet assay, couples with sperm DNA fragmentation <25% had a live-birth rate of 33%, whilst couples with >50% sperm DNA fragmentation had a live birth rate of 13%, following IVF. Significant differences in sperm DNA damage were not reported between any groups of patients following ICSI. High levels of DNA fragmentation were also observed in 39% of couples and 41% of men with idiopathic infertility (Simon et al., 2013).

Using Comet, sperm DNA fragmentation was found to be significantly higher in sperm from couples who failed to get pregnant, compared to both native semen and DGC (density gradient
centrifugation) sperm from couples who achieved a successful pregnancy following IVF (Lewis and Simon, 2010). They also studied the effect of the combination of the bifunctional DNA glycosylase, formamidopyrimidine-DNA glycosylase (FPG), (base excision repair enzyme) that recognizes and removes oxidized purines such as 8-OHdG thereby converting modified bases (MB) into strand breaks with the alkaline Comet assay (Simon et al., 2010; Collins, 2004). The addition of this enzyme and subsequent inclusion of modified bases in the assessment of DNA damage resulted in a marked difference in the level of DNA damage between the pregnant and non-pregnant groups (Lewis and Simon, 2010; Simon et al., 2010). By including modified bases, a strong relationship was observed between DNA damage and clinical pregnancy following ICSI, whilst increasing the sensitivity of the test in IVF (Lewis and Simon, 2010).

Even tests with high predictive values do not guarantee a correlation between test values and pregnancy outcome (Sakkas and Alvarez, 2010). This is mainly due to the sperm DNA repair capacity which lowers test specificity and maternal age. Collins et al., (2008) stated that “the small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles is not strong enough to provide a clinical indication for routine use of these tests in infertility evaluation of men. It is possible that yet to be determined subgroups of infertile couples may benefit from sperm DNA integrity testing.” This claim suggests that sperm DNA fragmentation may be limited to couples with a poor ART prognosis (Sakkas and Alvarez, 2010).

Whilst the measurement of sperm DNA damage may be useful as a prognostic biomarker in the evaluation of fertility potential, its implications may be even more far reaching (Lewis and Simon, 2010). Sperm have few repair mechanisms (Aitken and Baker, 2006), and oocytes are only capable of repairing a limited amount of sperm DNA damage (Ahmadi and Ng, 1999; Derijck et al., 2008). Nature does not necessarily prevent sperm containing damaged DNA from reaching the oocyte, achieving fertilization and contributing to mutations during embryonic development (Fraga et al., 1991). On the basis that fertilisation can still occur in the presence of sperm DNA fragmentation (Yamauchi et al., 2011; Fatehi et al., 2006; Fernandez-Gonzalez et al., 2008; Henkel et al., 2004; Ahmadi and Ng, 1999), embryo development can be greatly compromised in terms of rate and fidelity (Tamburrino et al., 2012).

Moreover, sperm DNA damage is pro-mutagenic and can induce mutations after fertilization (Aitken & Krausz, 2001). Damaged sperm DNA can be incorporated into the embryonic genome and lead to errors in DNA replication, transcription and translation during embryogenesis, contributing to a range of human diseases (Cooke et al., 2003). Such diseases are not limited to just one, but can be passed on to subsequent future generations (reviewed by Aitken et al., 2009). Continuing into
childhood, mutations incurred at early stages of embryo development due to poor sperm DNA integrity can result in childhood cancers, leukaemia and autism (reviewed by Aitken et al., 2009). These mutations can remain in the germline and cause imprinting diseases and infertility (Alukal & Lamb, 2008). Sperm DNA integrity is thought to impact the short- and long-term health of children born by ART (Lewis and Simon, 2010). Long term studies on the possible consequences of ICSI on the resulting offspring are necessary (Hamada et al., 2012) as children conceived by ICSI were found to have a higher incidence of disease than those conceived naturally (Basatemur & Sutcliffe, 2008; Katari et al., 2009). As yet, short term follow up studies of children born after ICSI compared to those born after standard IVF concerning the possibilities of congenital malformations, imprinting diseases and other health issues have been inconclusive (Hamada et al., 2012).

Such potential risks necessitate/dictate the practice of sperm function testing to better understand the basic sperm molecular defects. Ideally, once identified, they should be repaired using molecular targeted therapies prior to the utilization of dysfunctional sperm in ICSI. Whilst these therapies are still being researched, the recognition of abnormalities in these tests may also help. For example, identifying sperm DNA integrity defects may assist in counselling couple about the possible benefits, complications and failures of ART procedures, particularly pre-ICSI (Hamada et al., 2012).

The ability of DNA fragmentation tests to predict ART outcome depends on several factors (Sakkas and Alvarez, 2010). These include the type of DNA damage (single- versus double-stranded breaks), percentage of sperm with DNA damage (Sakkas and Alvarez, 2010; Spano et al., 2005), extent of DNA damage per spermatozoon (Ahmadi and Ng, 1999;), whether there is combined nucleotide damage and DNA fragmentation (Gu et al., 2010), whether DNA damage affects introns or exons (Sakkas and Alvarez, 2010), ability of the oocyte to repair sperm DNA damage in the fertilizing spermatozoon (Derijck et al., 2008), type of sperm DNA fragmentation test used (Simon et al., 2010), sperm processing in ART (Gosalvez et al., 2008; Dalzell et al., 2004; Donnelly et al., 2001; Tomlinson et al., 2001) and oocyte number (Duran et al., 2002).

Extrapolating the importance of DNA fragmentation tests to assess more subtle sperm anomalies in infertile couples may prove difficult due to its questionable nature (Beshay and Bukulmez, 2012). It is thought by some that sperm DNA fragmentation testing is merely a gross analysis and that detection of the subtle changes that pass through the paternal genome, leading to miscarriage and other congenital abnormalities may be beyond the scope of currently available diagnostic tools (Humm and Sakkas, 2013).
It is thought that of the couples undergoing ART or natural conception, those who have been exposed to toxicants or radio-chemotherapies that can cause significant DNA damage (O’Flaherty et al., 2010; Smit et al., 2010), diabetics (Agbaje et al., 2008) and those who have had recurrent, unexplained pregnancy losses (Zini et al., 2008) may benefit from sperm DNA fragmentation testing. Male cancer patients are thought to have increased DNA damage in their sperm, even before chemotherapy (O’Flaherty et al., 2010; Smit et al., 2010). This may be an issue when cryopreserving such semen samples for ART as this process may further increase the level of DNA damage (Tamburrino et al., 2012). In such cases, the prior analysis of sperm DNA damage may be useful to the clinician when providing counselling to the couple. Yet, since data establishing the cost-effectiveness of such tests in the clinical management of infertile couples is still lacking, this approach has not been approved in professional guidelines (Tamburrino et al., 2012).

Whilst several methodologies to assess DNA fragmentation in spermatozoa in the clinical setting are available, they are all unable to discriminate between the repairable or irreparable status of DNA damage (Sakkas and Alvarez, 2010). Furthermore, these tests result in the destruction of the spermatozoa being tested, preventing the selection of intact spermatozoa that could be used for fertilization. Recently, a novel peptide-based stain (DW1) capable of detecting DNA damage in individual sperm cells was developed (Enciso et al., 2012). Although this technique currently requires the removal of the sperm membrane using a detergent, it is hoped that further research will allow the application of this peptide to select viable spermatozoa containing intact DNA to use in ICSI and/or IMSI (intra-cytoplasmic morphologically selected sperm injection) (Enciso et al., 2012).

Given that standard tests are not reliable enough to discriminate between infertile men whose partners have difficulty conceiving and those who do not (reviewed by Barratt, 2010), new diagnostic tests for male factor infertility may be valuable clinically (Adamson and Baker, 2003). Considering that a few studies support the use of DNA damage assays clinically, robust methods must be used in the detection of DNA damage alongside rigorous examination of their application using standardized procedures as required with tests in other disciplines, prior to its possible routine employment in clinical practice (Gluud and Gluud, 2005).

**1.3d Human sperm aneuploidy and fluorescent *in situ* hybridization (FISH)**

Chromosomal aneuploidy refers to the alteration in chromosomal number from the normal diploid chromosomal complement found in somatic cells, or the haploid complement in gametes. Aneuploidy can be either numerical, involving either the gain or loss of an entire chromosome, or structural, which involves the gain or loss of a chromosomal segment (reviewed by Harton and
Most human aneuploidies originate from meiotic errors during chromosome segregation through non-disjunction and anaphase lag in gametes, untimely separation of chromatids (Fragouli et al., 2011; Gabriel et al., 2011; Kuliev et al., 2011), or a post-zygotic mitotic non-disjunction in the embryo (reviewed by Harton and Tempest, 2012; reviewed by Templado et al., 2011; Hassold and Hunt, 2001; Hassold et al., 1993; Hassold and Hunt, 2009; Yanowitz, 2010). With the exception of aneuploidy of the sex chromosomes which is paternally derived in 50%-100% of cases, most aneuploidy in early embryos originates from female non-disjunction (approximately 95%)(Hassold et al., 1993).

The technique most often used for the analysis of chromosomal aneuploidies is fluorescence in situ hybridization (FISH) (Griffin et al., 1992; Delhanty et al., 1993; Delhanty et al., 1997; Ruangvutilert et al., 2000; Ruangvutilert et al., 2000; Daphnis et al., 2005; Ziebe et al., 2003; Munne et al., 2010; DeUgarte et al., 2008; Griffin et al., 1994; Liu et al., 1998). It can be applied to single cells and can analyse chromosome number in both metaphase and interphase nuclei. FISH was originally applied in the evaluation of sperm chromosome aneuploidy, where semen smears were dried and sperm heads were slightly de-condensed to facilitate hybridization with 2-5 fluorescent probes (Marchetti et al., 2008; Martínez-Pasarell et al., 1997). Hybridization can be carried out either simultaneously or subsequently. Rehybridization with a second round of probes allows the analysis of 9-10 chromosomes. The development of FISH technology has allowed the evaluation of large numbers of interphase-stage chromosome aneuploidy, permitting the assessment of sperm from severely infertile men.

The main mechanism thought to lead to aneuploid spermatozoa is thought to be non-disjunction (Templado et al., 1996), but this has not yet been verified (Templado et al., 2011). In theory, errors in chromosome segregation in male meiosis may result from erratic homologous pairing or synopsis during early prophase I, altered recombination at pachytene, failure to maintain chromosome cohesion, or centromeric orientation at the anaphase stage (reviewed by Templado et al., 2011). Studies of polymorphic alleles in trisomic conceptuses revealed decreased recombination or repositioned recombination sites in 100% of the trisomies analysed (reviewed by Hassold et al., 2007). A study on metaphase I and II spermatocytes and spermatozoa from an infertile man showed that the two non-disjunction mechanisms found in meiosis I oocytes also existed in male meiosis (reviewed by Pelléstor, 2006; Uroz et al., 2008; Garcia-Cruz et al., 2010). Similar to oocytes, the prevalent method of non-disjunction in sperm was found to be premature separation of sister chromatids (PSSC), the other being achiasmate nondisjunction (whole chromosome nondisjunction). PSSC was found in MII spermatocytes in the form of one extra or missing chromatid, affecting...
chromosomes 21, X and Y (Uroz et al., 2008). Increased levels of disomy for chromosomes 21, X and Y were observed in the mature spermatozoa from the same individual (Uroz et al., 2008).

The estimated lower limit of total aneuploidy in spermatozoa is thought to be 4.5% (2 x disomy) (Templado et al., 2011). Studies assessing aneuploidy levels for different chromosomes in fertile and infertile men concluded that all men have a degree of aneuploid sperm in their ejaculate, and levels of aneuploid sperm in fertile men are reported to range from 3%-5% (Shi and Martin, 2000; Shi and Martin, 2001; Tempest and Griffin, 2004). Inter-donor variability and variations in values reported by different laboratories makes the baseline rates of sperm aneuploidy difficult to determine (Templado et al., 2011). In addition, since certain chromosomes have been researched more than others and sufficient data is lacking on 6 of the autosomes, a level of extrapolation is necessary when estimating total aneuploidy (Templado et al., 2011). Aneuploidy involving the sex chromosomes is more common than aneuploidy of the autosomes (reviewed by Templado et al., 2011). This observation was not limited to normal control individuals, but also those with paternally derived Down syndrome (DS) (Blanco et al., 1998; Soares et al., 2001), Turner syndrome (TS) (Martínez-Pasarell et al., 1999; Soares et al., 2001), Klinefelter syndrome (KS) (Arnedo et al., 2006) (reviewed by Templado et al., 2011). Age, smoking, alcohol and caffeine may also influence the occurrence of sex chromosome aneuploidy, suggesting that the sex chromosomes are more susceptible to non-disjunction than the autosomes (reviewed by Templado et al., 2011). Chromosomes X and Y may therefore be used as a marker of aberrant meiotic segregation. Further meiotic studies on spermatocytes and spermatozoa are required to establish the exact mechanisms resulting in meiotic missegregation and to evaluate the environmental influences on aneuploidy in human spermatozoa (Templado et al., 2011).

### 1.3c Human sperm aneuploidy, male infertility and risk to the offspring

The observation that men with impaired fertility displayed significantly higher levels of aneuploidy compared to their fertile counterparts was first reported in 1995 (Moosani et al., 1995). Most other studies investigating aneuploidy in infertile men reported similar findings (reviewed by Harton and Tempest, 2012; Shi and Martin, 2000; Shi and Martin, 2001; Tempest and Griffin, 2004). A comprehensive review of disomies for all chromosomes studied in fertile and infertile men found a correlation between sperm aneuploidy and male infertility (Tempest and Griffin, 2004). Immunocytogenetic techniques were used to analyse the recombination focal points during prophase in the synoptonemal complex (Sun et al., 2005). They observed that infertile males had decreased levels of recombination and high incidences of chromosomes lacking any recombination
foci (reviewed by Ioannou and Griffin, 2011). Overall, a relationship is thought to exist between the severity of infertility and increasing levels of disomy which can involve most chromosome pairs, particularly the XY bivalent (reviewed by Ioannou and Griffin, 2011; Tempest and Griffin, 2004). The clear correlation between increasing severity of infertility and increased frequencies of aneuploidy were strongest in men with severe oligoasthenoteratozoospermia (OATS) and sperm retrieved from testicular sperm extraction in cases of non-obstructive azoospermia (Shi and Martin, 2000; Shi and Martin, 2001; Tempest and Griffin, 2004). Patients with OATS were examined in the largest OAT cohort study to further assess the relationship between sperm disomy and male infertility (reviewed by Ioannou and Griffin, 2011; Durakbasi-Dursun et al., 2008). They reported increased rates of disomies for chromosomes 13, 18, 21, X and Y in OATS patients compared to fertile controls (Durakbasi-Dursun et al., 2008). Increased sperm aneuploidy has been linked with a range of other male infertilities including oligozoospermia (Faure et al., 2007), asthenozoospermia (Collodel et al., 2007), teratozoospermia (Faure et al., 2007) and azoospermia (Martin et al., 2003). Several studies reported a three-fold increase in aneuploidy in infertile men (reviewed by Harton and Tempest, 2012).

For the most part, chromosomal aneuploidy can be catastrophic for development and has been recorded for all chromosomes in spontaneous abortions (Harton and Tempest, 2012). Aneuploidy is surprisingly common in humans, occurring in around 4% of clinically recorded pregnancies (Hassold and Hunt, 1993). Approximated 60% of conceptions are thought to be aneuploid, but are spontaneously aborted, normally before the pregnancy is even clinically recognized (Hassold and Hunt, 1993). Understandably, monosomy (the loss of a chromosome), will be much more detrimental than trisomy (the gain of a chromosome). The only non-mosaic monosomic condition that is compatible with life is monosomy X (Lyon, 1971). Conceptions encompassing trisomies for chromosomes 13, 18, 21, X and Y can also survive to term, but not beyond except for trisomy 21 (reviewed by Harton and Tempest, 2012). However, whilst aneuploidy for the aforementioned chromosomes may be compatible with life, a majority of conceptions will spontaneously abort during early development (Harton and Tempest, 2012).

In PGD, it is possible to assess chromosomal integrity of an individual cell derived from an embryo. Unfortunately, there is no way to examine the chromosomal complement of a single spermatozoa destined to be used in IVF or ICSI, as it is destroyed in the process and will no longer be suitable for fertilization. It is unclear whether sperm aneuploidy assessments should be used to counsel patients, a predicament further complicated by the large maternal contribution to aneuploidy (Harton and Tempest, 2012). Several studies have attempted to understand the real relationship between sperm
aneuploidy level and the risk of aneuploid progeny (Blanco et al., 1998; Eskenazi et al., 2002; Martinez-Pasarell et al., 1999; Tang et al., 2004; Templado et al., 2011).

Sex chromosome aneuploidies have a combined frequency of 0.2% in live births (reviewed by Fonseka and Griffin, 2011) and unlike autosomal aneuploidies, are often paternally derived (Hassold and Hunt, 2001). Approximately 55% of all sex chromosome aneuploidies, 80% of Turner syndrome (45, X) aneuploidies, 6% of Triple X syndrome (47, XXX), 100% of Hyper Y syndrome (47, XYY) and 50% of Klinefelter syndrome (47, XXY) are paternal in origin (Wyrobek et al., 2000; reviewed by Fonseka and Griffin, 2011). The most common cause for male infertility in humans is Klinefelter syndrome, where approximately 1/500 male births are affected (Rolf et al., 1996). Around 1 in 1000 live births can be affected by the Hyper Y or Triple X syndrome (Sloter et al., 2004; Buwe et al., 2005). Turner syndrome occurs in approximately 1 in 5000 live female births (Sloter et al., 2004). The 45, X karyotype is highly lethal by 28 weeks gestation. Those that survive have relatively slight complications, possibly due to undetected mosaicism (Fonseka and Griffin, 2011).

Retrospective studies of men who previously fathered paternally derived aneuploid offspring suggest that the levels of sperm aneuploidy in these individuals were much higher than those observed in normal fertile men with no history of aneuploid offspring (Blanco et al., 1998; Eskenazi et al., 2002; Martinez-Pasarell et al., 1999; Tang et al., 2004). Templado et al., (2011) studied sperm aneuploidy using FISH on 15 fathers of offspring with paternally derived aneuploidy. The study group included Down syndrome fathers (Blanco et al., 1998; Soares et al., 2001), four fathers of Turner syndrome (45, X) patients of paternal origin (Martínez- Pasarell et al., 1999; Soares et al., 2001), and 9 Klinefelter (47, XXY) fathers (Arnedo et al., 2006). They reported significantly elevated frequencies of disomy for chromosome 21 and the sex chromosomes in all three groups of fathers compared to the control donors, although these findings were not universal (Templado et al., 2011). Furthermore, whilst the tendency for meiotic nondisjunction in Klinefelter fathers was limited to the XY chromosome pair, the Down syndrome and Turner syndrome fathers had additional increases in chromosomes 13, 21 and 22 (Templado et al., 2011). One study recorded the highest level of sperm aneuploidy observed in a single individual who produced four consecutive aneuploid offspring (Moosani et al., 1999). An interchromosomal effect was also observed, where reports of increased levels of aneuploidy were not limited to just the chromosomes involved, but also other chromosomes investigated (Blanco et al., 1998). Cytogenetic studies on trisomy recurrence determined that having a pregnancy with trisomy 21 increases the future risk of trisomies for the same or a different chromosome (Warburton et al., 2004; De Souza et al., 2009). Although this does not necessarily establish a direct link between sperm aneuploidy and the probability of aneuploid...
offspring, it suggests that certain individuals susceptible to chromosomal non-disjunction are likely
to have aneuploid conceptions (Harton and Tempest, 2012; Templado et al., 2011).

Other studies of fathers with Down (Hixon et al., 1998) and Klinefelter (Eskanazi et al., 2002)
syndrome children did not observe increases in sperm disomies for chromosomes 18 (Hixon et al.,
1998), 21 (Hixon et al., 1998; Eskanazi et al., 2002) and XY (Eskanazi et al., 2002). The inconsistencies
observed between these different studies may be due to heterogeneity among fathers (Templado et
al., 2011). Higher levels of sperm aneuploidy are also linked to recurrent ICSI failure (Nicopoullos et
al., 2008; Petit et al., 2005), increased chromosomal abnormalities in embryos (Gianaroli et al.,
2005), repeated abortions, and lower pregnancy and live birth rates (Nagvenkar et al., 2005; Rubio
et al., 1999; Tang et al., 2004).

Trisomy 18 (Edwards syndrome) is the second most common autosomal trisomy syndrome, after
trisomy 21, found in newborns with an incidence of approximately 1/7,000 (reviewed by Fonseka
and Griffin, 2011; Goldstein and Nielsen, 1988; Root and Carey, 1994; Rasmussen et al., 2003; Irving
et al., 2011; Parker et al., 2011). In several analyses to determine parent of origin, the extra
chromosome 18 is often maternally derived due to segregation errors in meiosis or postzygotic
mitosis (Kondoh et al., 1988; Kupke and Muller, 1989; Fisher et al., 1993, 1995; Nothen et al., 1993;
Ya-gang et al., 1993; Eggermann et al., 1996 Nicolaidis and Petersen, 1998). In the rare cases where
the additional chromosome was of paternal origin, it was usually due to a post-zygotic, mitotic error
(reviewed by Fonseka and Griffin, 2011; Fisher et al., 1995; Eggermann et al., 1996). Trisomy 18
pregnancies have a high risk of fetal loss and stillbirth (Morris and Savva, 2008; Won et al., 2005),
and considering early terminations (Irving et al., 2011) following early diagnosis, the prevalence
of trisomy 18 is probably higher than the observed live birth prevalence.

Epidemiological studies for sperm aneuploidies have not found substantial evidence to prove
paternal age effects (reviewed by Fonseka and Griffin, 2011; Templado et al., 2011), although some
studies have difficulty in discriminating between maternal and paternal effects (Sloter et al., 2004).
FISH studies on sperm nuclei have however observed slight increases in aneuploidy for
chromosomes 1 (Martin et al., 1995), 9 (Bosch et al., 2003), 21 (Rousseaux et al., 1998), X and Y,
often when there was a 2-fold difference between the youngest and oldest donors (Griffin et al.,
1995; Robbins et al., 1995; Kinakin et al., 1997; reviewed by Buwe et al., 2005; Rubes et al., 2005). If
there is in fact a paternal age effect of sperm aneuploidy, it is small and normally restricted to the
sex chromosomes (Templado et al., 2011).
Despite obvious increases in aneuploidy frequency and the potential risk to produce aneuploid conceptions, physicians rarely order aneuploidy screenings for a male indication (Harton and Tempest, 2012; Griffin et al., 2003). A possible reason may involve the uncertainty over how patients with increased levels of sperm aneuploidy should be counselled (Tempest and Martin, 2009; Carrell, 2008; Hann et al., 2011; Tempest, 2011). Some believe that screening for sperm aneuploidy should be a prognostic test for couples undergoing ICSI (Petit et al., 2005; Durakbasi-Dursun et al., 2008; Sanchez-Castro et al., 2009), and that there may even be possible paths for therapy (Tempest et al., 2008).

1.3f DNA fragmentation and aneuploidy in sperm

Factors implicated in sperm DNA damage, like age, obesity, smoking and cancer treatment, are not unlike those thought to increase sperm disomy (Aitken and De Iuliis, 2007). Most cases of male infertility may be explained by the presence of chromosomal abnormalities, DNA damage, or a combination of both. Several studies have reported elevated levels of both these parameters in the sperm of infertile or subfertile men involved in recurrent pregnancy loss (Carrell et al., 2003), men with globozoospermia (Brahem et al., 2011) or carriers of a constitutional chromosomal abnormality (Brugnon et al., 2006; Perrin et al., 2011). Furthermore, teratozoospermic males were found to display higher rates of chromosomal abnormalities and higher DFI values compared to fertile men (Tang et al., 2010). Male carriers of a structural chromosomal abnormality also demonstrated higher DFI values in their sperm than non-carriers (Perrin et al., 2009). Muriel et al., (2007) also found a correlation between the rates of sperm aneuploidy and DNA fragmentation. They observed that men with fragmented sperm DNA had a 4.4-fold increase in sperm aneuploidy compared to sperm with non-fragmented DNA. Although Liu et al., (2004) observed an increase in the levels of aneuploidy in DNA fragmentation in sperm from men with oligoasthenoteratozoospermia compared to normozoospermic men, the two parameters did not correlate.

Kovanci et al., (2001) reported a close relationship between the proportion of immature spermatozoa and disomies. Based on their analysis of chromosomes X, Y and 17, these immature sperm cells showed a 1.5- to 4-fold increase in the frequency of chromosomal abnormalities compared to mature spermatozoa. Furthermore, the rate of aneuploidies was significantly lower in sperm obtained from the 80% Percoll fraction containing concentrated mature spermatozoa compared to unprocessed semen samples. Both the SCSA and SCD test demonstrated higher levels of DNA fragmentation in immature spermatozoa (Ollero et al., 2001; Fernandez et al., 2003). Aneuploidies and DNA fragmentation therefore tend to appear in immature arrested sperm cells. It
has also been suggested that the degree of aneuploid sperm is associated with the degree of apoptotic sperm, reinforcing the hypothesis of a relationship between sperm DNA fragmentation and aneuploidy (Carrell et al., 2003; Schmid et al., 2003; Liu et al., 2004). Whether this is an epiphenomenon or a causal relationship is unclear.

In most cell types, apoptosis is thought to be related to a quality control mechanism to maintain DNA integrity in an attempt to prevent the production of aberrant genomes and neoplastic growth. In sperm, aneuploidy may trigger DNA fragmentation to genetically inactivate sperm nuclei with an abnormal genetic constitution (Muriel et al., 2007). This may prevent genetically abnormal spermatozoa from fertilizing the egg by arresting the sperm cell at an immature stage.

Since mature protaminated chromatin is not very prone to nuclease digestion (Sakkas et al., 1995), sperm DNA fragmentation is more likely to occur during chromatin maturation when the chromatin is not densely packed (Muriel et al., 2007). Sperm DNA fragmentation could also possibly occur as a passive process, secondary to partial arrest during chromatin remodelling (Muriel et al., 2007). Following the detection of chromosomal anomalies, the supposed genomic surveillance mechanism would continue to repair DNA breaks that initiate the removal of DNA supercoiling during the histone-protamine exchange (Muriel et al., 2007). If this alleged genomic surveillance mechanism failed to act, aneuploid spermatozoa may be produced containing intact DNA, increasing the probability of an aneuploid conceptus (Muriel et al., 2007). Consequently, aneuploid spermatozoa without DNA fragmentation would probably be even more detrimental than sperm with fragmented DNA (Muriel et al., 2007). In summary, Muriel et al., (2007) hypothesized that sperm DNA fragmentation occurs as a part of a genomic surveillance mechanism to genetically inactivate sperm with a defective genetic composition. Further studies of sperm aneuploidy frequencies in sperm with intact DNA are required to prove this theory. It is also possible that the possible relationship between sperm DNA fragmentation and aneuploidy is influenced by the origin of infertility (Muriel et al., 2007). The strength of the relationship between the two parameters may depend on the degree of infertility/infertility subgroup. In the case of sperm DNA fragmentation that occurs due to oxidative damage in the genital tract due to exogenous stresses, such associations may be undetectable (Muriel et al., 2007). Considering aneuploidy may trigger DNA fragmentation, and the observation that the rate of aneuploidy is higher in sperm samples of sub-optimal quality, it is possible that the level of sperm DNA fragmentation is correlated with sperm aneuploidy.
1.4 Impact of processes involved in ART on sperm DNA damage and PS translocation

1.4a Reactive oxygen species, sperm DNA fragmentation and male infertility

Containing at least one electron with unpaired spin, making them highly reactive, free radicals are short-lived chemical intermediates that oxidize lipids, amino acids and carbohydrates as well as generating DNA mutations (Sanocka and Kurpisz, 2004). Free radicals initiate a cascade of chain reactions when they attack the nearest stable molecule, converting it to a free radical itself. These then yield either a positive or negative influence on normal cell function by very rapidly oxidizing biomolecules encountered in their vicinity (Warren et al., 1987). Reactive oxygen species (ROS) formed by free radicals are oxidising agents that result from the metabolism of oxygen. ROS is produced via two main systems in sperm. The nicotinamide adenine dinucleotide-dependent oxidase system occurs at the level of the sperm plasma membrane and the NADH-dependent oxidoreductase (diphorase) system at the mitochondrial level (Aitken et al., 1997).

Normal aerobic metabolism depends on optimal levels of ROS because a balance exists between ROS production and antioxidants activity. The small amount of ROS produced by spermatozoa is necessary for physiological processes such as capacitation, hyperactivation and sperm-oocyte fusion (Makker et al., 2009; Lewis et al., 1995). However, to limit the amount of ROS to just the small amount necessary for normal cell function, ROS must be inactivated continuously. Oxidative stress occurs when the production of ROS by the sperm mitochondria overrides the gametes’ limited endogenous antioxidant defences due to electron leakage (Koppers et al., 2008), and when antioxidants are not able to successfully scavenge these free radicals (Sharma et al., 1999).

Because spermatozoa possess limited supplies of endogenous antioxidant protection, whilst containing copious amounts of unsaturated fatty acids and DNA open to free radical attack, they are particularly prone to oxidative stress (Alvarez et al., 1987; Aitken, 1999; Koppers et al., 2010). Cytoplasm, which is the main source of antioxidants, is extruded during spermatozoa maturation. Once this process ceases, residual cytoplasm forms a droplet in the sperm mid region. Spermatozoa that possess these cytoplasmic droplets are considered to be immature and functionally defective (Huszar et al., 1997). The positive correlation between immature spermatozoa and levels of ROS is in turn negatively correlated with sperm quality (Gomez et al., 1998). A high concentration of specific cytoplasmic enzymes which are a source of ROS, such as G6PDH and SOD, are found in the residual cytoplasm. Since the lack of cytoplasm leads to a decreased antioxidant defence, this process contributes to the relationship between poor sperm quality and elevated ROS. Also, it has been
observed that as the number of immature spermatozoa in the human ejaculate rises, so does the concentration of mature spermatozoa with fragmented DNA (Gil-Guzman et al., 2001).

Amongst the various types of cells such as mature and immature spermatozoa, round cells that result from different stages of the spermatogenic process, leukocytes and epithelial cells found in human ejaculate, peroxidise-positive leukocytes and abnormal spermatozoa produce free radicals continuously (Aitken and West, 1990; Hendin et al., 1999).

The sperm plasma membrane is made up of large amounts of polyunsaturated fatty acids which provide the fluidity required for membrane fusion events (acrosome reaction and sperm-egg interactions) and sperm motility. The unsaturated nature of these fatty acids makes spermatozoa particularly susceptible to free radical attack and lipid peroxidation resulting in oxidative stress and the loss of membrane integrity (Twigg et al., 1998; Griveau and Le Lannou, 1997; Gutteridge and Halliwell, 1990; Buettner, 1993). Lipid peroxidation of the sperm plasma membrane causes axonemal protein phosphorylation and protein immobilization, ultimately decreasing sperm mobility (Krishnamoorthy et al., 2007; de Lamirande and Gagnon, 1995).

The correlation between the lipid peroxide content of human spermatozoa and severe motility loss was first observed 25 years ago (Jones et al., 1979), and has subsequently been demonstrated in independent studies (Alvarez et al., 1987; Aitken and Clarkson, 1987; Aitken and Fisher, 1994; Sharma and Agarwal, 1996; Aitken, 1999, 2004). The main ROS involved in motility loss is hydrogen peroxide (du Plessis et al., 2010; Mahfouz et al., 2009). The exposure of human spermatozoa to extracellular ROS and its decrease in motility is directly related to its level of lipid peroxidation (Gomez et al., 1998). Exposure of human sperm to superoxide dismutase, which converts superoxide to hydrogen peroxide severely decreased sperm motility both in vivo and in vitro (Aitken et al., 1992), but motility was rescued through the subsequent addition of antioxidants such as tocopherol and catalase (Aitken et al., 1992; Aitken et al., 1989; Suleiman et al., 1996). This supports the role of lipid peroxidation as a cause of loss in sperm motility. Resveratrol was recently found to improve sperm motility, prevent lipid peroxidation and enhance antioxidant defences in the testes of hyperthyroid rats (Ourique et al., 2013).

Spermatozoa are thought to be particularly resistant to oxidative damage due to the manner in which sperm chromatin is packaged (Bennetts and Aitken, 2005; McKelvey-Martin et al., 1997). Despite this apparent resistance, various detection methods show abundant damage in the nuclear genome of ejaculated spermatozoa (Lewis and Aitken, 2005), and that this damage is usually induced by oxidative stress (Barroso et al., 2000; Duru et al., 2000). The vulnerability of DNA bases to
oxidative damage can result in base modifications, strand breaks and chromatin cross linking. The Y chromosome is particularly susceptible to DNA damage due to its genetic structure and inability to correct double-stranded DNA deletions. Although the levels of sperm-derived ROS measured in sperm preparations with minimal leukocyte contamination have been linked to sperm DNA damage, a ROS threshold above which sperm DNA damage is detected has not been established (reviewed by Zini et al., 2009; Irvine et al., 2000; Barroso et al., 2000; Saleh et al., 2003).

Men with idiopathic infertility tend to display significantly higher levels of seminal ROS along with decreased antioxidant potential compared to fertile controls (Pasqualotto et al., 2001). As oxidative stress can cause pathological effects, damaging cells tissues and organs (Aitken and Baker, 1995), ROS have been established as an etiological factor in a range of diseases (reviewed by Sanocka and Kurpisz, 2004). ROS-induced sperm cell dysfunction is dependent on the nature, quantity and duration of exposure to ROS, as well as surrounding environmental factors such as oxygen tension, temperature, concentrations of molecular components such as ions, proteins, and ROS scavengers (Agarwal and Saleh, 2002). Other mechanisms include ionizing radiation (Sadani and Nadkarni, 1997), bioactivation of xenobiotics (Akiyama, 1999), inflammatory cells (Villegas et al., 2003), increased cellular metabolism (Hollan, 1996), decompartmentalization of transition metal ions (Huang et al., 2001), activation of oxidases and oxygenases (Davydov, 2001) and a loss of antioxidant capacity (Aitken and Sawyer, 2003; Hsu et al., 1998). Severe pro-mutagenic change induced by oxidative damage, can affect the quality of the male germ line and prevent fertilization. Pathological outcomes such as reduced fertilization rates, aberrant preimplantation development, increased miscarriage and morbidity in the offspring (Bungum et al., 2004; Seli et al., 2004; Virro et al., 2004; Lewis and Aitken, 2005; Aitken et al., 2009; Zini and Sigman, 2009; Barratt et al., 2010) are thought to be due to oxidative stress related DNA damage.

1.4b Clinical diagnosis and assessment of seminal oxidative stress

The excessive release of nitric oxide, which causes spermatozoal dysfunction, has been observed within dilated spermatic veins in subfertile males with varicocele (Mitropoulos et al., 1996; Ozbek et al., 2000). A positive correlation has been observed between seminal ROS levels and varicocele grade where men with higher levels of seminal ROS were associated with varicocele grades 2 and 3 as opposed to men with varicocele grade 1 (Allamaneni et al., 2004). These patients also display low levels of total antioxidant capacity (TAC) in the seminal plasma and increased 8-hydroxy-2'-deoxyguanosine levels, which suggest a defective pro-oxidant defence system and increased oxidative DNA damage (Hendin et al., 1999; Chen et al., 2004). Compared to normal sperm donors,
varicocele patients have been shown to have significantly higher oxidative stress parameters such as ROS and lipid peroxidation, and substantially lower antioxidant concentrations (Agarwal et al., 2006). Supplementary antioxidants may therefore benefit the infertile population with varicocele. It has been reported that seminal plasma ROS and sperm DNA damage in infertile men can be reduced following a varicocelectomy (Zini et al., 2005) and is associated with increased seminal plasma concentrations of antioxidants such as superoxide dismutase, catalase, glutathione peroxidise and vitamin E (Mostafa et al., 2001).

Seminal leukocytes are the main source of ROS in the human ejaculate, and induce spermatozoal damage during ART procedures (Sharma et al., 2001). Leukocytospermia is characterized by unusually high seminal leukocytes, polymorphonuclear neutrophils, and macrophages (Saleh et al., 2002). Both enhanced ROS levels and leukocytospermia have been observed in patients with accessory gland infection, and sperm function defects in these patients are thought to be the result of abnormal lipid peroxidation induced by elevated ROS levels (Potts et al., 2000).

The occurrence of leukocytes in semen has been linked to decreased sperm mobility and fertilization capacity during genito-urinary (GU) tract infections (Wolff et al., 1990; Berger et al., 1982). Due to its lack of a pro-oxidant defence system, ROS produced as a result of infections that occur in the testis and epididymis, are particularly harmful to spermatozoa. Infections that induce ROS in the prostate gland and seminal vesicles may also have an indirect effect on sperm function. Although the underlying mechanism is not well understood (Pasqualotto et al., 2000), prostatitis, characterized by the presence of granulocytes in the prostatic fluid, has been linked to male infertility. In spite of the leukocytospermia status, men with chronic prostatitis and prostatodynia exhibited increased seminal oxidative stress (Pasqualotto et al., 2000). Many theories surround the relationship between male genital tract infections and their association with ROS. The leukocytes are thought to induce human spermatozoa to produce ROS. Although the mechanism has not been confirmed, it is thought to involve the direct contact of sperm and leukocytes or may be controlled by the release of soluble products by leukocytes (Aitken and West, 1990).

Harmful compounds found in the environment like alkaloids, nitrosamines, nicotine, cotinine and hydroxycotinine found in cigarettes are thought to produce free radicals (Gate et al., 1999; Traber et al., 2000), and a relationship between cigarette smoking and reduced seminal quality has been reported (Kunzle et al., 2003). When infertile men who smoked cigarettes were compared to those who did not smoke, smoking was linked to a significant increase in seminal leukocyte concentration and ROS levels (Saleh et al., 2002). The rise in industrialization has led to the increased deposition of highly toxic heavy metals into the atmosphere, and paternal exposure to metals such as lead, arsenic
and mercury, is associated with subfertility and pregnancy complications (Sallmen et al., 2000). It is believed that oxidative stress is involved in the onset of adverse health effects due to the occupational exposure to chemicals known as endocrine disruptors on the reproductive system (Kumar, 2004; Fowler et al., 2004).

1.4c Effects of sperm preparation involved in ART on sperm DNA damage and phosphatidylserine (PS) translocation

The sensitivity of ejaculated sperm cells to DNA fragmentation from both normospermic donors and infertile men is important to understand, since semen samples may be exposed to potentially damaging agents during assisted reproduction technologies (ART). Sperm processing during IVF and ICSI may affect DNA fragmentation. During an ART cycle, ejaculated spermatozoa are subjected to various handling procedures such as re-concentration, cooling, freezing and thawing, high-speed sorting, selection and incubation. Density gradient centrifugation is thought to produce sperm samples of higher sperm DNA integrity than sperm swim-up (Brahem et al., 2011; Zhang et al., 2011). Due to the varying resilience of spermatozoa to such practices, markers that can determine how extensive the consequent damages are during semen evaluation may be of use for a quick, detailed analysis of a large number of spermatozoa.

The extent of sperm DNA damage is related to sperm function and male infertility (Aitken, 1999). Sperm DNA fragmentation can occur by apoptosis, or programmed cell death, as the culmination of a complex cascade of biochemical events where the DNA is cleaved into oligonucleosomal sized fragments at the latter stages. It is caused by strand breaks during chromatin remodelling, exposure to free radicals during transition through the female reproductive tract, induction by endogenous endonucleases, radiotherapy and chemotherapy, and by environmental toxins (Sakkas and Alvarez, 2010).

Oxidative stress (OS) has been implicated as a cause for male infertility (Cocuzza et al., 2007). Reactive oxygen species can be beneficial to normal physiological function and hydrogen peroxide supposedly plays a role during sperm capacitation (Griveau et al., 1994). However, once the level of ROS exceeds the antioxidant defence system, thus over powering it and causing oxidative stress, pathological conditions are induced (Agarwal et al., 2006). Many studies have observed positive, significant correlations between the level of endogenous ROS and DNA strand breaks in the ejaculate (Barroso et al., 2000). Sperm preparation techniques for ART are potential causes for oxidative stress. For example, the removal of spermatozoa from seminal plasma during the washing
procedure can induce ROS. Whilst measuring the level of ROS directly may be of value, I considered the study of its effect to be of more importance.

Normal testicular function is dependent on temperature and requires the testes to be 2°C-8°C below core body temperature (Harrison & Weiner, 1948; Ivell, 2007), and men with increased scrotal temperatures have been associated with degrees of sub-fertility (Mieusset et al., 1987). Exposure of sperm to elevated temperatures of 41°C and 45°C have shown to increase the rate of DNA damage (Santiso et al., 2012) and concerns have been raised about the increasing level of DNA damage in sperm from men undergoing assisted reproduction (Paul et al., 2008). During an ART cycle, sperm samples are stored at 37°C for approximately 1 hour prior to its use in artificial insemination, ICSI or IVF.

The detrimental effects of freezing and thawing procedures can lead to a significant decrease in sperm viability and motility, and ultimately decrease the fertility potential of cryopreserved sperm compared to fresh samples (Chatterjee et al., 2001; Said et al., 2009, 2010). Although a cryoprotectant is used in sperm samples intended to be used in ART, it is hardly ever used during the storage of samples assessed for DNA fragmentation and basic scientific research. DNA fragmentation tests such as the SCSA test involve freezing sperm samples in the absence of cryoprotectant. Because freezing may induce apoptotic-like changes in the spermatozoa, subsequent assessments of DNA damage will likely be over-estimated.

Amongst the numerous techniques developed to isolate spermatozoa capable of fertilizing oocytes, the Annexin V assay was founded on the molecular binding characteristics of the sperm cell (reviewed by Henkel, 2012). Annexin V is a 35kDa protein that binds to negatively charged phosphatidylserine (PS) which accumulates on the inner membrane of viable cells (van Heerde et al., 1995). An early sign of apoptosis, PS migrates from the inner membrane to the outer membrane, highlighting the spermatozoa’s unsuitability for fertilization (Vermes et al., 1995) (Figure 1.4a). Once on the cell surface, PS is detected by staining with a fluorescent conjugate of Annexin V. The Annexin V- FITC Assay Protocol which was used in this study involves the suspension of the collected cells in Binding Buffer, the addition of Annexin V-FITC to the cell suspension, followed by a 5 minute incubation period in the dark (BioVision Incorporated, California, USA) . This one-step staining process takes only approximately 10 minutes, and detection can be analysed by flow cytometry or by fluorescence microscopy. Sperm PS translocation is thought to be positively correlated to DNA fragmentation, reflecting fertilization and pregnancy failure in assisted reproduction (Muratori et al., 2003; Henkel et al., 2004; Sun et al., 1997; Barroso et al., 2006).
Figure 1.4a: Illustration of annexin V binding to an apoptotic cell. In a normal cell, phosphatidylserine is exclusively localized in the inner leaflet of the plasma membrane. In apoptotic cells, phosphatidylserine begins to move to the outer leaflet of the plasma membrane. Annexin V sensitively binds to phosphatidylserine on the cell surface in a calcium ion-dependent manner. Reprinted from Annals of Nuclear Medicine, Volume 24, Issue 9, November 2010, Pages 629-638, Oku N et al., Nuclear neuroimaging in acute and sub-acute ischemic stroke, Copyright©2010, with kind permission from Springer Science and Business Media.

1.5 Unexplained female infertility and its evaluation

In couples with advanced female age (>35 years), it is common for practitioners to initiate diagnostic evaluation after the inability to conceive for 6 months (Qaas and Dokras, 2009). The prognosis is worse if the duration of infertility exceeds 3 years (Collins et al., 1995). Unexplained/idiopathic infertility in females refers to the diagnosis, or rather the lack of a diagnosis, reached in couples when tubal patency (hysterosalpingogram and/or laparoscopy), normal ovulatory function (basal body temperature, cervical mucus changes, serum LH surge or mid-luteal progesterone) and semen analysis are established (The Practice Committee of the American Society for Reproductive Medicine, 2006). Although estimates vary, the probability that a couple has unexplained infertility because such tests return normal in an infertile couple ranges from approximately 15%-30% (The Practice Committee of the American Society for Reproductive Medicine, 2006).

Abnormalities in ovulatory function have been reported in approximately 40% of infertile women and 15% of infertile couples (The Practice Committee of the American Society for Reproductive Medicine, 2006). Ovulatory defects are usually revealed as menstrual disturbances and such patients are then investigated for underlying causes such as polycystic ovarian syndrome, thyroid disease, hyperprolactinemia and hypothalamic causes resulting in weight change. Besides the assessment of the menstrual history, basal body temperature recordings, urinary luteinizing hormone (LH) ovulation kits, mid luteal serum progesterone testing and endometrial biopsy to test secretory endometrial development are used to assess ovulation (Quaas and Dokras, 2008).
Women with a history of ovarian surgery and advanced age risk a diminished ovarian reserve and/or function (Shah, 2013; Flyckt et al., 2013; Berlanda et al., 2013). Considering the relatively non-invasive nature of the test, practitioners usually evaluate ovarian reserve as the initial workup for infertility by measuring serum follicle-stimulating hormone (FSH) and estradiol level on day 3 of the cycle, anti Mullerian hormone, inhibin B, clomiphene citrate challenge test, and ultrasonographic ovarian antral follicle count (La Marca et al., 2012; Kunt et al., 2011; Anderson et al., 2012; The Practice Committee of the American Society for Reproductive Medicine, 2006). Whilst abnormal outcomes of these tests do not confirm infertility, they are associated with a low response to ovulation induction medications and decreased live birth rate following IVF.

The assessment of the uterine cavity and fallopian tubes can be performed by hysterosalpingography and hysteroscopy (Smith et al., 2012; Grimbizis et al., 2010; Pundir and Toukhy et al., 2010; Johnson et al., 2005). Hysterosalpingography involves the radiographic evaluation of the uterine cavity and fallopian tubes after injecting a radio-opaque medium through the cervical canal (Smith et al., 2012; Kodaman et al., 2004; Exacoustos et al., 2003). Hysteroscopy involves the insertion of a hysteroscope into the uterus to allow visualization of the endometrial cavity, including the tubal ostia, endocervical canal, cervix, and vagina (Smith et al., 2012). However, it is inadequate in the evaluation of fallopian tubal damage, requiring further tests to diagnose the cause of infertility (National Infertility Association, 2012; Grimbizis et al., 2010). Both these methods are thought to have little risk to the patient.

Transvaginal ultrasound involves the insertion of a probe into the vagina, which is moved to visualize the pelvic organs. Ultrasound tests in the follicular phase can identify uterine fibroids, polyps and congenital anomalies like a septate uterus, and assess ovarian volume and antral follicle counts (Smith et al., 2012; National Infertility Association, 2012; Grimbizis et al., 2010; Roma Dalfo et al., 2004).

Laparoscopy directly visualizes the pelvic reproductive anatomy and is used to identify unknown peritoneal factors that affect fertility, such as endometriosis and pelvic adhesions (Smith et al., 2012; Quaas and Dokras, 2008). It is usually performed in women with unexplained infertility, endometriosis or in whom reversible adhesive tubal disease is presumed (Bonneau et al., 2012; The Practice Committee of the American Society for Reproductive Medicine, 2006). The benefits of laparoscopy in patients with unexplained infertility and normal HSG findings are controversial (Tsuji et al., 2009; Lavy et al., 2004; Fatum et al., 2002).
Examination for basic infertility evaluation can usually be completed within one menstrual cycle. In the absence of a definite diagnosis when a couple have unexplained infertility, various treatments exist in the form of both pharmacological and non-pharmacological treatments (Smith et al., 2012). These include clomiphene citrate, GnRH antagonists, ART and expectant management with lifestyle changes (Smith et al., 2012). Individual patient traits including age, treatment effectiveness, side-effect profile such as multiple pregnancies, and cost considerations need to be respected prior to choosing a treatment plan.

### 1.6 Cumulus and granulosa cell DNA fragmentation as potential biomarkers of fertility potential

#### 1.6a The search for biomarkers to predict oocyte competence

In spite of significant progress in both the clinical and embryological aspect of ART, the take-home baby rate is still lower than wished for (Patrizio et al., 2007). Whilst pregnancy rates have not significantly increased, one of the major problems in IVF remains the high multiple pregnancy rate (Borini et al., 2005). Better criteria are therefore required to establish the viability of a transferable embryo. One study evaluated biological wastage in assisted reproduction recorded the number of oocytes inseminated, the number of euploid embryos produced and transferred, and the number of live births in patients undergoing pre-implantation genetic screening (PGS) (Patrizio et al., 2007). They found that only 8% of the oocytes inseminated produced euploid embryos for transfer, of which 1.5% implanted and only 1% of the total oocytes inseminated eventually resulted in live births (Patrizio et al., 2007). A majority of oocytes collected following ovarian stimulation are unable to produce viable embryos, possibly due to some form of inherent abnormality (Inge et al., 2005). These observations advocate the need to research how the few oocytes with the potential to produce a child can be identified from amongst the cohort retrieved. Whilst cytogenetic abnormalities can be a significant cause of implantation failure, several other factors may contribute to this issue. The current morphological criteria used to select and classify oocytes are thought to be insufficient when choosing the ideal oocyte for fertilization and the resulting embryo for transfer (Patrizio et al., 2007).

Oocyte quality is the single most important factor in fertilization, implantation and a successful pregnancy following IVF-ET. Due to the tendency to limit embryo overproduction, restrictive legislative rules in certain countries and the improving outcome of oocyte storage, oocyte assessment has fast become a major objective for embryologists in human IVF. Embryologists are constantly being challenged to identify characteristics to select the best oocyte to inseminate.
Research into the identification of optimal oocytes has been carried out in several research laboratories and some have yielded promising results (reviewed by Patrizio et al., 2007). Established methods of examining the oocyte, such as morphological evaluation of the oocyte (reviewed by Balaban and Urman, 2006; Ebner et al., 2006) and cytogenetic analysis of polar bodies using FISH have been joined by more advanced cytogenetic methods such as the use of CGH (comparative genomic hybridization) (Harper and Harton, 2010).

Certain morphological features are thought to be useful indicators of oocyte quality, and oocyte morphology is usually assessed following the removal of cumulus prior to ICSI (reviewed by Balaban and Urman, 2006; Ebner et al., 2006). Polarization light microscopy has shown that the absence of the metaphase II spindle can reduce the rate of fertilization and blastocyst formation (Wang et al., 2001; Moon et al., 2003; Rienzi et al., 2003). Reduced fertilization rates have also been associated with oocytes in which the spindle has shifted more than 90 degrees relative to the position of the first polar body (Rienzi et al., 2003). The reliability of using polar body morphology to judge oocyte quality, however, remains controversial. Variations in the oocyte's cytoplasmic density, viscosity and texture have also been associated with outcomes following IVF treatment (Kahraman et al., 2000; Meriano et al., 2001; Ebner et al., 2003). Whilst the occurrence of membrane vacuoles have been linked to reduced fertilization rates (de Sutter et al., 1996; Ebner et al., 2005), the aggregation of smooth endoplasmic reticulum is thought to decrease blastocyst formation and pregnancy rates (Otsuki et al., 2004). Although certain morphological abnormalities are associated with oocyte quality, definite quantification of the potential importance of different abnormalities is lacking (Patrizio et al., 2007). Additionally, only a fraction of morphologically normal oocytes produce pregnancies (Patrizio et al., 2007). The power of morphological observations and standard microscopic evaluation of the oocyte is therefore limited.

Oocytes can be analysed for aneuploidy by biopsying the first and second polar bodies through cytogenetic analysis (reviewed by Patrizio et al., 2007). An extra or missing chromosome in a polar body is indicative of a reciprocal loss or gain of chromosomes in the corresponding oocyte. Most chromosomal analyses have been carried out using FISH (Verlinsky et al., 1998; Kuliev et al., 2003; Pujol et al., 2003). Since then, CGH has been used to assess the copy number of chromosomes in polar bodies and oocytes (Wells et al., 2002; Gutierrez-Mateo et al., 2004; Fragouli et al., 2006). Despite its advantages, CGH is time consuming which makes it difficult to perform within the narrow time frame available for preimplantation testing (reviewed by Patrizio et al., 2007). Microarray-based CGH is thought to be a more suitable method, and is being used routinely in several PGD laboratories.
Recent advances in oocyte gene expression are thought to highlight the underlying processes involved in the determination of oocyte quality. Most cellular processes undergo some form of regulation, and changes in the utilization of a specific pathway can be observed in gene activity fluctuations. Increased expression of genes involved in the detection and response to cellular stress may be indicative of reduced oocyte competence. Studies using reverse transcription, followed by real-time PCR discovered that alterations in the activity of specific genes were related to oocyte and embryo quality (Wells et al., 2005; Dode et al., 2006; Russell et al., 2006). Morphologically abnormal preimplantation embryos were found to often display unusual patterns of gene expression (Wells et al., 2005). Variations in oocyte mRNA have also been associated with advanced maternal age (Steuerwald et al., 2001). These observations suggest that the analysis of oocyte gene expression is a worthwhile approach for the identification of viability markers. Unfortunately, the use of real-time PCR is limited by the number of genes that can be analysed per single oocyte (approximately <10).

A study comparing the oocyte transcriptome with that of the blastocyst to provide data on molecular pathways before and after embryonic genome activation used pooled metaphase II oocyte and blastocysts which were processed for RNA extraction, RNA amplification (Kakourou et al., 2013). The list of genes they identified may be useful in the understanding of processes involved in early embryo development and blastocyst implantation, and recognising any irregularities leading to infertility (Kakourou et al., 2013). Expression profiling of DNA repair genes in human oocytes and blastocysts using microarrays detected large numbers of repair genes, suggesting that all DNA repair pathways are possibly functional in human oocytes and blastocysts (Jaroudi et al., 2009). They observed higher mRNA levels for most repair genes in oocytes compared to blastocysts, indicating sufficient availability of template until the embryonic genome is activated (Jaroudi et al., 2009).

In an attempt to assess the influence of oocyte maturity on in vitro blastocyst development in patients undergoing IVF, 1278 oocytes were derived from 147-IVF cycles and retrospectively analysed (Lin et al., 2003). Oocyte maturity was based on the morphology of the ooplasm, cumulus mass, corona radiata, and granulosa cells. The study found that mature oocytes led to the highest fertilization rates. They noted that although cleavage rates were similar in both the mature and immature groups, the percentage of poor morphology, day-3 embryos was significantly higher in the latter group. Day-3 embryos of good morphology were derived from immature oocytes, but the chance of them developing into blastocysts was significantly lower compared to mature oocytes. Although blastocyst-stage embryos were derived from immature oocytes, these embryos rarely developed into top-grade blastocysts. They concluded that oocyte maturity may be able to
determine fertilization potential and subsequent blastocyst development quality \textit{in vitro} (Lin \textit{et al.}, 2003).

Whilst several methods have been proposed, thus far, the assessment of oocyte morphology during the maturation phase appears to be the most popular. Although being relatively quick and simple, it tends to be a more ‘negative’ than ‘positive’ predictor of oocyte quality. Furthermore, since there is no substantiated correlation between oocyte quality (based on morphology) and the outcome of IVF-ET (fertilization and pregnancy rates), this method may be unreliable (Veeck, 1988; Staessen \textit{et al.}, 1992; Balaban and Urman, 2006). It is therefore necessary to have additional biomarkers with the capacity to be both objective and predictive of successful fertilization.

1.6b The cumulus-oocyte complex (COC)

The mature cumulus oocyte complex (COC) comprises the secondary oocyte which is arrested at metaphase II following extrusion of the 1st polar body and the surrounding cumulus cells (CC). Granulosa cells (GC) are the primary cell type in the ovary, and provides the physical support and micro-environment necessary for the developing oocyte. They differentiate into two discrete phenotypes, the mural population coating the follicular antrum, and the cumulus population enclosing the oocyte (Diaz \textit{et al.}, 2007). As the follicle undergoes transition from the pre-antral to the antral stage, the GCs proliferate as follicular fluid accumulates and coalesces to generate a single follicular antrum (reviewed by Sanchez and Smitz, 2012; Hussein \textit{et al.}, 2005). Along with the change from primordial to primary follicle, the granulosa cell’s morphology changes from squamous to cuboidal (Lintern-Moore and Moore, 1979). Although the follicular antrum enlarges during the antral phase, the enclosed oocyte remains the same size (Durinzi \textit{et al.}, 2005).

Cumulus cells express many of the same genes as granulosa cells but they have a unique transcriptome in accordance with their specialized role. Murine cumulus cells are known to express the \textit{Amh} gene which codes for the Anti-Mullerian hormone at a higher level than in granulosa cells (Salmon \textit{et al.}, 2004). AMH regulates follicle development by curbing the effects of the follicle stimulation hormone (FSH) on follicle growth and inhibiting primordial follicle recruitment. \textit{Amh} expression may therefore be involved in intra- and inter-follicular coordination of follicle development (Salmon \textit{et al.}, 2004). The ability to undergo expansion is a unique characteristic of cumulus cell differentiation (Diaz \textit{et al.}, 2006), and is thought to be critical for normal oocyte development, ovulation and fertilisation (Elvin \textit{et al.}, 1999; Huang \textit{et al.}, 2002; Vanderhyden \textit{et al.}, 2003).
Originating from relatively undifferentiated granulosa cells, the CCs have highly specialised trans-zonal cytoplasmic projections that penetrate the zona pellucida and form gap junctions at their tips with the oocytes (Albertini et al., 2001). By metabolically linking the oocyte and cumulus cells, the extensive network of gap junctions act as gateways for several metabolites into the ooplasm, allowing an intimate association between the CCs and oocyte (Albertini et al., 2001; Depalo et al., 2003; Eppig, 1991). The primary functions of the cumulus cells include providing nutrients to the oocyte through these gap junctions and to protect the oocyte, for example, from oxidative stress (Tatemoto et al., 2000). Whilst the mural granulosa cells are closely associated with oocyte development, oestrogen production and follicular rupture, and is partly regulated by oocyte derived factors (Anderson et al., 2009), the cumulus cells contribute to oocyte maturation and consequent developmental potential (Eppig, 2001). Cumulus cells facilitate in vivo maturation during metaphase II and cytoplasmic maturation of the oocyte, allowing normal fertilization and consequent development (Saito et al., 1995; Quinn and Margalit, 1996; Zhang et al., 1995; Kim et al., 1996). They also support sperm selection, capacitation and acrosome reactions, thus ensuring male pronuclei formation and the acquisition of complete embryonic developmental competence (Tesarik and Kopecny, 1990; Van Soom et al., 2002; Liu et al., 2004; Takeuchi et al., 2005; Rijsdijk and Franken, 2007; Tanghe et al., 2002; Eisenbach, 1999).

The communication axis between the oocyte and cumulus cells is not merely in one direction, but bi-directional (Yeo et al., 2009; Nakahara et al., 1997; Albertini et al., 2001). This mutual dependency suggests that any apoptosis related process that influences the cumulus cells may have a similar impact on the oocyte itself. Because of the close interaction and shared microenvironment between CCs and the oocyte, a footprint of the follicular conditions experienced by the oocyte may be preserved in CCs. The close communication between CCs, the oocyte and surrounding GCs may have scientific relevance (the GC-CC-oocyte signalling pathway may reveal biological processes active in the oocyte/follicle) and can also be valuable clinically (Gasca et al., 2007; Huang and Wells, 2010). For example, if it is possible to detect and decode molecular alterations in the CCs, prognostically relevant information concerning the oocyte and its maturation may be revealed. Thus, a high degree of apoptosis in cumulus cells may be representative of diminished oocyte quality and developmental competence (Corn et al., 2005).

Morphologically abnormal oocytes have been shown to have significantly higher rates of cumulus cell apoptosis than the morphologically normal oocytes examined (Yang et al., 2009). An elevation in cumulus cell apoptosis has also been linked to immature human oocytes, impaired fertilisation (Host et al., 2002), suboptimal blastocyst development (Corn et al., 2005) and poor IVF outcome.
It is unclear as to whether cumulus cells with a high level of apoptosis result in the production of impaired oocytes or whether oocytes of poor quality induce apoptosis in the associated cumulus cells. What these observations do reveal is the interdependence between the oocyte and its cumulus cells. The adequate functioning of the latter ensures the survival and subsequent maturation of the oocyte, thus allowing it to achieve its full reproductive purpose (Tanghe et al., 2002).

1.6c Cumulus cells and their potential to predict fertility

The search for additional biomarkers that are both objective and predictive of successful fertilization is necessary. The greatest advances may originate from the evolution of molecular genetic technologies. However, as it is unlikely that any form of molecular analysis of the oocyte could provide such information while maintaining oocyte viability, alternative approaches must be sought. The identification of markers that can predict fertility potential may ultimately lead to non-invasive tests for oocyte quality. If validated, these new approaches may revolutionize oocyte and embryo selection, resulting in improved implantation rates and greater success rates using elective single embryo transfer. The search for non-invasive approaches has included the environment surrounding the oocyte; the analysis and function of the cumulus cells (McKenzie et al., 2004, Cillo et al., 2007, Feuerstein et al., 2007) and factors in the follicular fluid (Fanchin et al., 2007). Follicular fluid containing granulosa cells is aspirated along with the oocyte during oocyte pick-up, and is therefore readily available.

Feuerstein et al., (2007) studied the transcription of specific genes in cumulus cells harvested prior to intracytoplasmic sperm injection from pre-ovulatory follicles. Genes were chosen based on the induction of their expression by the LH peak ([Steroidogenic Acute Regulatory protein (STAR), Cyclooxygenase 2 (COX2 or PTGS2), Amphiregulin (AREG]) or because they played a role in oocyte lipid metabolism [Stearoyl-Coenzyme A Desaturase 1 and 5 (SCD1 and SCD5)] and gap-junctions [Connexin 43 (CX43 or GJA1)]. Messenger RNA levels in cumulus cells were analysed by real-time PCR. The observed upregulation of the genes investigated (except CX43) following the resumption of meiosis suggest that nuclear maturation was associated with increased expression of STAR, COX2, AREG, SCD1 and SCD5 by cumulus cells. In cumulus cells from metaphase II oocytes, gene expression had no relationship to embryo morphology at Day 2. However, transcript levels were lower and spread over a narrower range in cumulus cells enclosing oocytes that reached blastocyst level at Day 5/6, compared to cumulus cells surrounding oocytes that failed to develop beyond the embryo.
stage. They concluded that lower expression of these genes, in a narrower range, was required for embryos to develop from the embryo to blastocyst stage.

Cillo et al., (2007) carried out a retrospective study on cumulus cells derived from 90 oocytes from 45 patients to determine the relationship between human oocyte developmental competence and expression levels of certain cumulus genes. They reported that cumulus cells from oocytes that resulted in high quality embryos on Day 3 of culture had elevated levels of hyaluronic acid synthase 2 (HAS2) and gremlin1 (GREM1) than in oocytes that did not fertilize or developed into poor-quality embryos. Pentraxin 3 (PTX3) levels were not different between the two groups. Their results imply that HAS2 and GREM1 transcript levels may be suitable biomarkers to complement embryo morphology in the selection of oocytes with greater potential to successfully fertilize and develop in vitro. McKenzie et al., (2004) also investigated the relationship between cumulus gene expression and embryo development. They also used one-step real-time quantitative RT-PCR to study expression levels of PTGS2, HAS2 and GREM1, and their relationship with oocyte quality, fertilization and embryo morphology. They observed that PTGS2, HAS2 and GREM1 gene expression correlated to morphological and physiological characteristics of the embryo.

Fanchin et al., (2007) aimed to determine whether the production of AMH is reflective of embryo competence. They studied the possible relationship between serum and follicular fluid AMH and the fate of the respective oocyte, resulting embryos and results of IVF-embryo transfer in 118 infertile patients. Their clinical pregnancy and implantation rate results suggest that AMH concentration in the follicular fluid, but not serum, was a useful follicular marker of embryo implantation. Follicular fluid AMH levels were also negatively correlated with follicular fluid progesterone and estradiol levels.

A study was designed to determine the stage at which enclosed cumulus-oocyte complexes achieve developmental competence in primates, by examining the ability of oocytes in small antral follicles to spontaneously resume meiosis, fertilize, and support early embryo development (Peluffo et al., 2010). Ovaries were removed from adult rhesus monkeys during their early follicular phase of spontaneous cycles and small antral follicles were divided into five groups according to their diameter. Cumulus-oocyte complexes from healthy small antral were removed and cultured for 48 hours, following which, oocyte meiotic status and diameter were measured. They reported that cumulus oocyte complexes obtained from follicles with a diameter of 0.5-2mm contained oocytes that often resumed meiosis regardless of the presence of absence of gonadotrophins, and fertilized following IVF or ICSI. However, although the inseminated oocytes reached the morula stage, they
arrested. The potential for these oocytes to complete maturation was therefore suboptimal (Peluffo et al., 2010).

In an attempt to evaluate telomere length in cumulus cells as a potential biomarker of oocyte and embryo quality, DNA was extracted from the cumulus cells of 350 COCs and assessed for telomere length by real-time quantitative PCR (Cheng et al., 2013). Telomere length was analysed relative to a single copy marker gene (3684) to assess the effect of the real reproductive age of cumulus cells on oocyte and embryo development. The found that telomere length was longer in cumulus cells from mature oocytes compared to those from immature oocytes, and in cumulus cells from oocytes that developed into good-quality embryos compared to cumulus cells from oocytes that developed into poor-quality embryos (Cheng et al., 2013). The authors concluded that telomere length in cumulus cells at the time of oocyte collection is predictive of oocyte competency and embryo development, but may not be satisfactorily discriminating to be clinically useful (Cheng et al., 2013).

In an attempt to study the follicular environment of oocytes that become aneuploid and categorise non-invasive markers of oocyte chromosome status and competence, microarray comparative genomic hybridization was used to detect oocyte ploidy, and real-time PCR was used to study the expression of 96 genes in 51 cumulus cell samples (Fragouli et al., 2012). Of the genes assessed, 58 were found to be expressed in cumulus cells, and patterns of expression were mostly similar amongst all samples, irrespective of the respective oocyte’s chromosomal status. However, the SPSB2 (splA/ryanodine receptor domain and suppressor of cytokine signalling (SOCS) box containing 2) and TP53I3 (tumour protein p53 inducible protein 3) genes were found to be significantly down-regulated in cumulus cells derived from chromosomally abnormal oocytes. SPSB2 was also found to be up-regulated in the cumulus cells of oocytes that proceeded towards a live birth (Fragouli et al., 2012).

A study by Held et al., (2012) aimed to study the relationship between zona pellucida birefringence (ZPB) and bovine oocyte developmental capacity using scanning electron microscopy. They observed that ZPB is affected by alterations during in vitro maturation, and that in vivo-matured oocytes demonstrated significantly lower birefringence parameters and higher blastocyst rates compared to in vitro-derived oocytes. Similar observations of zona birefringence and developmental capacity were made for in vitro matured oocytes from COCs of better quality compared to those of sub-optimal quality. It was concluded that decreasing trends for ZPB during in vitro maturation were associated with good quality oocytes and successful cytoplasmic maturation. Accordingly, fully-grown immature oocytes reached higher blastocyst rates and lower XPB values compared to still-
To characterize and identify a non-invasive marker of oocyte competence, Ruvolo et al., (2013) studied oxygen consumption rates for individual oocytes before fertilization. They observed that higher rates of oxygen consumption was evidence of overall metabolic activity and related to better fertilization ability and embryo cleavage quality.

The sequential growth of 4042 embryos individually cultured from day 1-day 5/6 was recorded in a prospective study to determine the value of morphological assessment at days 1 and 2 to predict blastocyst development potential (Guerif et al., 2007). Pronuclear morphology on day 1 and early cleavage, cell number and fragmentation rate on day 2 were recorded for each zygote. Also, blastocyst transfers were analysed to assess their implantation ability and early embryo development. Whilst all four parameters analysed correlated to blastocyst development, early cleavage and cell number on day 2 were the strongest indicators to predict the development of a blastocyst with good morphology on day 5. Furthermore, although the transfer of a good morphology blastocyst usually resulted in high implantation and live birth rates, parameters of early development did not accurately predict implantation ability (Guerif et al., 2007). They concluded that the combination of all four parameters had a relatively low prediction of embryo viability (Guerif et al., 2007).

To determine if follicular free fatty acid (FFA) levels are associated with cumulus oocyte complex morphology, FFAs in serum and ovarian follicular fluid were measured in 102 women undergoing IVF (Jungheim et al., 2011). The study found that women with elevated levels of follicular FFAs were more likely to have COCs with poor morphology than others, but that further work was necessary to determine the factors that influence follicular FFA levels and whether those factors impact fertility (Jungheim et al., 2011).

1.6d Cumulus and granulosa cell DNA fragmentation as a predictor of fertility potential

The inherent role of the cumulus complex to ultimately achieve successful fertilization suggests that any form of dysfunction concerning their genetic or structural arrangement around the oocyte may lead to infertility. Whilst several studies (described above) of oocyte, cumulus and granulosa cells have searched for markers of oocyte quality, most of these techniques require expensive laboratory equipment, are complicated and time consuming procedures whose immediate clinical applicability...
is questionable. It is therefore necessary to have additional biomarkers with the capacity to be both objective and predictive of successful fertilization.

Since embryo quality depends on oocyte competence, which in turn depends on mutual interactions between the oocyte and its follicular environment (Balaban and Urman, 2006; Krisher, 2004; Blondin and Sirard, 1995; Mikkelsen et al., 2000), the incidence of cumulus cell DNA fragmentation on the outcome of IVF-ET may be used to establish an evaluation system for oocyte quality. Considering its closer proximity and more intimate associations with the oocyte, DNA fragmentation in cumulus cells may be a better predictor to determine oocyte quality, fertilization rate and embryo development than granulosa cells. Whilst most studies endorse the idea that a negative correlation exists between the level of apoptosis in cumulus cells and oocyte competence (Host et al., 2000; Saito et al., 2000; Saito et al., 2000; Lee et al., 2001; Yuan et al., 2005; Ruvolo et al., 2007), the subject remains controversial.

Several studies suggest an indirect relationship between cumulus DNA fragmentation and fertilizing potential of the corresponding oocyte (Díaz-Fontdevila et al., 2008; Raman et al., 2001; Hakuno et al., 1996). It has been stated that “The increase in cumulus cell apoptosis may produce a fall away of cumulus cells from the oocyte, and would be the signal that the oocyte has attained competence for fertilization” (Hakuno et al., 1996), and “probably this may be one of the mechanisms necessary to leave the surface of the developing embryo free of cumulus cells” (Moffatt et al., 2002). Research on porcine and bovine embryos suggest that the cumulus cells may play a role in easing oxidative stress on spermatozoa during fertilization, thus increasing the rate of apoptosis (Van Soom et al., 2002; Tanghe et al., 2003). Evidence also suggests that the rate of fertilization on day 1 of preimplantation embryo development is higher in oocytes originating from follicles with no or miniscule levels of apoptosis (Lee et al., 2001; Nakahara et al., 1997; Host et al., 2000; Host et al., 2002). A study of bovine cumulus DNA fragmentation using TUNEL also demonstrated the significantly higher level of cumulus cell apoptosis from unfertilized oocytes compared to those fertilized (Li et al., 2009). In contrast, Raman et al., (2001) demonstrated that fertilization rate of oocytes after ICSI was positively correlated with the cumulus cell DNA fragmentation using the Comet assay.

Host et al., (2000, 2002) observed that apoptosis in cumulus cells impaired the fertilization rate of metaphase II oocytes after ICSI. Similarly, Nakahara et al., (1997) observed that oocytes which actually fertilized were derived from follicles with granulosa cells containing the lowest incidence of apoptotic bodies. However, most of their research analysing apoptosis were conducted by detecting apoptotic bodies in cumulus and granulosa cells revealed as cytoplasmic fragments containing condensed chromatin or fragments of condensed chromatin when examined by fluorescence
microscopy. Several studies concluded that cumulus apoptosis had no effect on ICSI outcome (Abu Hassan et al., 2006 Corn et al., 2005; Díaz-Fontdevila et al., 2008). The studies by Corn et al., and Díaz-Fontdevila et al., both used the TUNEL assay to detect DNA fragmentation. Furthermore, studies suggest that whilst human cumulus cells contain a mechanism to undergo apoptosis, exposure to sperm may alter their profile (Moffat et al., 2002; Díaz-Fontdevila et al., 2008). Basing the selection of oocytes for ICSI on cumulus cell DNA fragmentation, to increase the chances of a positive outcome needs further work. Ruvolo et al., (2013) also observed that an apoptosis rate of cumulus cells lower than 25% was associated with higher rates of fertilization and better quality embryos at the cleavage stage.

Some studies found no correlation between cumulus cell apoptosis and embryo quality (Díaz-Fontdevila et al., 2008; Corn et al., 2005; Host et al., 2002). Still, Corn et al., (2005) stated that cumulus cells associated with oocytes that developed into normal blastocysts, a significantly lower rate of apoptosis was detected at day 5 of embryo cleavage. Host et al., (2002) suggest that cumulus complexes undergo a general decay or controlled cell destruction prior to ovum pick up, and that cumulus DNA fragmentation influences embryo development. Assuming the reason to be the transfer of apoptotic signals from the cumulus cells to the oocyte, the timing of gap junction closure between these two cell types and the initiation of apoptosis may have a crucial effect on oocyte and embryo development. Assuming the reason to be the transfer of apoptotic signals from the cumulus cells to the oocyte, the timing of gap junction closure between these two cell types and the initiation of apoptosis may have a crucial effect on oocyte and embryo development. As a result, LH induction of the final maturation phase of the immature oocyte may be compromised by apoptosis (Host et al., 2002). Whilst some studies have observed a correlation between granulosa cell apoptosis and embryo quality (Nakahara et al., 1997), others have found no difference in the incidence of cumulus cells apoptosis according to embryo quality (Lee et al., 2001). Lee et al., (2001) suggest that although oocytes with a high degree of cumulus cell DNA fragmentation may fail to fertilize, cumulus cell apoptosis has no impact on embryo quality and development.

A problem when studying the impact of granulosa and cumulus cell DNA fragmentation on pregnancy outcome is that these cells are usually pooled from all follicles prior to analysis, and is not collected on a per follicle basis. As indicated by a study by Corn et al., (2005) it is possible that there is substantial variance in the level of apoptotic cumulus cells within each follicle of the same patient. As a result, the study would probably be more accurate if the level of apoptosis was only considered in the cumulus cells from follicles from which corresponding embryos or blastocysts had been transferred. Even this may be difficult in the case of singleton pregnancies following multiple embryo transfer as it is not possible for embryologists to determine which embryo in particular implanted.
Several research groups did not observe a statistically significant difference in cumulus and granulosa cell apoptotic levels between pregnant and non-pregnant patients (Diaz-Fontdevila et al., 2009; Nakahara et al., 1997; Lee et al., 2001; Raman et al., 2001). It has been reported that high basal serum FSH levels in women undergoing IVF can increase apoptosis in granulosa cells (Seifer et al., 1996). A study by Oosterhuis et al., (1998) disputed this theory by describing how women with a lower percentage of apoptotic granulosa cells became pregnant than those who did not, despite having normal FSH level. They observed a cut-off level of 13%, where patients with ≤ 13% apoptosis became pregnant, but those with higher levels did not.

Several factors can affect cumulus and granulosa cell apoptosis, and contribute towards the discrepancies observed in different studies. For example, cumulus cell DNA fragmentation was correlated to the maturation stage of the corresponding oocyte where immature oocytes were associated with a higher proportion of apoptotic cumulus cells (Corn et al., 2005; Host et al., 2002; Host et al., 2000). No such relationship was observed by Moffatt et al., (2002), but they evaluated different, immunohistochemical markers to assess cellular apoptosis.

1.7 The assessment of murine embryo DNA fragmentation in response to culture medium containing GM-CSF

1.7a GM-CSF regulation of embryo development

The conceptus and its derivative placental and fetal tissue express paternal genes within the maternal uterus. For a successful pregnancy, semi-allogenic tissues need to co-exist within the implantation site, without immune rejection, in spite of the presence of leukocytes and normal immune competence. Cytokines and chemokines play a fundamental role in the immunological adjustments and tissue remodelling processes required to initiate and continue with a pregnancy. Their intercellular communication signals regulate leukocyte recruitment and proliferation, phenotypic differentiation and function and a suitable immune response to pregnancy. Cytokines also target cells in the pre-implantation embryo and placenta to facilitate tissue restructuring essential for normal fetal development. The hematopoietic cytokine GM-CSF (granulocyte macrophage colony-stimulating factor) is a key regulator of cell survival, proliferation and differentiation, and has a distinct influence on host defence and response to external insult (Gasson, 1991). Due to its presence in the in vivo environment, embryotrophic nature and ability to protect embryos from the detrimental effects of embryo culture, GM-CSF is being used as an additive to complement human IVF culture media.
The secretion of GM-CSF into the fallopian tube immediately after conception is thought to control growth of the pre-implantation embryo. As with leukocytes, cells of the developing embryo also act as targets for GM-CSF. Cells of the outer trophoderm layer and the inner cell mass both express GM-CSF, and receptor proteins have been found on trophoderm cell membranes (Robertson et al., 2001; Sjoblom et al., 2002). Whilst embryos can develop in in vitro culture medium lacking exogenous growth factors, increasing evidence suggests the need for cytokines, autocrine and paracrine growth factors in the regulation of blastomere development (Kaye, 1997; Hardy and Spanos, 2002). The concept behind commercially developed culture media is to meet with the changing metabolic needs of the developing embryo, and in theory, reduce environmental stress (Desai et al., 2007). Embryos cultivated under suboptimal conditions and devoid of growth factors may be particularly susceptible to in-vitro stress (Brison & Schultz, 1997).

In the human reproductive tract, the synthesis of GM-CSF is thought to be highest during the period related to conception and implantation (Giacomini et al., 1995; Zhao and Chegini, 1999). GM-CSF concentrations of up to 4ng/ml were observed in cultures of human endometria (Giacomini et al., 1995). The effect of GM-CSF has been evaluated at different concentrations doses (0.0625–16ng/ml) in different types of culture media and mouse strains (Behr et al., 2005; Karagenc et al., 2005; Robertson et al., 2001). Robertson et al., (2001) found that the maximum effect of GM-CSF on murine embryos was observed at 2ng/ml, and is equivalent to the concentration measured in uterine fluid during the preimplantation phase in mice (Robertson et al., 2001).

A study in our laboratory by Elaimi et al., (2012) investigated whether the addition of GM-CSF to culture media affected blastulation and the chromosomal integrity of murine embryos. Culture media supplemented with five different concentrations of GM-CSF was used to culture 2-cell stage murine embryos in vitro until they developed into blastocysts. The development of each embryo was recorded before chromosomal integrity was assessed for chromosomes 2, 11 and 16 using FISH. The study found that the addition of 1 and 2ng/ml GM-CSF to the culture medium had no effect on blastulation potential. With the addition of 5 and 10ng/ml, however, blastulation rate decreased significantly. The levels of aneuploidy detected in embryos cultured in medium supplemented with GM-CSF were slightly higher than those exhibited by embryos in the control group (0ng/ml GM-CSF). These increases were not statistically significant. When the mosaic embryos were further analysed, the percentage of aneuploid cells in embryos from the GM-CSF cultured group had a significantly higher level of aneuploid cells than embryos cultured in the absence of GM-CSF. The authors concluded that blastocyst development was negatively affected by higher concentrations of GM-CSF, and increased the rate of aneuploid cells within mosaic embryos.
The effect of supplementing in vitro culture medium with 2ng/ml GM-CSF on human oocyte ploidy rate, from fertilization until day 3, was assessed by Agerholm et al., (2010) in a study involving 73 women and 86 donated oocytes. Since previous studies on human embryos were performed on frozen-thawed 2- and 4-cell embryos, this was the first study in which GM-CSF has existed in the culture medium from the time of fertilization (day 0). They investigated the effect of GM-CSF on the chromosomal constitution of human embryos using FISH for chromosomes 13, 16, 18, 21, 22, X and Y, and the numbers of top quality embryos (TQE) and normally developed embryos evaluated morphologically on day 3 (Agerholm et al., 2010). They reported that no difference existed between the chromosomal constitution of human embryos cultured in medium containing 2ng/ml GM-CSF and those cultured in vitro without the supplementation of GM-CSF. Embryos cultured in vitro in the presence of GM-CSF produced 8/23 uniformly normal embryos (34.8%), whilst those cultured without GM-CSF produced 9/27 (33.3%) uniformly normal embryos. Due to a lack of TQE in the control group, the observed inclination towards TQE in the GM-CSF-cultured embryos was thought a chance observation. To reiterate, they did not observe any significant differences in the chromosomal constitution, fertilization rate, early cleavage rate or number of normally developed embryos when cultured with or without the addition of GM-CSF.

The evaluation of post-fertilization mosaicism may not be the most suitable methods for detecting abnormalities promoted by culture media, although several studies have used this approach when addressing the safety of new culture media supplements (Agerholm et al., 2010; Bergh et al., 2004; Loft et al., 2004, 2005; Ziebe et al., 2003). Culturing embryos to the blastocyst stage before analysing the inner cell mass and trophectoderm cells for ploidy rates may be a better form of evaluating the safety of such new additions to culture media (Agerholm et al., 2010).

The amount of GM-CSF secreted into culture medium containing human IVF embryos co-cultured with autologous endometrial cells was found to correlate with successful pregnancy following embryo transfer (de Moraes & Hansen, 1997). In human IVF where it is particularly difficult to achieve high rates of embryo development in vitro, these outcomes of GM-CSF are all the more profound (de Moraes & Hansen, 1997). GM-CSF action is not limited to just short-term embryo survival, but also long-term developmental competence. Adding GM-CSF to embryo culture before transfer was found to enhance embryo implantation, correct defects in placental structure and fetal growth curve, improve the long-term effects of embryo culture on postnatal growth (Sjoblom et al., 2005). These observations recognize the embryotrophic properties of GM-CSF and its importance in warranting optimal preimplantation and fetal development. The effect of GM-CSF in embryo culture medium on on-going implantation rate (OIR) was recently evaluated (Ziebe et al., 2013). Oocytes
from 1,149 women were fertilized, and the subsequent embryos were cultured and transferred to either control medium or test medium supplemented with 2ng/ml GM-CSF. They found that the addition of GM-CSF to embryo culture medium promotes the survival of transferred embryos to week 12 and live birth. Their results were compatible with the apparent protective effect of GM-CSF on culture-induced embryo stress. The authors also believed that GM-CSF may be particularly effective in women with previous miscarriage.

Its influence on increased cell accumulation suggests that GM-CSF is involved in the rates of cell division, differentiation and cell death that occur as an embryo develops from a zygote to a blastocyst. These events are susceptible to disruptions by environmental stresses (Xie et al., 2008) that include suboptimal culture media (Wang et al., 2005), growth factor and cytokine deficiency (O’Neill, 2008), metabolic and substrate deficiency (Leese et al., 1998; Leese, 2002) and oxidative stress (Nasr-Esfahani and Johnson, 1991). The stresses these factors exert on developing embryos can lead to an elevation in apoptosis (Xie et al., 2006, 2007) and hinder cell cycle progression resulting in fewer blastomeres. Experiments demonstrating the increased uptake of glucose in murine blastocysts cultured with GM-CSF support the theory that GM-CSF stimulates embryo metabolism (Robertson et al., 2001). Its role in the protection of murine and human blastomeres from apoptosis, particularly in the inner cell mass, has been established by TUNEL staining (Sjoblom et al., 2002; Behr et al., 2005; Desai et al., 2007).

Robertson et al., (2001) studied the effect of GM-CSF on glucose uptake and blastomere viability in murine preimplantation embryos from GM-CSF-null mutants (GM-/-) and GM+/+ embryos. In vitro culture of eight-cell embryos in recombinant GM-CSF accelerated blastocyst development to the hatching and implantation stages. A maximum response was observed at a concentration of 2ng/ml. Blastocysts derived from GM-/- mice on Day 4 showed retarded development compared with GM+/+ embryos. Blastocysts produced in vitro from two-cell GM-/- and GM+/+ embryos were larger when GM-CSF was added to the culture medium. Mouse embryos flushed from the reproductive tract and cultured in vitro in the presence of GM-CSF until the blastocyst stage developed faster, reached a higher proportion of viable blastomeres and implanted more frequently in vitro (Robertson et al., 2001; Robertson et al., 1991). GM-CSF also caused a 50% increase in embryo glucose uptake. However, the ‘quiet embryo hypothesis’ (Leese, 2002; Leese, 2012) states that ‘preimplantation embryo survival is best served by a relatively low level of metabolism; a situation achieved by reducing the concentrations of nutrients in culture media and encouraging the use endogenous resources’ (Leese, 2002). Furthermore, it is thought that blastocysts with poor quality have high
glycolytic activity (Gardner and Lane, 1996). On this basis, an increased uptake of glucose is not ideal for optimal embryo development.

Blastocyst development and differentiation were monitored in frozen-thawed one-cell mouse embryos cultured in medium containing GM-CSF (Desai et al., 2007). The total blastomere number and apoptosis were examined using TUNEL. Whilst ICM differentiation in thawed embryos was clearly improved with the supplementation of GM-CSF, no differences were observed in blastulation and hatching rates. GM-CSF was found to enhance continued cell survival and prevented apoptosis, but had no effect on the overall cell number in developing blastocysts.

### 1.7b GM-CSF and embryos apoptosis

An adaptive response to culture stresses, apoptosis is thought to remove damaged cells in embryos (Brison and Schultz, 1997; Hardy, 1999). The embryotrophic and programming influence of GM-CSF on embryos is thought to be governed by processes that protect the embryos from cellular stress. Apoptosis regulators include suppressor, inducers and executor molecule families that are represented by the Bcl-2, Bax and Trp53 genes respectively. Whilst the TRP53 protein induces cell cycle arrest along with apoptosis in cells containing DNA fragmentation, cell survival primarily depends on a balance between the BCL2 and BAX proteins (Agarwal et al., 1998). Stress induced by culture medium has been shown to activate Bcl-2 and Trp53 gene expression in human (Wells et al., 2005) and mouse embryos (Jurisicova et al., 1998; Li et al., 2007), inhibiting apoptosis. However, when the effect of GM-CSF on blastocyst expression of stress response and apoptosis genes was examined by microarray, qPCR and immunochemistry (Chin et al., 2009), varying results were observed. Chin et al., (2009) found that in spite of mRNA levels remaining constant, the apoptotic inhibitor BCL2 was barely detectable in embryos cultured in medium containing GM-CSF, thus possibly promoting apoptosis. The effect of GM-CSF on the pro-apoptotic factor BAX was quite different. Whilst Bax mRNA expression was inhibited by GM-CSF (preventing apoptosis), its protein levels remained unaffected (Chin et al., 2009). The authors concluded that the cellular stress response and apoptosis pathways were suppressed by GM-CSF to enable embryo growth and survival. Recombinant mouse GM-CSF inhibited expression of heat shock protein (HSP) and apoptosis pathway genes Cbl, Hspa5, Hsp90aa1, Hsp90ab1 and Gas5 in in vitro blastocysts (Chin et al., 2009). They also observed that the protective effects of GM-CSF were particularly apparent in embryos cultured in vitro, whereas other cytokines partly compensated for the absence of GM-CSF blastocysts developed in vivo (Chin et al., 2009).
To investigate the effects of GM-CSF on the development of preimplantation mouse embryos, 2-cell murine embryos were collected and cultured in medium supplemented with GM-CSF at different concentrations (Behr et al., 2005). Reverse transcription-polymerase chain reaction was used to assess the expression of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) mRNA in blastocysts in the GM-CSF group and control group. Blastocyst development was significantly higher in the group cultured in medium supplemented with GM-CSF (0.125ng/ml) than the control group. Bcl-2 expression was also upregulated in the GM-CSF supplemented group of embryos, inhibiting apoptosis, suggesting that GM-CSF might be an important regulator in embryo development.

Using reverse transcription-polymerase chain reaction and immunocytochemistry, Sjoblom et al., (2002) also demonstrated with in vitro experiments that blastulation rate in human embryos increased approximately twofold when GM-CSF was added to the culture medium. When embryos were analysed for apoptosis and cell number using TUNEL and propidium iodide staining, they observed that blastocysts cultured in GM-CSF contained 30% more viable inner cell mass cells and 50% fewer apoptotic nuclei. On average, these embryos were found to reach the blastocyst stage 14 hours faster, and the rate of embryos that develop to the blastocyst stage doubled (Sjoblom et al., 1999), along with improvements in the developmental competence of these blastocysts (assessed by the disintegration of the zona pellucida, and adherence to the extra-cellular matrix culture dishes).

Studies involving surplus human material demonstrated how the addition of GM-CSF to culture media promotes blastocyst development in vitro (Kawamura et al., 2008; Kawamura et al., 2009). A study by Kawamura et al., (2012) also confirmed the benefit of specific autocrine/paracrine growth factors to normally fertilized day 3 embryos, which increased the rate of blastocyst formation along with blastocyst quality. A 3.3-fold increase was observed in blastocyst formation rate and a 7.6-fold increase in high quality blastocysts compared to controls. These observations validate the theory that culturing embryos results in the dilution of autocrine/paracrine factors secreted by early embryos, thus requiring compensation by the addition of growth factors to ensure optimal blastocyst formation.

Researchers have attempted to optimize in vitro culture media by adjusting its formulations in an attempt to more closely imitate the female reproductive environment. Since it is apparent that stress induced by sub-optimal culture media can have an adverse effect on the developing embryo, fetal development and long-term health, the aforementioned attributes of GM-CSF may have significant implications on human IVF (Thompson et al., 2002).
The repeated observations that GM-CSF safeguards embryos from culture-induced stress have driven its application in human IVF culture media, especially in cases where embryo freezing and thawing that can elevate stress (Sjoblom et al., 1999; Papayannis et al., 2007). In spite of all these indications, a rather important concern is whether DNA fragmentation associated with apoptosis plays a physiological role in the specific removal of abnormal blastomeres (Hardy, 1999). However, human embryos cultured in media with GM-CSF were observed to have increased levels of aneuploidy (Elaimi et al., 2012; Agerholm et al., 2010), suggesting that the protective effects of this cytokine do not necessarily ‘rescue’ abnormal embryos (Chin et al., 2009). Although recent advancements in sequential culture media has promoted the selection of high quality blastocysts for a successful pregnancy following IVF (van Loendersloot et al., 2010; Spanos et al., 2000; Menezo et al., 1992), the efficacy of human blastocyst development in vitro has room for improvement. The possible side effects of supplementing embryo culture medium with endogenous growth factors on chromosomal and genetic integrity, proteomic changes and epigenetic modifications require thorough investigation before routine clinical use.

**1.7c Embryoscope™ and EmbryoSlide® Culture Dish**

Substantial work has been carried out on embryo culture conditions and embryo quality evaluation as a part of the strategies available to improve IVF cycle success rates (Freour et al., 2012). Conventional incubators are used in most IVF centres to select embryos based on brief “snap-shot” glimpses at defined time points, in order to minimise disturbances to culture conditions. However, since embryo development is a dynamic process, information regarding embryo history is lost to avoid potential damage to embryo quality that may be caused by the removal of embryos from a controlled temperature and gas environment.

Commercial devices employing more stable culture conditions and time lapse observation of embryo development have recently been developed in an effort to provide new insights into early embryo development following IVF. Amongst them, the Embryoscope™ is claimed to be the most user-friendly, and suited for routine daily practice, allowing better embryo selection according to kinetic parameters and observation of abnormal cleavage events, and continuing education and training, quality control and flexibility (Freour et al., 2012). The Embryoscope™ Time-lapse Imaging Monitoring System is being marketed as a revolutionary new system for embryo monitoring. It is said to provide a ‘safe, controlled culture environment, while allowing continuous monitoring and observation of up to 72 embryos at a time’ (Unisense® Fertilitech, Denmark). Its application may
provide new parameters for future embryo scoring and assist embryologists to identify viable embryos for transfer, increasing the success rate of IVF.

The EmbryoSlide® Culture Dish comprises a ‘tray’ that can hold 12 embryos which ‘automatically sink to the bottom of the well. Conical sides of the well automatically place the embryo in a central depression with a diameter of 0.2mm for direct thermal contact with a heated tray holder’ (Unisense® Fertilitech, Denmark). Micro-numerals next to the bottom of each well, and that are visible under a dissection microscope during embryo handling, allows for the unique identification of each embryo. The EmbryoSlide® Culture Dish has a standard slide format (25 x 75mm) and is therefore compatible with standard and inverted microscopes. It also apparently allows the testing of spent media for subsequent analysis of proteome or secretome. A safe, non-humidified condition is provided by the polymer slide impermeable to water and cover of immersion oil which prevent dehydration during handling processes that take place in low humidity laboratory air and dry incubators (Unisense® Fertilitech, Denmark).

Due to its presence in the in vivo environment, embryotrophic nature and ability to protect embryos from the detrimental effects of embryo culture, GM-CSF is being used as an additive to complement human IVF culture media. It is currently being used to supplement the culture medium EmbryoGen® (Origio, Denmark) at a concentration of 2ng/ml, and is being marketed as an ‘advanced cleavage-stage medium containing GM-CSF growth factor for patients with previous miscarriages’ (Origio, Denmark).

1.7d Apoptosis in preimplantation embryos

As with all mammalian somatic cells, blastomeres of mammalian preimplantation embryos are capable of undergoing ‘programmed cell death’, or apoptosis. It has been proposed to describe the elimination of single cells at a specific location and time, and is vital for embryonic development and homeostasis (Hardy et al., 1989; Brison & Schultz, 1997; Kamjoo et al., 2002). The characteristics of apoptosis are the culmination of a complex cascade of biochemical events. Morphologically the cell shrinks, and displays cytoplasmic and chromatin condensation and fragmentation. Biochemically, vital proteins are degraded and nuclear DNA is cleaved into oligonucleosomal sized fragments at the later stages (reviewed in Fabian et al., 2005).

The frequency of apoptotic processes during the blastocyst stage can result in either beneficial or detrimental effects, and is a crucial mechanism controlling the health of the future offspring. By removing genetically abnormal cells, it provides a protective function. However, if the rate of
apoptosis is elevated, the occurrence of massive cell death can lead to embryo arrest or death. The precise threshold frequency for apoptosis to be detrimental has not yet been established. The preimplantation embryo also does not experience morphogenetic changes that require the elimination of a large number of cells. It is therefore likely that apoptosis is involved in the selective removal of cells that carry inappropriate phenotypes or are damaged in some form, particularly because preimplantation development stimulates numerous important developmental transitions that requires the regulation of cell quality control.

The examination of embryo development following fertilization with X-ray irradiation-induced DNA damage suggested that apoptosis may play a role in the elimination of cells with DNA damage from the embryo, probably via a p53-mediated pathway (Haines et al., 1997). TUNEL analysis demonstrated a high level of labelling, indicative of an apoptotic response. Four days after oocytes were fertilized with DNA damage-induced sperm, a majority of the embryos recovered showed severe developmental damage, and most of the embryonic material recovered was from pre-blastocyst stages. A very small proportion of these experimental embryos reached the blastocyst stage compared to the controls. This study concluded that DNA damage introduced by sperm can have severe implications for the embryonic developmental processes and thus, in an attempt to eradicate cells with damaged DNA, generate apoptotic responses in the embryo (Haines et al., 1997). Naturally, this has serious implications for the genetic integrity of offspring produced through IVF, and particularly ICSI due to the lack of sperm selection, especially since the proportion of DNA-damaged sperm found in infertile men is relatively high (Sun et al., 1997).

It has been observed that the first wave of apoptosis across a range of species roughly coincides with the period of embryonic genome activation (Jurisicova et al., 1998). It is possible that apoptosis gets rid of the cells that fail to competently activate the embryonic genome, probably as a response to a severely compromised paternal genome. This action may ensure the survival of just the embryos with a competent genome beyond early cleavage.

In humans, the initial phase of apoptosis occurs during formation of the blastocyst, but decreases as the blastocyst expands and the number of cells increase (Hardy, 1997). Ordinarily confined to the ICM, apoptosis may be responsible for the plateau in the number of ICM cells by the late blastocyst stage, in the absence of a decline in mitotic activity, thus restricting the number of cells. This physiological process has also been associated with the elimination of ICM cells that retain the ability to develop into the TE, hence preventing any ectopic development of this tissue. Whilst the mechanism of selective cell death has not yet been established, anti-oxidant protective mechanisms
and oxidative stress have been proposed to play a role (Parchment, 1993; Pampfer and Donnay, 1999). However, there is no direct evidence to support these theories.

Apoptotic processes, and DNA fragmentation, are not observed prior to embryo genome activation but can be chemically induced. Peaking during the blastocyst stage, the frequency of apoptosis can be influenced by many internal and external factors. Possible causes for apoptosis in the pre-implantation embryo include inappropriate developmental protection (Handyside & Hunter, 1986), an imbalance in ‘survival’ factors (Fabian et al., 2004; Byrne et al., 2002; Makarevich & Markkula, 2002) and hormones (Hardy & Spanos, 2002), and the exposure to damaging agents (Yang et al., 1998; Herrler et al., 1998; Paula-Lopes and Hansen, 2002). The occurrence of apoptosis in embryos cultured in vitro can also be influenced by culture conditions, such as culture medium composition (Levy, 2001) and the culture density of embryos (O’Neill, 1998). Growth factors have been suggested as modulators of apoptosis, as they have a significant influence on embryonic metabolism, blastocyst development and differentiation (reviewed in Hardy & Spanos, 2002, Huppertz & Herrler, 2005).

1.8 Effect of pre-tested toxins on human embryonic stem cell DNA fragmentation and aneuploidy

1.8a Human embryonic stem cells as a model to study apoptosis in the preimplantation embryo

The search for a novel, more physiological model that closely mimics normal development of the pre-implantation embryo is still in its early stages. It is expected that hESC-based systems may potentially be the most suited model to investigate certain facets of this process. In anticipation of the ability to manipulate hESCs to produce suitable models to screen compounds potentially toxic to the pre-implantation embryo, hESCs may also help ascertain the optimal molecular composition of the embryo microenvironment. This information may potentially assist us to further understand certain aspects of infertility.

Embryonic stem cells are transiently found during embryo development, in preimplantation embryos and fetal gonads, they can be maintained as established cell lines (Eiges and Benvenisty, 2002). Under suitable conditions, they can be kept in culture in their pluripotent state indefinitely, where one embryonic stem cell line represents a potentially unlimited source of cells. Human ESCs are representative of a special type of pluripotent stem cells that are derived from the inner cell mass of blastocyst stage embryos. They are thought to have the greatest developmental potential with the
ability to differentiate into a wide range of cell types (Thomson et al., 1998; Reubinoff et al., 2000), both in vivo and in vitro.

1.8b Aneuploidy in preimplantation embryos

Aneuploidies have been observed in women of different ages, in arrested and developing embryos, fresh and frozen-thawed embryos, in fragmented embryos and those of good morphology (Fragouli and Wells, 2012; Fragouli and Wells, 2011; Fragouli et al., 2011; Baart et al., 2006; Bielanska et al., 2002; Daphnis et al., 2008; Delhanty et al., 1997; Iwarsson et al., 1999; Munne et al., 1994; Ruangvutilert et al., 2000; Santos et al., 2010). The occurrence of mitotic aneuploidies in human preimplantation embryos has been deduced from the frequency of mosaic embryos (reviewed in Mantikou et al., 2012). The reported frequency of mosaic embryos in the literature is varied, ranging from 15% to 90% (Bielanska et al., 2002; Daphnis et al., 2005; Harper et al., 1995]. A recent systematic review and meta-analysis of studies on the chromosomal constitution of preimplantation embryos revealed that 73% of all human preimplantation embryos after IVF are mosaic, 22% are diploid and 5% contain other abnormalities (van Echten-Arends et al., 2011). Mitotic aneuploidies were observed at all developmental stages of preimplantation embryo development, where the first 3 mitotic divisions were the most error-prone (Katz-Jaffe et al., 2004; Katz-Jaffe et al., 2005).

The most common mechanisms leading to mitotic aneuploidy in human embryos include anaphase lagging and non-disjunction (Daphnis et al., 2008; Delhanty et al., 1997; Daphnis et al., 2005; Katz-Jaffe et al., 2005, Coonen et al., 2004). Other mechanisms include chromosome demolition, premature cell division, errors in cytokines, cell fusion and chromosome breakage (reviewed in Mantikou et al., 2012). Preimplantation mitotic aneuploidy may result from several interrelated factors of maternal and/or paternal origin, and the effect of in vitro factors.

Activation of the human genome occurs at the 4- to 8-cell stage, and most proteins controlling chromosome segregation are provided by the oocyte (Braude et al., 1988). Defective maternal mRNA and protein production can result in failure of the mechanisms that regulate cell division. These mechanisms involve microtubule kinetics, cell cycle checkpoints, DNA repair proteins, chromosome cohesion, telomere shortening and mitochondrial function (Baumann et al., 2010; Jaroudi et al., 2009; Li et al., 2010; Liu et al., 2002; Treff et al., 2011; Wells et al., 2005; Wilding et al., 2003; Zheng and Dean, 2009). Free radicals that accumulate within the oocyte through the years until fertilization, exposure to external environmental factors like radiation, and poor vascularization of the antral follicle during oocyte maturation may affect the oocyte protein pool (reviewed in Mantikou et al., 2012).
Paternally inherited, the organizing centre of the mitotic spindle, the centrosome consists of two centrioles from which the spindle microtubules are inherited (Sathananthan et al., 1991). An abnormal number of centrioles can lead to defective spindle formation and chromosomal mal-segregation. Dispermic embryos were found to have a higher frequency of mosaicism originating from abnormal spindle organization, compared to monospermic or digynic embryos (Palermo et al., 1994). Severe sperm defects may also cause aneuploidy in embryos. Patients with non-obstructive azoospermia (NOA) undergoing testicular sperm extraction (TESE) were found to generate embryos with an increased rate of mosaicism compared to embryos derived from ejaculated sperm (Silber et al., 2003). Similar observations strengthen the hypothesis that organization of the first mitotic spindle in testicular spermatozoa may be difficult (Magli et al., 2009).

The high percentage of mitotic errors observed in preimplantation embryos may be different from those conceived in vivo, and possibly influenced by IVF procedures. Different rates of mosaicism have been detected amongst embryos obtained cultured under different protocols from different IVF clinics (Munne et al., 1997). Temperature fluctuations, oxygen concentration, culture medium and hormonal stimulation protocols may affect spindle assembly and chromosomal segregation. Ovarian stimulation was found to influence embryo quality and chromosomal integrity, including post-zygotic errors (Katz-Jaffe et al., 2005; Baart et al., 2007). A higher daily dose of FSH was linked to an increase in mitotic divisions of chromosome 21 in mosaic embryos (Katz-Jaffe et al., 2005). Chromosomal abnormalities have also been observed in embryos from un-stimulated cycles in young women with an average of 31 years (Verpoest et al., 2008). The observed increase in non-disjunction events in embryos in the presence of increased oxygen concentration suggests that subtle changes in the IVF environment can seriously affect chromosomal segregation (Bean et al., 2002). In spite of these observations in the IVF setting, the high frequency of mosaicism detected in early human abortions reflects the high incidence of mosaicism in in vivo conceptuses (Lebedev et al., 2004; Vorsanova et al., 2005).

Unlike the higher frequency of aneuploidy observed in early preimplantation embryos, lower numbers of blastomeres with mitotic aneuploidies are observed in the later stages of human preimplantation development, subsequent pregnancies and live births (van Echten-Arends et al., 2011). This suggests a natural selection against aneuploid blastomeres and embryos. This is reflected in the relative decrease in the number of aneuploid blastomeres from the cleavage to the blastocyst and implantation stages (van Echten-Arends et al., 2011). The main underlying mechanisms are thought to be cell cycle arrest or apoptosis of aneuploid blastomere and/or embryos, active correction via anaphase lag, non-disjunction or chromosome demolition and the preferential
allocation of diploid/aneuploid blastomeres to embryonic or extra-embryonic tissues (reviewed in Mantikou et al., 2012).

The methodology used to analyse aneuploidy in human preimplantation embryos contributes to the variation in the reports of aneuploid frequencies (van Echten-Arends et al., 2011). Karyotype analysis was used in the original studies of human aneuploidy (Clouston et al., 1997; Jamieson et al., 1994; Pellestor et al., 1994). Allowing the analysis of all chromosomes, this technique requires dividing, metaphase-stage cells. As arrested cells do not produce metaphases, karyotyping can only be used on developing cells and embryos. It is also difficult to produce optimal chromosomal banding, making individual chromosomes hard to identify, chromosomes may also be lost during fixing of the nuclei (Clouston et al., 1997; Pellestor et al., 1994). Karyotyping is no longer used in the analysis of chromosomal aneuploidies in human preimplantation embryos.

A limitation of FISH is the number of probes that can be used simultaneously, as only a few spectrally distinct fluorochrome probes are available. Multiple sequential FISH rounds can be used, although hybridization efficiency declines with each round (Liu et al., 1998). Another limitation of FISH is the possible chromosomal loss and scoring errors that arise from split signal, overlapping signals, damage to the nucleus, loss of micronuclei, hybridization failure and probe inefficiency (Ruangvutilert et al., 2000).

The introduction of array CGH (aCGH) and single-nucleotide polymorphism-based (SNP) microarrays (Vanneste et al., 2009; Vouillaire et al., 2000; Wells and Delhanty, 2000; Handyside et al., 2004; Le Caignec et al., 2006; Iwamoto et al., 2007) has allowed the examination of the copy number of all 24 chromosomes. With aCGH, DNA from an individual cell is extracted and the entire genome is amplified (whole-genome amplification). This ‘test’ DNA and chromosomally normal ‘reference’ DNA are differentially labelled with fluorochromes and hybridized to an array. The fluorescence ratios of the ‘test’ and ‘reference’ signals are scored to determine any additional or missing chromosomes. Array CGH uses hybridization to microarray chips covered by thousands of probes that encompass the entire genome and reveals changes in chromosome number. The analytical output of microarray chips provides data within 24 hours, allowing the screening and the timely transfer of embryos. Similar to aCGH, SNP-based microarrays contain probes to detect SNPs. This test is therefore not limited to detecting numerical aberrations, but can also provide data on crossovers and determine parental origin of aneuploidy (Handyside, 2011). SNP-based arrays are capable of testing of specific genetic diseases and aneuploidy in each embryo simultaneously (reviewed by Harper and Harton, 2010). This allows the selective transfer of genetically and chromosomally normal embryos for patients undergoing IVF with PGD for monogenic diseases. Embryos that have not inherited the
affected chromosome can be selected by haplotyping the SNPs surrounding and set in the disease-causing gene (reviewed by Harper and Harton, 2010). Furthermore, the haplotyping of various markers throughout the genome in live-born children will help determine which embryo(s) implanted in any given ART cycle (reviewed by Harper and Harton, 2010). These techniques can be used on cells at any phase of the cell cycle. An obstacle when using genome-wide, single-cell analysis is the possible failure to amplify one of the parent alleles or over-amplifying specific regions, thus introducing bias to the results (Handyside et al., 2004; Iwamoto et al., 2007; Piyamongkol et al., 2003; Renwick et al., 2006). Recent advances in the use of array technology may provide new insights into the occurrence of preimplantation aneuploidy.

1.8c Human embryonic stem cells as a model for embryo toxicity assays

It is now possible to use undifferentiated hESCs, thus filling the gap in the analysis of the influence of environmental factors, drugs and xenobiotics may have on human cell lines and cell lines that have undergone terminal differentiation (reviewed by Rohwedel et al., 2001). Assessing the reaction of specific agents on hESCs will help establish their toxicology profiles at the earliest stages of development and generate information that may be vital for comprehending both short and long term effects of exposure.

The dominant-lethal assay (Russell and Russell, 1954; Ehling et al., 1978) and the locus-specific test (Ehling, 1978, 1981; Russell et al., 1981) were applied to identify germline mutations induced by mutagenic compounds in vivo, but they are expensive, labour intensive and time consuming. The reproductive toxicity and teratogenecity studies performed in vivo involving multigenerational studies brought forth similar issues (Rutledge, 1997). In an attempt to reduce the number of experimental animals and develop systems that more likely represent human development, in vitro alternative such as whole embryo cultures and cellular models employing primary cultures and permanent cell lines were developed (reviewed in Schwetz et al., 1991; Brown et al., 1995; Spielmann, 1998). Unfortunately, cell lines derived from specialized somatic cells fail to mimic embryonic properties and prevent the analysis of early embryonic development. Thus, undifferentiated pluripotent cells, capable of developing into endodermal, ectodermal and mesodermal lineages, were introduced. These cell lines, which represent early embryonic stages, are now used to analyse mutagenic, cytotoxic and embryotoxic effects of chemical compounds in vitro.
1.8d The Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS)

The Embryonic Stem cell-based Novel Alternative Testing Strategies (ESNATS) aims to develop a novel toxicity platform that uses hESCs to develop in vitro test systems for developmental and acute toxicity. The pluripotent nature of ESCs circumvents problem of interspecies differences and since these cells are a unique tissue culture model for developmental processes, they can also be used to study developmental toxicity.

In an attempt to enhance drug development, decrease R and D related costs and put forth a strong proxy to animal tests, ESNATS intends to design a battery of toxicity tests using hESCS lines subjected to standardised culture and differentiation protocols. The toxicity tests will include reproductive toxicity, neurotoxicity, metabolism and toxicokinetics before being incorporated into an ‘all-in-one’ test system. Once the required hESC lines are cultured and automated, they can be scaled-up to allow for their potential industrial use of the developed toxicity assays. ESNATS results aim to increase patient safety by being more predictive than animal tests, thus reducing, refining and replacing (3Rs principle) animal tests, decrease the cost of drug testing and allowing rapid to medium high throughput testing substances. Adult stem cells can have distinct properties depending on their source, so in its attempt to develop an ‘all in one’ in vitro test system, ESNATS employed a single cell type that has the potential to generate all cell types of the body, ES Cs. Whilst mouse ESCs are similar to human ESCs developmentally, physiological differences between the two cell types deem the mouse an unsuitable model for toxicological assays. These discrepancies may lead to a substance being tested negative in the mouse system whereas it is significantly toxic to humans, giving rise to a potentially dangerous situation and so the ESNATS project intends to use hESCs for its research. In the development of test systems, it is important to use well characterised reference substances that have known modes of action. The ESNATS test system has established an initial list of reference substances with the aid of industrial partners. This study will use 5-azacytidine, cadmium chloride, cisplatin and sodium arsenite, at four concentrations each.

A DNA demethylating agent, 5-azacytidine is widely used as a chemotherapeutic drug to reverse the irregular promoter hypermethylation, and therefore the silencing, of tumour suppressor genes (TSG) thought to be involved in carcinogenesis (Esteller, 2005; Stresemann and Lyko, 2008). Although it has not been established whether DNA methyltransferase (DNMT) inhibitors induce cell maturation versus apoptosis, the latter is considered the typical mechanisms for 5-azacytidine induced cell death (Vicencio, 2008).
The heavy metal cadmium is found in environmental and industrial processes and is absorbed from cigarette smoke. The incorporation of cadmium into the chromatin of gametes is thought to contribute towards ovulation failure, and inhibition of trophoblastic invasion and development can result in implantation delay and early pregnancy loss. Exposure to higher dosages can retard progression to the blastocyst stage with apoptosis and breakdown in cell adhesion. The promoter activity of cadmium can induce oxidative stress (Pearson and Prozialech, 2001) and increase the levels of hydrogen peroxide, which produces free radicals capable of breaking or cross-linking DNA or trigger lipid peroxidation. Generally, the mechanisms by which apoptosis is provoked by cadmium is determined by the dosage (Pulido and Parrish, 2003) and the type of cell exposed (Watjen et al., 2002).

Cisplatin is a platinum-based chemotherapy drug used to treat many types of cancers. These platinum complexes react in vivo, binding to and causing the crosslinking of DNA, ultimately triggering apoptosis (Stordal and Davey, 2007).

Low doses of sodium arsenite led to a lack of compaction due to the dissociation of adhesive cells in the morula (Riethmacher et al., 1995), which can then lead to aberrant embryo development (Houghton et al., 2002). In a study on the effect of sodium arsenite on preimplantation embryos, whilst the control group had fewer fragmented DNA, the group treated with sodium arsenite resulted in the death of nearly every blastomere suggesting that arsenic-induced toxicity to preimplantation stage embryos is partly due to DNA damage, although it is difficult to determine whether it is due to apoptosis or necrosis.

The aim of this study is to examine if hESCs exposed to pre-tested toxins exhibit signs of DNA damage and aneuploidy and if a correlation exists between these two parameters. DNA damage was detected using TUNEL, and chromosome aneuploidy using FISH. Numerical aberrations in chromosomes 13, 18 and 21 are thought to lead to viable yet affected pregnancies, whilst errors involving chromosomes 15, 6 and 22 tend to cause implantation failure. Human ESCs were provided by the MRC Centre for Regenerative Medicine, University of Edinburgh, in collaboration with their ESNATS study on toxicity assays. The application of totipotent or pluripotent cell lines may revolutionize our understanding of pre-implantation development at a molecular level, thus leading to the recognition of specific markers that can be used to screen likely toxic compounds.
1.9 Aims

Overall, the aims of this thesis were to examine the variability of DNA fragmentation, aneuploidy and phosphatidylserine translocation at different stages of gametogenesis and preimplantation embryo development, and the factors that affect these potential biomarkers.

1.9a The assessment of DNA fragmentation and aneuploidy in spermatozoa

It is known that abnormal semen parameters and aneuploidy can result in unfavourable ART outcomes. It has been suggested that ‘normal’ semen samples can hold significant DNA damage and those with apparently poor sperm quality can carry minimal DNA fragmentation. Whilst it is suspected that DNA fragmentation may also result in ART failure, it has not been confirmed. It is also unknown if a correlation really does exist between sperm DNA fragmentation and aneuploidy. Over the last 20 years, a number of DNA fragmentation assays have been introduced for both clinical and research purposes. These tests include Comet, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL), SCSA and SCD. Sperm DNA fragmentation testing has been proposed to stratify patients based on the degree of damage, and whilst cut-off levels have been recommended to predict risk, it is unknown whether these values are applicable to the different types of tests available. It has been claimed that the SCD-FISH tests can be used to measure the degree of DNA fragmentation and aneuploidy in the same sperm cell, but this has not been validated. In order to address these issues, I tested the hypotheses that:

- A correlation exists between DNA fragmentation and conventional sperm parameters.
- Any potential correlation that exists between conventional sperm parameters and DNA fragmentation is the same when using the different DNA fragmentation tests.
- Sperm DNA fragmentation and aneuploidy results differ by analysis technique.
  - Currently available DNA fragmentation tests are not comparable to one another as they do not measure the same underlying quantity.
  - The degree of DNA fragmentation observed using the SCD and SCD-FISH tests is the same when using first principles.
  - The SCD-FISH test is not comparable to the FISH test.
- A correlation exists between DNA fragmentation and aneuploidy.
1.9b The assessment of processes involved in ART on sperm DNA damage and PS translocation

Exogenous stresses such as oxidative stress and thermal stress are known to contribute to male infertility, and ejaculated, viable spermatozoa selected for ART may be undergoing abortive apoptosis in spite of their normal appearance. Sperm preparation techniques in ART are thought to be potential generators of these exogenous stresses, and DNA fragmentation tests such as the sperm chromatin structure assay, involve freezing sperm samples in the absence of cryoprotectant. Programmed cell death has been implicated as a possible contribution to the decrease in sperm quality following cryopreservation. However, the resilience of sperm to these agents in terms of membrane and nuclear integrity is unknown. Phosphatidylserine translocation (PST) and DNA fragmentation is an early and late sign of apoptosis respectively, but whether a correlation exists between these two parameters is not known. The significance of Annexin V positivity in viable sperm is controversial. Increased staining in most motile fractions of sperm populations imply that the membrane modification observed is due to capacitation, but this phenomenon may not be limited exclusively to this process. Furthermore, whilst the oocyte is known to degenerate with time, the effect of male age on sperm DNA fragmentation integrity is not clear. In order to address these issues, I tested the hypotheses that:

- A correlation exists between conventional sperm parameters and DNA fragmentation.
- A correlation exists between conventional sperm parameters and PST.
- The degree of sperm DNA fragmentation increases with age.
- Oxidative stress through the exposure of sperm to hydrogen peroxide causes DNA fragmentation and PST.
- Thermal stress and freezing cause sperm DNA fragmentation and PST.
- A correlation exists between sperm PST and DNA fragmentation.

1.9c The assessment of cumulus and granulosa cell DNA fragmentation as a biomarker for fertility potential

As an oocyte cannot be investigated for biochemical or molecular markers whilst maintaining its viability, the investigation of markers in the cumulus cells which surround the oocyte is important. It is thought that cumulus and granulosa cells contain a ‘follicular footprint’ of what goes in the oocyte due to the close proximity of these different cells. However, the degree of DNA fragmentation within these cells is unknown. It is also unclear as to whether the variable degree of DNA fragmentation in the cumulus and granulosa cells is representative of oocyte fertility potential. In ICSI, the cumulus
cells are stripped before a single sperm cell is injected into the oocyte. In IVF, the cumulus-oocyte-complex is incubated with spermatozoa overnight. Discrepancies exist as to whether the exposure of cumulus cells to sperm influences the degree of DNA fragmentation due to the potential production of ROS. Whilst it is known that maternal age affects oocyte quality, it is not known whether cumulus and granulosa cells are also affected. In order to address these issues, I investigated the hypotheses that:

- The degrees of DNA fragmentation in cumulus and granulosa cells differ with distance from the oocyte.
- A correlation exists between maternal age and the degree of DNA fragmentation in cumulus and granulosa cells.
- The degree of DNA fragmentation in cumulus cells is influenced by the use of IVF versus ICSI.
- The degree of DNA fragmentation in cumulus and granulosa cells is indicative of fertilization rates, blastocysts development and pregnancy rates.

1.9d The assessment of murine embryo DNA fragmentation in response to culture medium containing GM-CSF

GM-CSF is currently being used to supplement the culture medium EmbryoGen® (Origio, Denmark), at a concentration of 2ng/ml as it has been shown to promote embryo development. If the protective effect exerted is through the reduction of apoptosis, it may also have an impact on DNA fragmentation. However, the effect of GM-CSF on embryo DNA fragmentation is unknown. In order to address these issues, I tested the hypotheses that:

- GM-CSF has a dose-dependent effect on murine embryo DNA fragmentation
- Using a standard culture dish where embryos are cultured in a group, or an EmbryoSlide® where embryos are cultured singly, will not influence the potential concentration dependent effect of GM-CSF on murine embryo DNA fragmentation

1.9e The assessment of human embryonic stem cell DNA fragmentation and aneuploidy following exposure to pre-tested toxins

Several environmental toxins are known to affect fertility potential in both males and females. Whilst the oocyte repair capacity is thought to be significantly more efficient than that of sperm, the effect of toxins on human embryo development is unknown, mainly due to ethical reasons in conducting such research. Human embryonic stem cells are widely being used for toxicity assays in lieu of animal models. However, the resilience of human embryonic stem cells to such toxins, in
terms of DNA fragmentation and aneuploidy is unknown. In order to address these issues, I tested the hypotheses that:

- Human embryonic stem cells contain variable degrees of DNA fragmentation and aneuploidy
- Toxins found in the environment have a concentration dependent effect on the degree of DNA fragmentation and aneuploidy in human embryonic stem cells
2. MATERIALS AND METHODS
2.1 The assessment of DNA fragmentation and aneuploidy in spermatozoa

The aim of this study was to test the following hypotheses:

- Sperm DNA fragmentation and aneuploidy results differ by analysis technique.
- Currently available DNA fragmentation tests are not comparable to one another.
- The degree of DNA fragmentation observed using the SCD and SCD-FISH tests is the same when using first principles.
- The SCD-FISH test is not comparable to the FISH test.
- A positive correlation exists between DNA fragmentation and aneuploidy.
- The relationship between conventional semen parameters and DNA fragmentation is the same when using the SCSA, SCD, SCD-FISH and Halosperm® DNA fragmentation tests.

2.1a Research committee ethics approval

This study was approved by the Joint UCL/UCLH Ethics Committee (REC Number: 05/Q0502/143). The license covers the analysis of human gametes (including cumulus and granulosa cells) and embryos for aneuploidy and DNA fragmentation.

2.1b Preparation of poly-L-lysine-coated microscope slides

Super frost microscope slides were washed in 100ml methanol and 3 drops of HCl for 2 minutes. The slides were air-dried before being washed for 5 minutes in 100ml H₂O containing 10ml of poly-L-lysine solution. The slides were dried overnight in the dark at room temperature, and stored at 4°C.

2.1c Preparation of semen samples

Twenty semen samples collected by Barbara Speyer from patients attending the CRGH for IVF/ICSI were used for analysis in this study. These samples were stored as 0.1ml aliquots in Eppendorf tubes at -80°C. These samples were previously analysed to record sperm concentration, DFI and HDS levels (Speyer et al., 2010).

2.1d Optimization of protocols to determine if the SCD and SCD-FISH tests can be performed in the laboratory via first principles

A modified version of the SCD test can apparently be used to simultaneously determine the level of DNA fragmentation and aneuploidy on the same sperm cell (Muriel et al., 2007). The reproducibility of the method using first principles was assessed by replacing just one factor/reagent from the
commercially available kit with a laboratory produced substitute [(i.e. lysing solution prepared in the lab vs. lysing solution provided in the kit) whilst using all other components (i.e. agarose pre-coated slides, acid denaturant)] from the kit. The results were then compared to slides processed entirely using the commercial kit. This ensured that the results observed were identical regardless of whether the Halosperm® kit or materials prepared in the laboratory using first principles were employed.

The level of DNA fragmentation and aneuploidy were scored in the same semen samples subjected to the SCD test, SCD-FISH, and conventional FISH. The spermatozoa were scored to determine the frequency of cells with DNA damage (demonstrated by the absence of a halo), aneuploidy, and both.

2.1e Analysis of DNA fragmentation using the SCD test

An aliquot of frozen semen sample, stored at -80°C, was thawed in a water bath at 37°C for 40 seconds. The sample was diluted in PBS (Phosphate buffered saline tablets, Sigma) solution to a concentration of 5-10 million cells/mL. Eppendorf tubes containing gelled aliquots of low-melting point aqueous agarose (to obtain a 0.7% final agarose concentration at 37°C) were placed in an oven at 90°C for 5 minutes to melt the agarose, and then placed at 37°C. After 5 minutes incubation at 37°C to allow for equilibrium, 40 µL of the diluted semen sample was added to the Eppendorf tube containing agarose and mixed well. Aliquots of 20 µL of the semen–agarose mixture were pipetted onto a 22mm glass coverslip and inverted onto a 0.65% agarose precoated glass slide and outlined by a diamond marker. The slide was placed on a cold plate in a 4°C fridge for 5 minutes. This allows the agarose to produce a microgel with the trapped sperm inside. The coverslip was gently removed and the slide was immediately immersed horizontally in 0.08N HCl solution at 7 minutes at room temperature. The slide was then immersed in 30 mL of lysing solution 1 (0.4 M Tris, 0.8 M DTT, 1% SDS, and 50 mM EDTA, pH 7.5) for 10 minutes and then 30 mL of lysing solution 2 (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 5 minutes at room temperature. Following a 5 minute wash in distilled water, the slides were dehydrated in an ethanol series of increasing concentration (70%, 90%, 100%) for 2 minutes each. The slides were air-dried, and those on which only DNA fragmentation was assessed were mounted in 30 µL of DAPI (49, 6-diamidino-2-phenylindole, 2 mg/mL) in Vectarshield (1 mL Vectarshield (Vectar Laboratories, CA, USA), 6 µL DAPI) and stored in a tightly closed box in the dark at room temperature.

2.1f Analysis of DNA fragmentation using the SCD-FISH test

Note: the slides were kept horizontal during all stages until the post-washes.
The sperm samples were processed according to the SCD procedure until the final dehydration series (refer section 2.1e). Following step 2.1e, FISH analysis was performed on those slides to be assessed for aneuploidy.

Slides were immersed in 1% paraformaldehyde /PBS (1.34 mL paraformaldehyde, 49 mL PBS) at 4°C for 10 minutes, washed in excess PBS and then distilled water for 1 minute each. They were dehydrated in an ethanol series of increasing concentration (70%, 90%, and 100%) for 2 minutes each and air-dried. Aliquots of 4 µL DNA probe mix (probe:buffer 1:1) were pipetted onto a 13 mm coverslip and inverted onto the area defined by a diamond marker. The DNA probes used were for the alphoid centromeric regions of X chromosome (DXZ1 Locus, SpectrumGreen; Vysis, Inc, Izasa, Spain), Y chromosome (DYZ3 locus, SpectrumOrange; Vysis), and chromosome 18 (D18Z1 locus, SpectrumAqua; Vysis). The slides were denatured at 75°C for 5 minutes. They were transferred to a moist chamber and incubated at 37°C for an hour, allowing hybridization of the probes. The post-washes comprised washing the slides twice for 5 minutes each in 60% formamide/2 x SSC (30 mL formamide, 5 mL 20xSSC, 15 mL distilled H2O) at 42°C. This was followed by 5 minutes in 4xSSC/20% Tween (20 mL 20xSSC, 80 mL distilled H2O, 0.25 mL 20% Tween 20) at room temperature. All post washes need to be performed in the dark, but the slides no longer need to be horizontal. Dehydration in an increasing ethanol series (70%, 90%, and 100%) for 3 minutes each preceded air drying. Cells were counterstained with 30 µL of DAPI in Vectashield (1 mL Vectashield (Vectar Laboratories, CA, USA), 6 µL DAPI) and stored in the dark at 4°C.

2.1g Analysis of DNA fragmentation using the Halosperm® test

The semen sample was diluted in PBS (Sigma-Aldrich, Gillingham, UK) to a concentration of 5-10 million per millilitre. 25µl of the semen sample was added to the melted agarose and the cell suspension from the agarose Eppendorf was placed on the super coated slides and covered with a glass coverslip. Ensuring the slide was in a horizontal position throughout the entire process, the slide was placed on a cold surface at 4°C for 5 minutes. The coverslip was removed and the slide was immersed in acid denaturant for 7 minutes. It was then incubated in tempered lysis solution at room temperature for 25 minutes. Following a 5 minute wash in distilled water, the slide was dehydrated in an ethanol series of increasing concentration (70%, 90%, 100%) for 2 minutes each before air drying and mounting in 30µl of DAPI (49, 6-diamidino-2-phenylindole, 2 mg/ml) in Vectashield (1 ml Vectashield (Vectar Laboratories, CA, USA), 6µl DAPI). The slides were stored in a tightly closed box in a dark at room temperature (Halosperm®, Halotech DNA, SL).
2.1h Analysis of DNA fragmentation using the SCSA® test

The analysis of sperm DNA fragmentation using the SCSA test was carried out by Barbara Speyer as described elsewhere (Evenson and Jost, 1994; Evenson et al., 2002). A 100–200 µl aliquot of frozen neat semen was rapidly thawed at 37°C and diluted with TNE buffer (150 mM NaCl, 10 mM Tris–HCl and 1.0 mM disodium EDTA, pH 7.4) to reach a final sperm concentration of 1-2 x 10^6. Diluted semen sample aliquots of 0.20 ml were mixed with 0.40 ml of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2). After 30 seconds, the cells were stained by adding 1.2 ml acridine orange (AO) stain solution (A6014, Sigma-Aldrich Company Ltd, Poole, Dorset, UK). At 3 min after the staining procedure started, fluorescence measurements were collected on 5000 cells per sample. A Beckman-Coulter Epics Elite cytometer (High Wycombe, UK) was used to carry out flow cytometry.

2.1i Validation of the SCD, SCD-FISH and Halosperm® tests

The values obtained following the SCD, SCD-FISH and Halosperm® tests in the measure of DNA fragmentation were compared to the previously recorded DFI values obtained by Barbara Speyer in 2010 using the SCSA test.

2.1j Analysis of sperm aneuploidy using conventional FISH

*Sperm preparation*: 5ml of phosphate buffered saline (Sigma-Aldrich, Gillingham, UK) was added to 0.1ml aliquots of semen and spun at 750g (2200 revs/minute) for 5 minutes in a falcon tube. The pellet was re-suspended in 10ml PBS (Sigma-Aldrich, Gillingham, UK) and spun again. This wash was repeated. The pellet was re-suspended in 10ml fix (methanol:acetic acid 3:1), incubated at room temperature for 10 minutes and spun again. The pellet was re-suspended in enough fresh fix to give a moderately opalescent suspension of sperm and stored at -20°C.

*Preparation of slides*: 6µl of prepared sperm was spread on a glass slide, delineated with a diamond marker and air dried. The slides were then dehydrated through an ethanol series (70%, 90%, 100%) for 3 minutes each.

*Decondensation*: the fresh slides were incubated in 10mM dithiothreitol (DTT) solution in 0.05M tris for 6 minutes, followed by two 2-minute washes in 2xSSC (0.3M sodium chloride, 0.03M tri sodium citrate, pH 7.2) at room temperature. The wet slides were then dehydrated through another ethanol series for 3 minutes each.
Denaturation: Aliquots of 4µl DNA probe mix (probe:buffer 1:1) were pipetted onto a 13mm coverslip and inverted onto the area defined by a diamond marker. The DNA probes used were for the alphoid centromeric regions of X chromosome (DXZ1 Locus, SpectrumGreen; Vysis, Inc, Izasa, Spain), Y chromosome (DYZ3 locus, SpectrumOrange; Vysis), and chromosome 18 (D18Z1 locus, SpectrumAqua; Vysis). The slides were denatured at 75°C for 5 minutes. They were transferred to a moist chamber and incubated at 37°C for an hour, allowing hybridization of the probes.

Post hybridization washes: All post-hybridization washes were performed in the dark. The coverslips were removed and the slides were submerged in 60% formamide/2 x SSC at 42°C, twice for 5 minutes each. This was followed by 5 minutes in 4xSSC/20% Tween at room temperature. The slides were dehydrated through an ethanol series for 3 minutes each, air dried, mounted in Vectarshield (30µl DAPI) and stored in the dark at 4°C.

2.1k Analysis of aneuploidy using FISH on cells processed for the SCD test (SCD-FISH)

Note: the slides were kept horizontal during all stages until the post-washes.

Following step 2.1e, FISH analysis was performed on those slides to be assessed for aneuploidy.

Slides were immersed in 1% paraformaldehyde /PBS (1.34 mL paraformaldehyde, 49 mL PBS) at 4°C for 10 minutes, washed in excess PBS and then distilled water for 1 minute each. They were dehydrated in an ethanol series of increasing concentration (70%, 90%, 100%) for 2 minutes each and air-dried. Aliquots of 4 µL DNA probe mix (probe:buffer 1:1) were pipetted onto a 13 mm coverslip and inverted onto the area defined by a diamond marker. The DNA probes used were for the alphoid centromeric regions of X chromosome (DXZ1 Locus, SpectrumGreen; Vysis, Inc, Izasa, Spain), Y chromosome (DYZ3 locus, SpectrumOrange; Vysis), and chromosome 18 (D18Z1 locus, SpectrumAqua; Vysis). The slides were denatured at 75°C for 5 minutes. They were transferred to a moist chamber and incubated at 37°C for an hour, allowing hybridization of the probes. The post-washes comprised washing the slides twice for 5 minutes each in 60% formamide/2 x SSC (30 mL formamide, 5 mL 20xSSC, 15 mL distilled H2O) at 42°C. This was followed by 5 minutes in 4xSSC/20% Tween (20 mL 20xSSC, 80 mL distilled H2O, 0.25 mL 20% Tween 20) at room temperature. All post washes need to be performed in the dark, but the slides no longer need to be horizontal. Dehydration in an increasing ethanol series (70%, 90%, 100%) for 3 minutes each preceded air drying. Cells were counterstained with 30 µL of DAPI in Vectarshield (1 mL Vectarshield (Vector Laboratories, CA, USA), 6 µL DAPI) and stored in the dark at 4°C.
2.1l Fluorescence microscopy and scoring criteria

The slides were examined with a Nikon fluorescence microscope, comprising a triple-band pass filter and with monochrome filters for DAPI, SpectrumGreen (X), SpectrumOrange (Y), and SpectrumAqua (18). Approximately 500 spermatozoa were scored per sample. Those sperm nuclei that overlapped or showed nullisomy were disregarded. A sperm nucleus was considered disomic when it showed 2 fluorescent domains of the same chromosome, similar in size and brightness and separated by at least one-half diameter of the domain of 1 signal in nucleoids with large and medium halo size (i.e., those without DNA fragmentation) or by a distance of at least 1 domain in those sperm nucleoids with small halo or without halo (i.e., those with DNA fragmentation). Diploidy was established when 2 distinct chromosome 18 signals and also 2 signals for X and/or Y chromosomes were present in the same sperm nucleus. Although FISH signals in sperm nucleoids with halos may be spread, their dispersion starts from a restricted location from the core. Moreover, this origin from which the DNA fiber spread usually has a stronger intensity than that of the diffused fibers (Klaus et al., 2001). This may help overcome ambiguity that can arise. During FISH analysis on conventional sperm spreads, sperm were considered disomic when displaying duplicated domains with similar size, shape and intensity, and separated by a distance of at least 1 domain. Sperm nuclei were considered only if they were intact, non-overlapping, had a distinct border, and had not been decondensed to more than twice the size of a condensed sperm head.

2.1m Relationship between sperm DNA fragmentation, sperm aneuploidy, sperm concentration, total percentage motility, patient age, and days of abstinence

Sperm concentration and total percentage motility at 20 minutes was analysed and recorded by embryologists at the CRGH. Patients provided information regarding their age and days of abstinence prior to semen analysis. These parameters were compared to the levels of sperm DNA fragmentation as determined by the SCD, SCD-FISH, Halosperm® and SCSA tests, and sperm aneuploidy determined by the FISH and SCD-FISH tests.

2.1n Data analysis

The data was analysed using SPSS 14.0 for Windows package software (SPSS Inc, Chicago, Ill). The Pearson correlation test which is designed for continuous variables was used to describe the strength and direction of the 2 variables being analysed.
Due to the small sample size (n=20), the Wilcoxon Ranks test, a statistical test designed for use with repeated measures was used to analyse the data and identify any significant differences between the levels of DNA fragmentation and aneuploidy in semen samples analysed by different tests from the same patient. A paired-sample t-test was used to compare the mean scores of sperm DNA fragmentation and aneuploidy to reaffirm the findings.

2.2 The assessment of processes involved in ART on sperm DNA damage and PS translocation

The aim of this study was to test the following hypotheses:

- A correlation exists between conventional sperm parameters and DNA fragmentation assessed by TUNEL.
- A correlation exists between conventional sperm parameters and PS translocation.
- The degree of sperm DNA fragmentation increases with age.
- Oxidative stress through the exposure of sperm to hydrogen peroxide causes DNA fragmentation and PST.
- Thermal stress and freezing cause sperm DNA fragmentation and PST.
- A correlation exists between sperm PST and DNA fragmentation.

2.2a Research committee ethics approval

This study was approved by UCLH Ethics Committee, Rec Number: 05/Q0502/143. The license covers the analysis of human gametes (including cumulus and granulosa cells) and embryos for aneuploidy and DNA fragmentation.

2.2b Sperm study population

Semen was obtained from 40 different men attending the Centre for Reproductive and Genetic Health for IVF treatment. Patients collected semen by masturbation into 110 ml BD Falcon™ sterile containers (VWR (Jencons), East Grinstead, UK) following a minimum 2-3 day period of abstinence. Five patients were oligozoospermic, three showed signs of asthenospermia and one was oligoasthenozoospermic. The majority of samples were suitable for IVF and/or ICSI.

Aliquots of the 40 samples were used to test for DNA fragmentation and PS translocation, and these parameters were related to sperm concentration, total percentage motility, patient age, days of abstinence, alcohol consumption and cigarette smoking. Aliquots of the 40 samples were also frozen.
and thawed to determine the effect of freezing on sperm DNA fragmentation. Aliquots from 20 samples were used to study the effect of hydrogen peroxide on sperm DNA fragmentation and PS translocation. Aliquots from the remaining 20 samples were used to study the effect of thermal stress on sperm DNA fragmentation and PS translocation.

Flow diagram 2.2a: Sperm PS translocation and DNA fragmentation were analysed in each experimental group. Sperm PS translocation and DNA fragmentation in the untreated groups of 40 samples were compared to concentration, motility, patient age, days of abstinence, alcohol consumption and no. of cigarettes smoked.

2.2c Analysis of sperm PS translocation using Annexin V

Phosphatidylserine (PS) is an amphiphilic phospholipid universally present in the inner membrane of prokaryotic and eukaryotic cells. The translocation of sperm PS from the inner to the outer leaflet of the plasma membrane is associated with sperm apoptosis and male infertility. Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis (Arur et al., 2003). Once the apoptotic cells are bound with FITC-labeled Annexin V, they can be visualized with fluorescent microscopy.

Sperm PS translocation was detected using the Annexin V-FITC Detection Kit (Promokine, Heidelberg, Germany) according to the manufacturer’s instructions. The supernatant containing sperm was adjusted to 1-5 x 10^5 and the cells were collected by centrifugation. These cells were re-suspended in 500µl of 1 x Binding Buffer. 5µl of Annexin-V FITC was added and incubated at room temperature for 5 minutes in the dark. The cell suspension was pipetted onto a poly-L-lysine-coated microscope slide (refer to Section 2.1b) and gently smeared with a clean slide/glass cover slip. The slides were mounted in 30µl of DAPI (49, 6-diamidino-2-phenylindole, 2mg/ml) in Vectarshield (1ml
Vectarshield) (Vectar Laboratories, CA, USA), 6μl DAPI). The slides were stored in a tightly closed box in the dark at 4°C.

2.2d Analysis of sperm DNA fragmentation using TUNEL

Sperm DNA fragmentation was detected using the DeadEnd™ Fluorometric TUNEL (TdT-mediated dUTP Nick-End Labeling (Promega Corporation, Madison, USA), according to the manufacturer’s instructions. Approximately 2 x 10^7 cells/ml were collected by centrifugation. The cell suspension (50-100µl) was pipetted onto a poly-L-lysine-coated microscope slide (refer to Section 2.1b) and gently smeared with a clean slide/glass cover slip. Slides were immersed in 1% (v/v) paraformaldehyde in PBS for 25 minutes at 4°C and washed in PBS (phosphate buffered saline) (Sigma-Aldrich, Gillingham, UK) twice. Cells were permeabilised in 0.2% (v/v) Triton X-100 (Sigma-Aldrich, Gillingham, UK) in PBS for 5 minutes and washed in PBS twice. Samples were then covered in 100µl of equilibration buffer and equilibrated at room temperature for 10 minutes before labelling with rTdT (Terminal Deoxynucleotidyl Transferase, Recombinant enzyme) reaction mix. Slides were incubated for 60 minutes at 37°C in a humidified chamber. The slides were immersed in 2 x SSC (0.3M sodium chloride, 0.03M tri sodium citrate, pH 7.2) for 15 minutes to terminate the reaction, and washed in PBS three times. The slides were mounted in 30µl of DAPI (49, 6-diamidino-2-phenylindole, 2mg/ml) in Vectarshield [1ml Vectarshield (Vectar Laboratories, CA, USA), 6μl DAPI]. The slides were stored in the dark at 4°C.

2.2e Positive controls

Spermatozoa treated with DNase I was used as positive controls. 100µl of DNase I buffer (New England BioLabs, Ipswich, Massachusetts, U.S) was added to the fixed cells and incubated at room temperature for 5 minutes. The excess liquid was tapped off and 100µl of DNase I buffer containing 10units/ml of DNase I (RQI DNase; Promega Corporation, Madison, USA) was added to the slide and incubated at room temperature for 10 minutes. Excess liquid was tapped off and the slides were washed extensively 3–4 times in deionized water in a coplin jar dedicated for the positive control. The positive control slides were then processed continuing with the TUNEL protocol, at the 10 minute equilibration step. DNase I treatment of the fixed cells results in fragmentation of the chromosomal DNA and exposure of multiple 3’-OH DNA ends for labelling. These positive control slides were processed at the same time as the experimental sperm slides, and resulted in the majority of the treated cells exhibiting green fluorescence.
2.2f Relationship between sperm DNA fragmentation and PS translocation

Sperm DNA fragmentation and PS translocation were determined in 40 untreated semen samples from different patients and the relationship between the two parameters was analysed.

2.2g Relationship between sperm DNA fragmentation, sperm PS translocation, sperm concentration, total percentage motility, patient age, and days of abstinence

Sperm concentration and total percentage motility at 20 minutes was analysed and recorded by embryologists at the CRGH. Patients provided information regarding their age and days of abstinence prior to semen analysis. These parameters were compared to the levels of sperm DNA fragmentation and PS translocation determined by TUNEL and Annexin V, respectively. When examining the effect of sperm concentration on sperm DNA fragmentation, patients were grouped based on those with a sperm concentration <15M/ml and those with a sperm concentration >15M/ml. When assessing the effect of patient age on sperm DNA fragmentation and PS translocation, patients were also grouped by those <40 years and >40 years.

2.2h Assessment of the effect of alcohol consumption and cigarette smoking on sperm DNA fragmentation and sperm PS translocation

Patients provided information regarding their smoking and drinking habits. These parameters were compared to the levels of sperm DNA fragmentation and PS translocation determined by TUNEL and Annexin V, respectively.

2.2i Effect of freezing on sperm PS translocation and DNA fragmentation

Forty unprocessed samples of neat semen were stored in Eppendorf tubes at -80°C on the day of production for approximately 2 months. They were prepared in the same way as if they were being sent for DNA fragmentation testing by SCSA (i.e. no cryoprotectant was used). On the day of analysis, these samples were left to thaw for 15 minutes at room temperature prior to the assessment of DNA fragmentation using TUNEL. Sperm DNA fragmentation following thawing was compared to the level of DNA fragmentation detected in all 40 untreated semen samples on the original day of production (refer to Section 2.2d).
2.2j Effect of oxidative stress on sperm PS translocation and DNA fragmentation

Following liquefaction, 0.1ml aliquots of fresh semen from 20 different patients were treated with 0mM, 0.1mM, 1mM, 10mM, 100mM H₂O₂ [30% (w/w) ; Sigma Aldrich, Gillingham, UK] and incubated for one hour at room temperature. DNA fragmentation and PS translocation were determined by TUNEL and Annexin V respectively, and the relationship between the two parameters was analysed.

2.2k Effect of thermal stress on sperm PS translocation and DNA fragmentation

Following liquefaction, 0.1ml aliquots of fresh semen from 20 different patients were incubated at 25°C, 34°C and 37°C for one hour. Normal testicular temperature is 34°C whilst IVF procedures are performed at 37°C. These temperatures were investigated to determine if an increase in temperature, from physiological and room temperature, has a detrimental effect on sperm. DNA fragmentation and PS translocation were determined by TUNEL and Annexin V respectively, and the relationship between the two parameters was analysed.

2.2l Fluorescence microscopy and scoring criteria

The slides were examined with a Nikon fluorescence microscope, comprising a triple-band pass filter and with monochrome filters for DAPI and Spectrum Green. The standard filter was set to view the green fluorescence of fluorescein at 520+/-20 nm. Five hundred spermatozoa were scored per slide at a magnification of x100. Sperm cells displaying green fluorescence were considered to show signs of DNA fragmentation.

2.2m Data analysis

The data was analysed using SPSS 14.0 for Windows package software (SPSS Inc, Chicago, Ill). The Pearson correlation test which is designed for continuous variables was used to describe the strength and direction of the 2 variables being analysed.

Due to the small sample size, the Wilcoxon Ranks test, a statistical test designed for use with repeated measures was used to analyse the data and identify any significant differences between the levels of DNA fragmentation and PS translocation in semen samples from the same patients.
exposed to damaging agents. A paired-sample t-test was used to compare the mean scores of sperm DNA fragmentation and PS translocation to reaffirm the findings.

### 2.3 The assessment of cumulus and granulosa cell DNA fragmentation as a biomarker for fertility potential

The aim of this study was to test the following hypotheses:

- The cumulus cells closer to the oocyte contain a greater degree of DNA fragmentation than the granulosa cells further away from the oocyte.
- Increasing maternal age is associated with higher levels of DNA fragmentation in cumulus and granulosa cells, and decreased rates of fertilization.
- Cumulus cells from IVF cycles contain more DNA fragmentation than cumulus cells from ICSI cycles (In IVF, the cumulus-oocyte-complex is incubated with spermatozoa overnight. It was hypothesized that spermatozoa increase the level of oxidative stress, potentially inducing DNA damage in the cells within the same culture medium).
- Increasing levels of DNA fragmentation in cumulus and granulosa cells result in a decrease in fertilization rates, blastocysts development and pregnancy rates.

#### 2.3a Research committee ethics approval

This study was approved by the Joint UCL/UCLH Ethics Committee (REC Number: 05/Q0502/143). The license covers the analysis of human gametes (including cumulus and granulosa cells) and embryos for aneuploidy and DNA fragmentation.

#### 2.3b Patients

Forty eight female patients undergoing IVF/ICSI at the Centre for Reproductive and Genetic Health, University College London Hospital donated their follicular fluid and cumulus cells for research. Granulosa cells were obtained from 36 patients and cumulus cells were obtained from 38 patients. Study end points were percentage DNA fragmentation in cumulus and granulosa cells, treatment outcome, number of oocytes collected, number of oocytes fertilized, number of embryos transferred, status of sperm, reason for infertility, period infertile, whether patient was a smoker and age. All patients provided informed consent for their follicular fluid and cumulus cells to be used in this study.
2.3c Controlled Ovarian Hyperstimulation and IVF Procedures

Following evaluation of the ovarian reserve by antral follicle count, FSH, estradiol and anti-Mullerian hormone, the stimulation protocol was individualized for each patient. Patients with a normal ovarian reserve underwent a long down-regulation protocol. Treatment with antagonist drug and a high starting dose of gonadotrophins was used to stimulate patients with reduced ovarian reserves. Ultrasounds scans were used to monitor follicular growth from Day 7 of stimulation. hCG was administered when at least two follicles reached a diameter of 18mm. Ultrasound guided vaginal oocyte retrieval was carried out 34-36 hours after hCG administration. Depending on patient parameters and requirements, IVF or ICSI was carried out 40 and 41 hours post-hCG, respectively. Fertilization was assessed 18-20 hours following insemination. Embryos were cultured in IVF medium (GIII series, Vitrolife, UK).

2.3d Procedure for the analysis of granulosa cell suspensions by fluorescent microscopy

This procedure was always performed on the same day that follicular fluid was aspirated from the patient. Thirty-four to 36 hours after hCG administration, follicular fluid (FF) was collected by means of transvaginal ultrasound-guided oocyte retrieval by a specialist. Following follicular aspiration, the cumulus-oocyte complexes were separated for IVF and ICSI by embryologists, and the follicular fluid containing granulosa cells was immediately transported to the laboratory in a 150ml volume polypropylene urine container with screw cap (Falcons urine, PP 150ML; #5641, Kartell disposlab®, Australia). The follicular fluid was decanted in small volumes into a petri dish. A light microscope under highest power was used to identify granulosa cells that appeared as sheaths. A micro-pipette was used to aspirate these ‘sheaths’ of granulosa cells and collected in an Eppendorf tube. The cells were flushed with 0.1% PVA/PBS (Sigma-Aldrich, Gillingham, UK) solution and mixed using a vortex for a few seconds to disperse the cells. The supernatant was removed and approximately 50-100µl of the cell suspension was pipetted onto a poly-L-lysine-coated microscope slide (refer Section 2.1b) and gently smeared with a clean slide/glass cover slip. Cells were processed to assess DNA fragmentation using TUNEL (refer Section 2.3g).

2.3e Procedure for the analysis of cumulus cell suspensions by fluorescent microscopy

In the case of IVF, COCs were incubated with sperm for approximately 18 hours at 37°C, before cumulus cell removal/denudation of oocytes on the day of the fertilisation check after insemination.
(Day 1). For ICSI, cumulus cells were removed/oocytes denuded on Day 0 (the day of egg collection) prior to ICSI injection. Following denudation of oocytes by the embryologists, Nunc 4-well dishes containing cumulus cells were immediately transported to the laboratory. Cumulus cells obtained from ICSI patients were incubated overnight at 37°C in the laboratory to maintain similar environmental conditions between cumulus cells retrieved from both IVF and ICSI patents. A light microscope was used to identify cumulus cells in each well. Cells were aspirated using a Pasteur pipette and transferred to an Eppendorf tube. The cell suspension was centrifuged at 300 x g for 10 minutes at 4°C to obtain a concentrated pellet. The culture medium was removed and the cells were washed in PBS (Sigma-Aldrich, Gillingham, UK) by centrifugation, then re-suspended in PBS (Sigma-Aldrich, Gillingham, UK) at a concentration of approximately 2 x 10⁷ cells/ml. The cell suspension (50-100µl) was pipetted onto a poly-L-lysine-coated microscope slide and gently smeared with a clean slide/glass cover slip. Cells were processed to assess DNA fragmentation using TUNEL (refer Section 2.3g).

2.3f Positive controls

Cumulus cells treated with DNase I was used as positive controls. 100µl of DNase I buffer (New England BioLabs, Ipswich, Massachusetts, U.S) was added to the fixed cells and incubated at room temperature for 5 minutes. The excess liquid was tapped off and 100µl of DNase I buffer containing 10units/ml of DNase I (RQI DNase; Promega Corporation, Madison, USA) was added to the slide and incubated at room temperature for 10 minutes. Excess liquid was tapped off and the slides were washed extensively 3–4 times in deionized water in a coplin jar dedicated for the positive control. The positive control slides were then processed continuing with the TUNEL protocol, at the 10 minute equilibration step. DNase I treatment of the fixed cells results in fragmentation of the chromosomal DNA and exposure of multiple 3'-OH DNA ends for labelling. These positive control slides were processed at the same time as the experimental sperm slides, and resulted in the majority of the treated cells exhibiting green fluorescence.

2.3g Analysis of cumulus and granulosa cell DNA fragmentation using TUNEL

Refer section 2.3d for the fixing of granulosa cells, and 2.3e for the fixing of cumulus cells. The slides with attached cells were immersed in 1% (v/v) paraformaldehyde in PBS for 25 minutes at 4°C and washed in PBS (phosphate buffered saline) (Sigma-Aldrich, Gillingham, UK) twice. Cells were permeabilised in 0.2% (v/v) Triton X-100 (Sigma-Aldrich, Gillingham, UK) in PBS for 5 minutes and
washed in PBS twice. Samples were then covered in 100µl of equilibration buffer and equilibrated at room temperature for 10 minutes before labelling with rTdT (Terminal Deoxynucleotidyl Transferase, Recombinant enzyme) reaction mix. Slides were incubated for 60 minutes at 37°C in a humidified chamber. The slides were immersed in 2 x SSC (0.3M sodium chloride, 0.03M tri sodium citrate, pH 7.2) for 15 minutes to terminate the reaction, and washed in PBS three times. The slides were mounted in 30µl of DAPI (49, 6-diamidino-2-phenylindole, 2mg/ml) in Vectashield [1ml Vectashield (Vectar Laboratories, CA, USA), 6µl DAPI]. The slides were stored in the dark at 4°C.

2.3h Fluorescence microscopy and scoring criteria

The slides were examined with a Nikon fluorescence microscope, comprising a triple-band pass filter and with monochrome filters for DAPI and Spectrum Green. The standard filter was set to view the green fluorescence of fluorescein at 520+/−20 nm. Five hundred cumulus and granulosa cells were scored per slide at a magnification of x100. Cells displaying green fluorescence were scored as showing signs of DNA fragmentation.

2.3i Relationship between cumulus and granulosa cell DNA fragmentation with patient age, ovarian stimulation protocol, exposure to spermatozoa, fertilization rate, blastocyst development and pregnancy outcome

Cumulus and granulosa cell DNA fragmentation was compared to patient age, ovarian stimulation protocol, exposure to spermatozoa (ICSI vs. IVF), fertilization rate, rate of blastocyst development, and pregnancy outcome.

The level of DNA fragmentation in granulosa and cumulus cells from the same patient (n=31), were compared to determine if the there is a difference between the level of DNA damage in the cells closer to the oocyte (cumulus) and those further away within the follicle (granulosa).

Female patients were grouped by age in the following manner by those <37 years and ≥37 years, and the effect of patient age on the level of granulosa and cumulus cell DNA fragmentation and fertilization rate was examined.

The potential influence of incubating the cumulus oocyte complex with sperm, overnight as in the case of IVF, on DNA fragmentation was studied. This was examined by recording the level of DNA fragmentation in cumulus cells from IVF and ICSI patients, and determining if there was a difference
between the two groups. To maintain experimental consistency, cumulus cells from ICSI patients were incubated at 37°C in the absence of spermatozoa.

The effect of granulosa and cumulus cell DNA fragmentation on fertilization rate will be investigated, along with the influence of patient age. Finally, the degree of DNA fragmentation in granulosa and cumulus cells and its association with blastocyst rate and pregnancy outcome will be studied.

2.3j Data analysis

The data was analysed using SPSS 14.0 for Windows package software (SPSS Inc, Chicago, Ill). The Pearson correlation test which is designed for continuous variables was used to describe the strength and direction of 2 variables being analysed.

Due to the small sample size, the Wilcoxon Ranks test, a statistical test designed for use with repeated measures was used to analyse the data and identify any significant differences between the levels of DNA fragmentation and PS translocation in semen samples from the same patients exposed to damaging agents.

A paired-sample t-test was used to compare the mean scores of sperm DNA fragmentation in granulosa and cumulus cells from the same patient. An independent-samples t-test was used to analyse the level of DNA fragmentation in cumulus and granulosa cells from patients grouped below and above the age of 37. A one-way analysis of variance (ANOVA) test was used to analyse the relationship between cumulus or granulosa cell DNA fragmentation vs. patient age, and fertilization rate vs. patient age.

2.4 The assessment of murine embryo DNA fragmentation in response to culture medium containing GM-CSF

The aim of this study was to test the following hypotheses:

- GM-CSF has a dose-dependent effect on murine embryo DNA fragmentation.
- The use of a standard culture dish or an EmbryoSlide® does not influence the potential concentration dependent effect of GM-CSF of murine embryo DNA fragmentation.

2.4a Superovulation and embryo collection

This study used MF1 mice, approximately 5-7 weeks old (Charles River, UK). All processes involving superovulation, mating and embryo collection were carried out by Katayoon Gardner at the Institute
of Child Health, University College London. Standard superovulation was achieved by injecting MF1 female mice with 7IU of pregnant mare serum gonadotrophin (Intervet), followed by 5IU of human chorionic gonadotrophin (HCG; Intervet) 48 hours later. The females were then immediately mated with male MF1 mice. The pregnant females were sacrificed 48 hours post-hCG, and embryos at the 2-cell stage were flushed from the oviduct.

2.4b Embryo culture

All processes involving embryo culture were performed by Aisha Elaimi at the Institute for Women’s Health, University College London. The procured embryos were randomly divided into 2 groups. In the first, 192 embryos were consigned to group culture in a standard petri dish loaded with 50µL of culture media. In the second group, 67 embryos were culture singly using the EmbryoSlide® Culture Dish which contained 12 micro-wells, each loaded with 25µl of culture media. In both groups, the embryos were further divided into 5 subcategories. Of these subgroups, the embryos of one group were cultured in potassium simplex optimization media containing 1g/l BSA (bovine serum albumin) (KSOM, Millipore), which was used as the untreated control group (0ng/ml GM-CSF). The other 4 subgroups containing the same culture medium of were supplemented with recombinant mouse GM-CSF (R&D system, Abingdon, UK) of 4 different concentrations; 1ng/ml, 2ng/ml, 5ng/ml and 10ng/ml. According to the manufacturer’s instructions, the GM-CSF was dissolved in PBS (Sigma-Aldrich, Gillingham, UK) prior to its use. The concentrations of GM-CSF were decided upon following the review of applicable studies researching the effect of GM-CSF on embryo development (Sjoblom et al., 1999; Behr et al., 2005; Karagenc et al., 2005; Sjoblom et al., 2005; Agerholm et al., 2010). All embryos (groups) were cultured in their respective medium in a standard incubator for 3 days at 37°C and 5% CO₂ until they reached the blastocyst stage (120 hours post-hCG/day 5). The entire process was repeated 3 times.
Flow diagram 2.4a: Experimental plan to determine the concentration dependent effect of the culture medium additive GM-CSF on the level of DNA fragmentation in murine blastocysts, and the potential influence of the method of culture (standard petri dish/group culture versus EmbryoSlide®/single culture) on the degree to which GM-CSF acts as an apoptosis inhibitor.

2.4d Embryo fixation/spreading and slide preparation

Processes involving embryo fixation/spreading were performed by Aisha Elaimi at the Institute for Women’s Health, University College London. To prevent the loss of nuclei, entire embryos at the blastocyst stage were spread on poly-L-lysine slides (refer section 2.1b). A diamond marker was used to draw a circle on the underside of the glass slide, and a drop of spreading solution was deposited on the demarcated area. A dissecting microscope and hand pipette were used to select one embryo at a time and wash it in a drop of PBS (Sigma-Aldrich, Gillingham, UK) on the corner of a slide prior to its transfer into the spreading solution. Fresh spreading solution was added and the hand pipette was used to apply gentle stimulation if required, until the zona pellucida dissolved, cells lysed and cytoplasm dissolved.

The slides were air dried, washed in PBS (Sigma-Aldrich, Gillingham, UK) for 5 minutes and dehydrated in increasing concentrations of ethanol (70%, 90% and 100%) for 5 minutes each. The slides were air dried again and stored at 4°C until analysis. When required, nuclei location was recorded using an England Finder (Graticules, UK).
2.4e Preparation of control slides

Murine blastocysts were spread on poly-L-lysine slide as before (See 2.4d; by Aisha Elaimi) and treated with DNase I to induce DNA fragmentation in the cells. TUNEL was performed on the positive control slides to ensure that the majority of cells or all cells were damaged.

2.4f Analysis of embryo DNA fragmentation using TUNEL

Refer sections 2.2d or 2.3g.

2.4g Data analysis

The data was analysed using SPSS 14.0 for Windows package software (SPSS Inc, Chicago, Ill). A one-way analysis of variance (ANOVA) test was used to determine if there is a significant difference in the mean level of DNA fragmentation in embryos exposed to five concentrations of GM-CSF. Post-hoc tests were used to establish where the differences lay.

2.5 The assessment of human embryonic stem cell DNA fragmentation and aneuploidy following exposure to pre-tested toxins

The aim of this study was to test the following hypotheses:

- Human embryonic stem cells contain variable degrees of DNA fragmentation and aneuploidy.
- Toxins found in the environment have a concentration dependent effect on the degree of DNA fragmentation and aneuploidy in human embryonic stem cells.

2.5a Human embryonic cell lines

Human embryonic stem cells were obtained from the MRC Centre for Regenerative Medicine, University of Edinburgh. Cytotoxicity tests were performed in their laboratory by Eirini Koutsouraki under the supervision of Dr. Paul De Sousa, where RH1 undifferentiated human embryonic stem cells were exposed to at least 8 different concentrations of a compound (at a 3 log-fold dilution series) for six days, 24 hours after plating the cells. Four biological replicates were maintained. Following treatment, the CellTiter-Blue Cell Viability Assay (Promega Corporation, Madison, USA) was used to determine the proportion of viable cells and compared to the control group of untreated cells. Thus, the toxin concentration that reduced cell viability by 10% was used as the IC10 dose.
2.5b Induction of DNA fragmentation and aneuploidy by exposure to toxins

Four toxic compounds at 4 different concentrations each were used in this study. Human ESCs of the RH1 line at passage 53 were used in the undifferentiated state. Approximately 50,000 undifferentiated RH1 cells were plated per well (24-well format) and the specific toxins were added to their respective wells after 24 hours. RH1 cells plated at the same density but not exposed to any toxins were used as a control. These processes were all performed by Eirini Koutsouraki and her colleagues. On day 7 of the culture, the hESCs were removed and fixed for FISH and TUNEL.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>hESC Control</th>
<th>Dose 1</th>
<th>Dose 2</th>
<th>Dose 3</th>
<th>Dose 4 (IC10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-azacytidine</td>
<td>Untreated</td>
<td>3.3x10^{-12}</td>
<td>3.3x10^{-11}</td>
<td>3.3x10^{-10}</td>
<td>3.3x10^{-9}</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>Untreated</td>
<td>1x10^{-9}</td>
<td>1x10^{-8}</td>
<td>1x10^{-7}</td>
<td>1x10^{-6}</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Untreated</td>
<td>1x10^{-11}</td>
<td>1x10^{-10}</td>
<td>1x10^{-9}</td>
<td>1x10^{-8}</td>
</tr>
<tr>
<td>Sodium arsenite</td>
<td>Untreated</td>
<td>3.3x10^{-14}</td>
<td>3.3x10^{-13}</td>
<td>3.3x10^{-12}</td>
<td>3.3x10^{-11}</td>
</tr>
</tbody>
</table>

Table: Concentrations of the four toxins used. All concentrations are displayed as Molar. (Dose 4=IC10 is the toxicant concentration at which 10% of the initial cell number is killed)

2.5c Fixing the human embryonic stem cells for FISH analysis

The culture media was changed 24 hours before harvesting the culture to stimulate cell division. Pre-warmed Trypsin-EDTA was added and cell detachment was monitored to ensure detachment of both the feeder cells and ESCs. The cells were gently titrated to obtain a single cell suspension. The cell suspensions were transferred to a labelled tube and centrifuged for 8 minutes at 1000 rpm. The supernatant was discarded and the pellet gently dislodged. KCl hypotonic solution (0.075M, 2ml) was added to the tube, gently swirled to mix, and the total volume adjusted to 8 ml with KCl. The tube was mixed by inversion and incubated at room temperature for 20 minutes. Following treatment with hypotonic solution, the cells were fragile and treated gently. Ten drops of fixative (3:1 methanol:acetic acid) were added to the tube, mixed by inversion and incubated at room temperature for 10 minutes. The centrifugation step was repeated and supernatant discarded. Freshly prepared fixative (2ml) was added, gently swirled to mix, the total volume was adjusted to 8 ml with fixative, mixed by inversion and incubated at room temperature for 30 minutes. The centrifugation step was repeated and supernatant discarded. Freshly prepared fixative (2ml) was
added, gently swirled to mix, the total volume was adjusted to 8 ml with fixative, and mixed by inversion. The centrifugation step was repeated and supernatant discarded. For short term storage, 4 ml of fixative was added and stored at 4°C until slide preparation.

2.5d Fixation of human embryonic stem cells for TUNEL

Non-attached cells were isolated from the culture medium and centrifuged at 300 x g for 10 minutes at 4°C. The culture medium was removed and the cells were washed in PBS (Sigma-Aldrich, Gillingham, UK) by centrifugation as described before re-suspension in PBS (Sigma-Aldrich, Gillingham, UK). 50-100 µl of the cell suspension was pipetted onto a poly-L-lysine-coated microscope slide. The cell suspension was gently smeared with a clean slide/glass cover slip. The fixed slides were permeated by immersing the slides in 0.5% Triton X-100 for 10 minutes at room temperature.

2.3e Analysis of human embryonic stem cell DNA fragmentation using TUNEL

Apoptotic cells were identified by the DeadEnd™ Fluorometric TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) system (Refer section 2.2d or 2.3g). Approximately 300 cells were scored per slide.

2.5f Fluorescence in-situ hybridization (FISH) on interphase nuclei using direct labelled DNA probes for chromosomes 13, 18 and 21

Following fixation, the cells were spread on poly-L-lysine slides and air dried. They were washed in PBS (Sigma-Aldrich, Gillingham, UK) and dehydrated in an ethanol series of 70%, 90% and 100% ethanol. The slides were incubated in pepsin (10mg/ml) in pre-heated HCl at 37°C for 15 minutes. The slides were washed in PBS (Sigma-Aldrich, Gillingham, UK) and incubated in 1% paraformaldehyde/PBS for 10 minutes at 4°C. They were washed in PBS (Sigma-Aldrich, Gillingham, UK), distilled water and dehydrated in an ethanol series before air drying. The slides were inverted onto coverslips containing 100µl of 70% deionised formamide solution and placed in the denaturation oven at 75°C for 5 minutes. The coverslips were removed and the slides incubated for 5 minutes in 70% ice cold ethanol, followed by dehydration for 3 minutes each in 90% and 100% ethanol. The slides were air dried and inverted onto coverslips containing 5µl of probe mix. They were placed in a dark humid chamber in the hybridization incubator at 37°C and left overnight.
Post-hybridization washes were performed the following day. The coverslips were removed and the slides incubated for 3 minutes in 50% formamide at 41°C. This wash was repeated twice more for a total of three washes. The slides were then incubated in 2xSSC for 3 minutes at 41°C. This wash was repeated twice more. The slides were dehydrated in an ethanol series for 3 minutes each and air dried. They were mounted in 10μl of Vectarshield DAPI and stored in the dark at 4°C. FISH signals were examined (refer Section 2.5h) and recorded prior to re-probing for chromosomes 15, 16 and 22 (refer Section 2.5g). Approximately 300 cells were scored per slide.

2.5g Fluorescence in-situ hybridization (FISH) on interphase nuclei using direct labelled DNA probes for chromosomes 15, 16 and 22

Before reprobing the slides, the coverslips were removed. The slides were incubated for 1 minute in PBS (Sigma-Aldrich, Gillingham, UK) at room temperature on a rocking plate under bright light. They were then incubated for 5 minutes in 4xSSC/0.5% Tween 20 at room temperature on a rocking plate under bright light. The slides were dehydrated in an ethanol series for 3 minutes each at 70%, 90% and 100% ethanol before air drying. The probe mixes were prepared separately; probe mix 1 for chromosomes 15 and 22, and probe mix 2 for chromosome 16. The slides were denatured as described when setting up the first round of FISH. The two probe mixes were denatured at 75°C for 5 minutes and then mixed. The probe mix (5μl) was pipetted onto glass coverslips and the slides inverted. The slides were placed in a dark humid chamber inside a hybridization incubator and left overnight.

Post-hybridization washes were performed the following day. The coverslips were removed and the slides incubated for 3 minutes in 40% formamide at 40°C. This wash was repeated once more for a total of two washes. The slides were then incubated in 2xSSC for 3 minutes at 40°C. This wash was repeated once more. The slides were dehydrated in an ethanol series for 3 minutes each and air dried. They were mounted in 10μl of Vectarshield DAPI and stored in the dark at 4°C. FISH signals were scored as described in Section 2.5h. Approximately 300 cells were scored per slide.

2.5h FISH scoring criteria

The slides were examined with a Nikon fluorescence microscope, comprising a triple-band pass filter and with monochrome filters for DAPI, SpectrumGreen, SpectrumOrange, and SpectrumAqua. Signal scoring was performed on the basis that cells were scored as ‘normal’ if FISH clearly indicated two separate signals for each probe, while ‘abnormal’ cell showed derivation from the normal signal pattern (Munne et al., 1998). Two signals represented two homolog chromosomes when their
distance apart was at least two domain diameters (Munne et al., 1996). Two signals that are less than two domains apart were considered as one duplicated signal and represented a single homolog chromosome.

2.5i Data analysis

Statistical analysis could not be carried out on this set of data as mean scores were unavailable; the experiments were only carried out once and not repeated.
3. Results
3.1 The assessment of DNA fragmentation and aneuploidy in spermatozoa

Refer Aims Section 1.9a, and Materials and Methods Section 2.1

3.1a The assessment of sperm DNA fragmentation and aneuploidy using the SCD, Halosperm®, SCSA, SCD-FISH and FISH tests

Semen samples from twenty males undergoing treatment at the Centre for Reproductive and Genetic Health (CRGH), London, UK, for IVF/ICSI were collected by Barbara Speyer and frozen at -80°C, before being analysed for sperm DNA fragmentation and aneuploidy. The SCSA test was used to determine level of DNA fragmentation in these frozen semen sample aliquots by Barbara Speyer. Sperm DNA fragmentation levels observed using the Halosperm®, SCD and SCD-FISH tests were compared to those obtained using the gold standard SCSA test. Aneuploidy for chromosomes X, Y and 18 was assessed using SCD-FISH, and values compared to those obtained using the established method of fluorescence-in-situ hybridization (FISH).

3.1b Preliminary optimization of the SCD assay

A significant portion of this project involved optimizing the procedures involved in the SCD assay to enable the use of first principles as opposed to the costly Halosperm® kit. This was executed by first using the kit on a sample of semen to establish a point of reference when comparing the results following the testing of various parameters using first principles (Table 3.1a).

Each factor involved in the SCD test was individually verified (Table 3.1a; Refer Materials and Methods Section 2.1d)

<table>
<thead>
<tr>
<th>Laboratory prepared reagent being tested</th>
<th>Main outcome observed</th>
<th>Method used in final study</th>
</tr>
</thead>
<tbody>
<tr>
<td>The effect of the super-coated glass slides was tested by fixing sperm cells on:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) Frosted glass slides</td>
<td>i) No sperm observed under the microscope; cell washed off</td>
<td></td>
</tr>
<tr>
<td>ii) Slides coated with 1% standard agarose and dried at room temperature overnight</td>
<td>ii) No sperm observed under the microscope; cell washed off</td>
<td></td>
</tr>
<tr>
<td>iii) Slides coated with 0.65% agarose solution and dried at 70°C for 5 minutes</td>
<td>iii) Sperm cells present on slide</td>
<td>✓</td>
</tr>
<tr>
<td>Agarose solution to produce microgel</td>
<td>Results observed consistent with those with those generated by the Halosperm® kit</td>
<td>✓</td>
</tr>
<tr>
<td>0.08N acid denaturant</td>
<td>Results observed consistent with those</td>
<td>✓</td>
</tr>
</tbody>
</table>
The effect of the lysing solution was tested by comparing sperm cells processed using:

| i) Lysing solution from the kit for 25 minutes | i) Clear cells; compact cores and distinct halos |
| ii) Lysing solution 1 for 25 minutes | ii) Very faint DAPI signals and unclear halos |
| iii) Lysing solution 1 (10 minutes) and 2 (5 minutes) | iii) Clear cells; compact cores and distinct halos |

Table 3.1a: Summary of results following the optimization procedure. The procedures eventually used in the final study are marked with a ✓ in the column on the far right.

Image 3.1a: Spermatozoa processed by the SCD test. When observed under the microscope, these cells did not display halos, demonstrating the presence of DNA fragmentation. Magnification: x600

Image 3.1b: Spermatozoa processed by the SCD-FISH test. When observed under the microscope, these cells did not display halos, demonstrating the presence of DNA fragmentation. Magnification: x1000.
Image 3.1c: Spermatozoa processed by the Halosperm® assay. When observed under the microscope, halos were visible around the cellular core, demonstrating the lack of DNA fragmentation. Magnification: x600.

3.1c Validation of SCD, Halosperm® and SCD-FISH in the assessment of DNA fragmentation in spermatozoa

To investigate the accuracy of DNA fragmentation analysis using SCD, Halosperm® and SCD-FISH, these assays were used to compare the proportion of sperm cells without halos (i.e. displaying signs of DNA fragmentation) with samples processed by the SCSA test. The levels of DNA fragmentation based on the different DNA fragmentation assays were SCSA; 4-43% (n=20, mean=16.1, SD=8.49), Halosperm®; 6-64% (n=20, mean=19.64, SD=14.23), SCD; 0.4-40.6% (n=20, mean=6.14, SD=9.06) and SCD-FISH; 7.6-99% (n=20, mean=52.38, SD=34.04) (Table 3.1b).

<table>
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<th>Sample no.</th>
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<tbody>
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</tr>
<tr>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>
Table 3.1b: Percentage sperm DNA fragmentation observed using the SCSA, SCD, Halosperm and SCD-FISH tests in samples from 20 male patients.

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
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<tr>
<td>16</td>
<td>4</td>
<td>15.2</td>
<td>6</td>
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</tr>
<tr>
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<td>13.4</td>
<td>15.2</td>
<td>98.6</td>
</tr>
<tr>
<td>18</td>
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<tr>
<td>20</td>
<td>14</td>
<td>7.4</td>
<td>10</td>
<td>10.4</td>
</tr>
</tbody>
</table>

Figure 3.1a: Comparison of mean percentage DNA fragmentation recorded using the SCSA, SCD, Halosperm® and SCD-FISH tests. Statistically significant differences were observed between the levels of DNA fragmentation when analysed by the SCSA and SCD tests (n=20, P=0.001), SCSA and SCD-FISH tests (n=20, P=0.001), SCD and SCD-FISH tests (n=20, P=0.000) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed). Error bars indicate standard error.

When using the Wilcoxon Signed Ranks tests, a statistically significant difference was observed between the levels of sperm DNA fragmentation in the samples subjected to SCSA and SCD (P=0.001), SCSA and SCD-FISH (P=0.001), SCD and SCD-FISH (P=0.000), SCD and Halosperm® (P=0.003), SCD-FISH and Halosperm® (P=0.001) (Figure 3.1a and Table 3.1c). A statistically significant difference was not observed when comparing SCSA and Halosperm® (P=0.211) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed) (Figure 3.1a and Table 3.1c). It can therefore be assumed that whilst our laboratory-prepared SCD test was not accurate in assessing DNA fragmentation, the Halosperm® assay produced results similar to those generated by the SCSA.
Table 3.1c: Statistically significant differences between the levels of sperm DNA fragmentation as assessed by different sperm DNA fragmentation tests. NS=not statistically significant; Wilcoxon Ranks Test, P ≤ 0.05, two-tailed.

A statistically significant positive correlation was observed between SCSA and SCD (n=20, r=0.628, P=0.003) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.1b and Table 3.1d). This observation suggests that the same underlying quality (i.e. percentage of sperm cells with DNA fragmentation) is being measured, and probably on the same scale. A very small correlation of statistical significance was observed between SCD-FISH and Halosperm® (n=20, r=0.123, P=0.016) (Pearson Correlation, P ≤ 0.05, two-tailed). Small positive correlations of no significance were observed between SCSA and SCD-FISH (n=20, r=0.123, P=0.606), and SCSA and Halosperm® (n=20, r=0.407, P=0.075) (Pearson Correlation, P ≤ 0.05, two-tailed). Correlations were also observed between DNA fragmentation measured by SCD-FISH and SCD (n=20, r=0.269, P=0.252), and SCD and Halosperm® (n=20, r=0.121, P=0.612) (Pearson Correlation, P ≤ 0.05, two-tailed), but were not statistically significant (Table 3.1d).

Figure 3.1b: Relationship between percentage sperm DNA fragmentation assessed by the SCD and SCSA tests. A significant correlation was observed between the levels of DNA fragmentation when measured by these two tests (n=20, r=0.628, P=0.003; Pearson Correlation, P ≤ 0.05, two-tailed).
Table 3.1d: Statistically significant correlation between the levels of sperm DNA fragmentation as assessed by different sperm DNA fragmentation tests. NS=not statistically significant: Pearson Correlation, P ≤ 0.05, two-tailed.

### 3.1d Validation of the SCD-FISH test in the assessment of aneuploidy in spermatozoa

To establish the accuracy of aneuploidy analysis using SCD-FISH, the level of chromosomal abnormality was compared in samples subjected to SCD-FISH and conventional FISH (Table 3.1e). A difficulty that arose when scoring the SCD-FISH processed cells was that some sperm cells appeared destroyed. Instead of a compact core and peripheral halo, they appeared similar to astrocytes. Thus it was difficult to distinguish between cells with halos and those without and these samples were avoided during the scoring procedure. It is possible that this phenomenon occurred due to the intensity of the lysing solution. Another problem concerned the FISH probes; the signals tended to diffuse making scoring tedious and inaccurate (Images 3.1d and 3.1e). When the cells appeared fragmented, so did the FISH signals. This was rectified to an extent by using 2μl of probe with 2μl buffer instead of 2.5μl each.
Image 3.1d: Spermatozoa processed by SCD-FISH. When observed under a microscope, the cell displayed a halo, signifying the lack of DNA fragmentation. This sperm cell demonstrated aneuploidy for chromosome Y (YY, 18). This image highlights the dispersion of FISH signals and the difficulty in the scoring of FISH signals following the SCD-FISH test.

Image 3.1e: Spermatozoa processed by SCD-FISH. When observed under a microscope, the cell displayed a halo, signifying the lack of DNA fragmentation. This sperm cell was normal (X, 18) for the chromosomes analysed. This image highlights the difficulty in the scoring of FISH signals following the SCD-FISH test.

Image 3.1f: Spermatozoa processed by conventional FISH. This cell was aneuploid for the Y chromosome (YY, 18).
The levels of aneuploidy ranged from 2.4-9.0% following FISH (n=20, mean=5.63, SD=1.64) and 11.0-28.2% following SCD-FISH (n=20, mean=17.94, SD=4.38) (Table 3.1e). In the researched literature, whilst the frequency of chromosomal aberrations in the general population was estimated at 0.6% (Berger, 1975), disomy per autosome was approximately 0.1% but ranged from 0.03 (chromosome 8) to 0.47 (chromosome 22) (reviewed by Templado et al., 2011). The majority of studies found that chromosome 21 (0.18%-0.29%) and the sex chromosomes (0.27%-0.43%) had significantly elevated frequencies of disomy (Tempest and Griffin, 2004; Martin, 2006; reviewed by Templado et al., 2011). The total disomy in FISH studies was 2.26% and the estimated aneuploidy (2x disomy) was 4.5% (reviewed by Templado et al., 2011). The odegree of aneuploidy observed was consistently higher when analysed using the SCD-FISH test compared to the FISH assay (Figure 3.1c).

<table>
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<th>Patient no.</th>
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</tr>
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</table>
Table 3.1e: Percentage aneuploidy in spermatozoa recorded by conventional FISH and SCD-FISH.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>FISH (%)</th>
<th>SCD-FISH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
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</tr>
</tbody>
</table>

Figure 3.1c: Percentage sperm aneuploidy in each patient was consistently higher when analysed by SCD-FISH test compared to conventional FISH. Differences between the mean levels of aneuploidy when using FISH and SCD-FISH were statistically significant (n=20, P=0.000; Paired Samples T-Test, P ≤ 0.05, 2-tailed) (P=0.000; Wilcoxon Ranks test; P ≤ 0.05, 2-tailed).
Figure 3.1d: Comparison of mean percentage aneuploidy recorded using the FISH and SCD-FISH tests. Differences between the levels of aneuploidy when using FISH and SCD-FISH were statistically significant (n=20, P=0.000; Paired Samples T-Test, \( P \leq 0.05 \), 2-tailed) (P=0.000; Wilcoxon Ranks test, \( P \leq 0.05 \), 2-tailed).

The Paired Samples T-Test generated a statistically significant difference between the levels of aneuploidy when using FISH and SCD-FISH; \( P=0.000 \) (n=20, SD=3.81, \( P \leq 0.05 \), 2-tailed) (Figure 3.1d).

The Wilcoxon Ranks test was also used to statistically analyse the two sets of data generated; \( P=0.000 \) (\( P \leq 0.05 \), 2-tailed), confirming the statistically significant difference between the level of aneuploidy detected by SCD-FISH and conventional FISH (Figure 3.1d). These results suggest that SCD-FISH does not provide the same results as FISH. However, they could be equivalent, but on different scales. It was therefore interesting to test the correlation between these two tests.

The correlation between the level of aneuploidy when the same twenty sperm samples were assessed by the established FISH method and SCD-FISH was statistically significant (n=20; \( r=0.51 \), \( P=0.021 \)) (Pearson Correlation, \( P \leq 0.05 \), 2-tailed) (Figure 3.1e). The high correlation coefficient of \( r=0.51 \) suggests that the same underlying quantity (i.e. percentage of cells displaying aneuploidy) was being measured, but not on the same scale. A lower correlation (\( r<0.2 \)) would imply that the same quantity is not being measured.
3.1e Investigating the relationship between DNA fragmentation and aneuploidy in spermatozoa

A statistically significant negative correlation was generated when comparing the relationship between sperm aneuploidy and DNA fragmentation using FISH and SCD-FISH respectively (n=20, r=-0.541, P=0.014) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.1f). Samples processed by FISH and SCD also demonstrated a statistically significant negative correlation (n=20, r=-0.457, P=0.043) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.1g). The correlation between DNA fragmentation and aneuploidy in sperm cells processed by FISH and Halosperm® generated a small positive correlation, which was not statistically significant (n=20, r=0.355, P=0.125) (Pearson Correlation, P ≤ 0.05, two-tailed). Surprisingly, the samples subjected to the gold standard analysis techniques of FISH and SCSA also generated a small negative correlation of no significance (n=20, r=-0.078, P=0.742) (Pearson Correlation, P ≤ 0.05, two-tailed). SCD-FISH was used on twenty samples to simultaneously determine the relationship between aneuploidy and DNA fragmentation in the same sperm cells. Statistical significance was not established (n=20, r=-0.191, P=0.419) (Pearson Correlation, P ≤ 0.05, two-tailed).
3.1f Relationship between sperm concentration, total percentage motility, patient age, days of abstinence, and sperm DNA fragmentation

Sperm concentration and total percentage motility, at room temperature 20 minutes following ejaculation, were assessed by embryologists at the CRGH as a part of standard semen analysis on the day of semen production. Patients provided information regarding their age and days of abstinence prior to ejaculation for semen analysis.
**Sperm concentration**: Although a relatively strong negative correlation was observed between sperm concentration (n=20, mean=38.35, SD=21.94) and percentage DNA fragmentation using the SCSA test (n=20, mean=16.10, SD=8.71), the relationship was not statistically significant (n=20, r=-0.399, P=0.081) (Pearson Correlation, P ≤ 0.05, two-tailed). Still, considering the relatively high r value of 0.399, it can be assumed that sperm DNA fragmentation increases with decreasing levels of sperm concentration.

The relationship between sperm concentration (n=20, mean=38.35, SD=21.94) and DNA fragmentation assessed by the SCD test (n=20, mean=6.14, SD=9.06) was also not statistically significant (n=20, r=-0.150, P=0.529) (Pearson Correlation, P ≤ 0.05, two-tailed).

When sperm concentration (n=20, mean=38.35, SD=21.94) was compared to DNA fragmentation measured by the Halosperm® test (n=20, mean=19.64, SD=14.23), a statistically significant negative correlation was observed (n=20, r=-0.485, P=0.030) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.1h). It can therefore be assumed that sperm DNA fragmentation (when analysed using the Halosperm® test) increases in proportion to a decline in sperm concentration.

![Relationship between sperm concentration and percentage DNA fragmentation measured by the Halosperm® test](image)

**Figure 3.1h**: Relationship between sperm concentration and DNA fragmentation measured by the Halosperm® test. A statistically significant correlation was observed between these two parameters (n=20, P=0.030; Pearson Correlation, P ≤ 0.05, two-tailed).

The weak relationship (n=20, r=0.088, P=0.712) (Pearson Correlation, P ≤ 0.05, two-tailed) observed between sperm concentration (n=20, mean=38.35, SD=21.94) and percentage DNA fragmentation assessed by the SCD-FISH test (n=20, mean=52.38, SD=34.04) was not statistically significant.
**Total percentage motility:** The relationship between sperm motility (n=20, mean=54.35, SD=14.05) and percentage DNA fragmentation measured by the SCSA test (n=20, mean=16.10, SD=8.71) was not statistically significant (n=20, r=-0.339, P=0.144) (Pearson Correlation, P ≤ 0.05, two-tailed).

A negative correlation (n=20, r=-0.164, P=0.490) (Pearson Correlation, P ≤ 0.05, two-tailed) was also observed between total percentage motility (n=20, mean=54.35, SD=14.05) and DNA fragmentation measured by the SCD test (n=20, mean=6.14, SD=9.06) but was not statistically significant.

Comparison of total percentage motility (n=20, mean=54.35, SD=14.05) and sperm DNA fragmentation using the Halosperm® test (n=20, mean=19.64, SD=14.23) generated a relatively strong negative correlation, although it was not statistically significant (n=20, r=-0.427, P=0.061) (Pearson Correlation, P ≤ 0.05, two-tailed). It is possible that a larger sample size would have resulted in a statistically significant outcome, demonstrating how sperm motility decreases with an increase in DNA fragmentation. Taking the previous observation of the relationship between sperm concentration and DNA fragmentation evaluated by the Halosperm® test into consideration, it appears that this test may have some potential in the study of sperm parameters and DNA fragmentation.

A negative relationship was observed between total percentage motility (n=20, mean=54.35, SD=14.05) and sperm DNA fragmentation (n=20, mean=52.38, SD=34.04) when measured by SCD-FISH. This observation was not statistically significant (n=20, r=-0.261, P=0.266) (Pearson Correlation, P ≤ 0.05, two-tailed).

**Patient age:** The relationship between patient age (n=20, mean=35.45, SD=4.96) and sperm DNA fragmentation measured by the SCSA test (n=20, mean=16.10, SD=8.71) was not statistically significant (n=20, r=0.064, P=0.790) (Pearson Correlation, P ≤ 0.05, two-tailed).

Likewise, patient age (n=20, mean=35.45, SD=4.96) had no effect on sperm DNA fragmentation measured by the SCD test (n=20, mean=6.14, SD=9.06) (n=20, r=0.051, P=0.831) (Pearson Correlation, P ≤ 0.05, two-tailed).

A negative correlation (n=20, r=-0.237, P=0.314) (Pearson Correlation, P ≤ 0.05, two-tailed) was observed between patient age (n=20, mean=35.45, SD=4.96) and sperm DNA fragmentation measured by the Halosperm® test (n=20, mean=19.64, SD=14.23), but again, was not statistically significant.
Patient age (n=20, mean=35.45, SD=4.96) did not correlate with sperm DNA fragmentation measured by the SCD-FISH test (n=20, mean=52.38, SD=34.04) (n=20, r=-0.027, P=0.911) (Pearson Correlation, P ≤ 0.05, two-tailed).

**Days of abstinence**: Surprisingly, a relatively strong negative correlation of statistical significance (n=16, r=-0.594, P=0.015) (Pearson Correlation, P ≤ 0.05, two-tailed) was observed between the days of abstinence (n=16, mean=3.38, SD=1.20) and sperm DNA fragmentation when analysed by the gold standard SCSA test (n=16, mean=15.68, SD=9.24) (Figure 3.1i). This observation suggests that as the days of abstinence decreases, the level of sperm DNA fragmentation increases.

![Relationship between days of abstinence and percentage sperm DNA fragmentation measured by the SCSA test](image)

Figure 3.1i: A statistically significant negative correlation was observed between the days of abstinence and sperm DNA fragmentation recorded using the SCSA test (n=16, P=0.015; Pearson Correlation, P ≤ 0.05, two-tailed).

A negative correlation existed between the days of abstinence (n=16, mean=3.38, SD=1.20) and sperm DNA fragmentation measured by the SCD test (n=16, mean=6.86, SD=10.05). This observation was not statistically significant (n=16, r=-0.344, P=0.193) (Pearson Correlation, P ≤ 0.05, two-tailed)

A negative correlation (n=16, r=-0.194, P=0.472) (Pearson Correlation, P ≤ 0.05, two-tailed) was also observed between days of abstinence (n=16, mean=3.38, SD=1.20) and sperm DNA fragmentation detected by the Halosperm® test (n=16, mean=17.44, SD=9.84) but was not statistically significant.

Patients provided information regarding the days of abstinence prior to semen analysis. A correlation of no statistical significance (n=16, r=-0.277, P=0.300) (Pearson Correlation, P ≤ 0.05, two-tailed) was observed between the days of abstinence (n=16, mean=3.38, SD=1.20) and sperm DNA fragmentation analysed by SCD-FISH (n=16, mean=53.66, SD=34.46).
<table>
<thead>
<tr>
<th></th>
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<th>Halosperm</th>
<th>SCD-FISH</th>
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<td>NS</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td><strong>Days of abstinence</strong></td>
<td>P=0.015</td>
<td>NS</td>
<td>NS</td>
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</tr>
</tbody>
</table>

Table 3.1f: Statistically significant correlations observed when comparing the relationship between sperm concentration, total percentage motility, patient age, and days of abstinence with sperm DNA fragmentation analysed by the SCSA, SCD, Halosperm and SCD-FISH assays. NS=not statistically significant; Pearson Correlation, $P \leq 0.05$, two-tailed.

3.1g **Relationship between sperm concentration, total percentage motility, patient age, days of abstinence, and sperm aneuploidy**

The levels of aneuploidy detected using FISH and SCD-FISH were compared to sperm concentration, total percentage motility at 20 minutes, days of abstinence and patient age.

**Sperm concentration**: A positive correlation of no significance ($n=20$, $r=0.233$, $P=0.323$) (Pearson Correlation, $P \leq 0.05$, two-tailed) was observed between sperm concentration ($n=20$, mean=38.35, SD=21.94) and percentage sperm aneuploidy measured by FISH ($n=20$, mean=5.63, SD=1.64). The relationship between sperm concentration ($n=20$, mean=38.35, SD=21.94) and percentage sperm aneuploidy measured by SCD-FISH ($n=20$, mean=17.94, SD=4.38) was also not statistically significant ($n=20$, $r=-0.088$, $P=0.714$) (Pearson Correlation, $P \leq 0.05$, two-tailed).

**Total percentage sperm motility**: The relationship between total % motility ($n=20$, mean=54.35, SD=14.05) and percentage sperm aneuploidy measured by FISH ($n=20$, mean=5.63, SD=1.64) was not statistically significant ($n=20$, $r=0.058$, $P=0.810$) (Pearson Correlation, $P \leq 0.05$, two-tailed). The relationship between total % motility ($n=20$, mean=54.35, SD=14.05) and percentage sperm aneuploidy measured by SCD-FISH ($n=20$, mean=17.94, SD=4.38) was also not statistically significant ($n=20$, $r=-0.224$, $P=0.343$) (Pearson Correlation, $P \leq 0.05$, two-tailed).

**Patient age**: The potential effect of patient age on sperm aneuploidy was assessed. There appeared to be no relationship ($n=20$, $r=0.058$, $P=0.809$) (Pearson Correlation, $P \leq 0.05$, two-tailed) between patient age ($n=20$, mean=35.45, SD=4.96) and sperm aneuploidy assessed by FISH ($n=20$, mean=5.63, SD=1.64). A similar pattern ($n=20$, $r=0.035$, $P=0.884$) (Pearson Correlation, $P \leq 0.05$, two-tailed) was observed between patient age ($n=20$, mean=35.45, SD=4.96) and sperm aneuploidy when measured by SCD-FISH ($n=20$, mean=17.94, SD=4.38).
**Days of abstinence prior to semen analysis:** A positive correlation \( (n=16, r=0.467, P=0.068) \) (Pearson Correlation, \( P \leq 0.05, \) two-tailed) existed between the days of abstinence \( (n=16, \text{mean}=3.38, \text{SD}=1.20) \) and the level of sperm aneuploidy when assessed by FISH \( (n=20, \text{mean}=35.45, \text{SD}=4.96) \), but was not statistically significant. A correlation co-efficient of 0.467 is relatively strong, and considering \( P=0.068 \), the lack of statistical significance may once again be due to the small sample size. A negative correlation of no significance \( (n=16, r=-0.296, P=0.266) \) (Pearson Correlation, \( P \leq 0.05, \) two-tailed) was generated when comparing the days of abstinence \( (n=16, \text{mean}=3.38, \text{SD}=1.20) \) and sperm aneuploidy using SCD-FISH \( (n=16, \text{mean}=18.11, \text{SD}=4.20) \).

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Table 3.1g: Statistically significant correlations observed when comparing the relationship between sperm concentration, total percentage motility, patient age, days of abstinence with sperm aneuploidy analysed by the FISH and SCD-FISH assays. NS=not statistically significant; Pearson Correlation, \( P \leq 0.05, \) two-tailed.

### 3.1h Study conclusions

The assessment of DNA fragmentation and aneuploidy in spermatozoa using the SCSA, SCD, Halosperm® and SCD-FISH assays concluded that:

- When analysing sperm DNA fragmentation using the SCSA, SCD, Halosperm® and SCD-FISH assays, only the Halosperm® assay is comparable to the gold standard SCSA test.
- The SCD and SCD-FISH tests are unsuitable to analyse sperm DNA fragmentation.
- The SCD-FISH test consistently over-estimates the degree of sperm aneuploidy and is an unreliable assay.
- A negative correlation exists between sperm DNA fragmentation and aneuploidy when assessed using the SCD-FISH and FISH tests, and the SCD and FISH tests.
- The degree of sperm DNA fragmentation increases as sperm concentration decreases, as assessed by the Halosperm® assay.
- A negative correlation exists between the days of abstinence and sperm DNA fragmentation when analysed by the gold standard SCSA test.
3.2 The assessment of processes involved in ART on sperm DNA damage and PS translocation

Refer Aims 1.9b, and Materials and Methods 2.2

The Annexin V and TUNEL tests were used on semen samples from 40 male patients attending the CRGH for IVF/ICSI treatment to determine the level of PS translocation and DNA fragmentation following processes involved in sperm handling (refer Flow Diagram 2.2a). Phosphatidylserine (PS) is a phospholipid present in the inner membrane of cells. The translocation of sperm PS from the inner to the outer leaflet of the plasma membrane is associated with sperm apoptosis and male infertility.

Image 3.2a: Spermatozoa processed by Annexin V. The image on the left is of sperm cells stained with DAPI. The image on the right is of the same sperm cells observed under the microscope filter for Spectrum Green. The absence of fluorescent signals suggests that the cells in view do not have any PS translocation. Magnification: x600

Image 3.2b: Spermatozoa processed by TUNEL. The image on the left is of sperm cells stained with DAPI. The image on the right is of the same sperm cells observed under the microscope filter for Spectrum Green. The presence two fluorescent signals (TUNEL positivity) marked by arrows, suggest that those two specific cells contain DNA fragmentation. Magnification: x600
3.2a Relationship between sperm PS translocation and DNA fragmentation

Percentage PS translocation and DNA fragmentation in 40 untreated sperm samples ranged from 0.2-33.6% (n=20, mean=7.19, SD=5.53) and 0-37.6% (n=20, mean=7.63, SD=7.88) respectively (Table 3.2a). A paired-samples t-test suggested that there was no significant difference between the levels of DNA fragmentation and PS translocation in the 40 samples analysed (P=0.774) (Paired Samples T Test, P ≤ 0.05, two-tailed). This observation was further verified by the Wilcoxon Signed Ranks Test (P= 0.977) (Wilcoxon Signed Ranks Test, P ≤ 0.05, two-tailed).

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Table 3.2a: Percentage sperm DNA fragmentation and PS translocation assessed by TUNEL and Annexin V respectively.

When sperm PS translocation was compared to sperm DNA fragmentation, no correlation was observed. This result was not statistically significant (n=40, r=-0.009, P=0.957) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.2a).

3.2b Relationship between sperm concentration, total percentage motility, male age and days of abstinence with sperm DNA fragmentation and PS translocation

Sperm concentration and total percentage motility, at room temperature 20 minutes following ejaculation, were assessed by embryologists at the CRGH as a part of standard semen analysis on the day of semen production. Patients provided information regarding their age and days of abstinence
prior to ejaculation for semen analysis. These values were compared to the level of phosphatidylserine translocation and DNA fragmentation in fresh/neat, untreated sperm samples from each patient (Table 3.2b).

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Table 3.2b: Percentage PS translocation assessed by Annexin V, percentage DNA fragmentation assessed by TUNEL, sperm concentration, total percentage motility, male age and days of abstinence prior to ejaculation on the day of semen analysis.

**Sperm concentration:** The relationship between sperm concentration and sperm PS translocation and DNA fragmentation was investigated. Sperm concentration ranged from 0.5-115M/ml (n=40, mean=32.53, SD=24.97) (Table 3.2b). The correlation between percentage PS translocation and sperm concentration was not statistically significant (n=40, r=-0.224, P=0.167) (Pearson Correlation, P ≤ 0.05, two-tailed). As with PS translocation, whilst DNA fragmentation increased as sperm concentration decreased, the correlation between these two parameters was also not statistically significant (n=40, r=-0.205, P=0.205) (Pearson Correlation, P ≤ 0.05, two-tailed).

When 40 patients were grouped according to sperm concentration, percentage DNA fragmentation in samples with concentrations <15M/ml (n=10, mean=13.4, SD=12.62) was significantly higher than those with concentrations >15M/ml (n=30, mean=5.79, SD=3.75) (P=0.009 if equal variances assumed) (Independent-samples t-test, P ≤ 0.05, two-tailed) (Figure 3.2b).

**Figure 3.2b:** Difference in percentage sperm DNA fragmentation between sperm samples with a concentration <15M/ml (n=10) and >15M/ml (n=30). The difference in the level of DNA fragmentation between these two groups was statistically significant (P=0.009) (Independent-samples t-test, P ≤ 0.05, two-tailed).
**Sperm motility:** Total percentage motility following a 20 minute incubation at room temperature, ranged from 38-86% (n=40, mean=55.55, SD=10.40) (Table 3.2b). Whilst a decline in sperm motility as PS translocation increased was expected, the negative correlation observed was not statistically significant (n=40, r=-0.153, P=0.347) (Pearson Correlation, P ≤ 0.05, two-tailed). The negative correlation between percentage DNA fragmentation and percentage motility was also not statistically significant (n=40, r=-0.090, P=0.582) (Pearson Correlation, P ≤ 0.05, two-tailed).

A weakly positive correlation was observed between sperm concentration (n=40, mean=55.55, SD=10.53) and total percentage motility at 20 minutes (n=40, mean=32.53, SD=24.97) but was not statistically significant (n=40, r=0.147, P=0.364) (Pearson Correlation, P ≤ 0.05, two-tailed).

**Male age:** The effect of patient age on sperm PS translocation and DNA fragmentation was investigated. Male patient age ranged from 28-56 years (n=40, mean=38.65, SD=6.45) (Table 3.2b). The correlation between percentage PS translocation and patient age failed to reach statistical significance (n=40, r=-0.007, P=0.967) (Pearson Correlation, P ≤ 0.05, two-tailed). Although percentage DNA fragmentation increased with patient age, this relationship was also not statistically significant (n=40, r=0.244, P=0.129) (Pearson Correlation, P ≤ 0.05, two-tailed).

Patients were categorized by those <40 years and those ≥40 years of age, and mean percentage PS translocation and DNA fragmentation of the two groups was compared. Percentage PS translocation in the sperm samples from patients above and equal to the age of 40 years (n=16, mean=7.61, SD=7.60) was higher than those below the age 40 years (n=24, mean=6.90, SD=3.73), but not statistically significant (P=0.695) (Independent-samples t-test, P ≤ 0.05, two-tailed) (Figure 3.2c). Percentage DNA fragmentation in the cohort above and equal to the age of 40 years (n=16, mean=9.91, SD=8.19) was higher than those below the age 40 years (n=24, mean=6.11, SD=6.12), but not statistically significant (P=0.137) (Independent-samples t-test, P ≤ 0.05, two-tailed) (Figure 3.2d).
Figure 3.2c: Difference in percentage sperm PS translocation between sperm samples from patients <40 years (n=24) and those ≥40 years (n=16). The difference was not statistically significant (P=0.695; Independent-samples t-test, P ≤ 0.05, two-tailed).

Figure 3.2d: Difference in percentage sperm DNA fragmentation between sperm samples from patients <40 years (n=24) and those ≥40 years (n=16). The difference was not statistically significant (P=0.137; Independent-samples t-test, P ≤ 0.05, two-tailed).

Days of abstinence: The potential effect of the period of abstinence prior to semen analysis was compared to PS translocation and sperm DNA fragmentation. Days of abstinence ranged from 1-9 days (n=34, mean=3.74, SD=1.66) (Table 3.2b). Data concerning the period of abstinence was not available for 6 patients (patient no. 1, 12, 19, 28, 32, and 33).

The negative correlation observed between PS translocation and days of abstinence was not statistically significant (r=-0.065, P=0.715) (Pearson Correlation, P ≤ 0.05, two-tailed). The relationship between percentage DNA fragmentation and days of abstinence generated a positive
correlation but was also not statistically significant (n=34, r=0.199, P=0.259) (Pearson Correlation, P ≤ 0.05, two-tailed).

When total % motility at 20 minutes was compared to days of abstinence, the very weak positive correlation observed was not statistically significant (n=34, r=0.51, P=0.774) (Pearson Correlation, P ≤ 0.05, two-tailed). As expected, a positive correlation was observed between sperm concentration and days of abstinence. This relationship was statistically significant (n=34, r=0.422, P=0.013) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.2e).

![Relationship between sperm concentration and days of abstinence](image)

**Figure 3.2e**: Relationship between period of abstinence and sperm concentration. A statistically significant positive correlation was observed between these two parameters (n=34, r=0.422, P=0.013; Pearson Correlation, P ≤ 0.05, two-tailed).

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**Table 3.2c**: Statistical significance observed when comparing the correlation between sperm concentration, total percentage motility, patient age, and days of abstinence with sperm PS translocation and DNA fragmentation analysed by the Annexin V and TUNEL assays respectively. NS=not statistically significant; Pearson Correlation, P ≤ 0.05, two-tailed.
3.2c Effect of cigarette smoking and alcohol consumption on sperm DNA fragmentation and PS translocation

To determine the effect of cigarette smoking on sperm membrane and nuclear damage, the number of cigarettes smoked a day (by the patient’s own admission) was compared to sperm PS translocation and DNA fragmentation.

Information regarding their smoking habits was only available for 21 patients. The number of cigarettes smoked a day ranged from 0-30 (n=21, mean=3.19, SD=7.88). Smoking did not appear to have a notable effect on either sperm PS translocation or DNA fragmentation. A negligible correlation was observed between the number of cigarettes smoked per day and sperm PS translocation (n=21, r=0.05, P=0.829) (Pearson Correlation, P ≤ 0.05, two-tailed). This observation was not statistically significant. A relationship of no statistical significance was also observed between the number of cigarettes smoked per day and sperm DNA fragmentation (n=21, r=-0.038, P=0.87) (Pearson Correlation, P ≤ 0.05, two-tailed).

To determine the effect of alcohol intake on sperm membrane and nuclear damage, units of alcohol consumed per week (by the patient’s own admission) was compared to sperm PS translocation and DNA fragmentation. Information regarding their drinking habits was only available for 24 patients. Alcohol consumption ranged from 0-40 units/week (n=24, mean=12.56, SD=9.78). A weak negative correlation was observed between the levels of alcohol consumption and PS translocation (n=24, r=-0.253, P=0.232) (Pearson Correlation, P ≤ 0.05, two-tailed). This observation was not statistically significant. A relationship of no statistical significance was observed between the levels of alcohol consumption and DNA fragmentation (n=24, r=0.114, P=0.594) (Pearson Correlation, P ≤ 0.05, two-tailed).

3.2d Effect of sperm freezing on sperm DNA fragmentation and PS translocation

To determine the effect of sperm freezing on DNA damage, 40 fresh samples were frozen at -80°C for a period of 2 months, and DNA fragmentation was analysed in thawed samples using TUNEL. These results were then compared to the level of DNA fragmentation recorded on the day of production in the original untreated sperm samples from the same 40 patients. Percentage DNA fragmentation ranged from 3.8-57% (mean=16.65%, SD=11.59) in frozen-thawed samples and 0-37.6% (mean=7.63%, SD=7.88) in the control group of 40 untreated sperm samples (Table 3.2d).
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Table 3.2d: Percentage DNA fragmentation in 40 sperm samples before and after freeze-thawing, as assessed by TUNEL.

The difference in the level of DNA fragmentation between the untreated control group (mean=7.63%, SD=7.88) and frozen-thawed cohort (mean=16.65%, SD=11.59) was statistically significant (P=0.000; Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed) (Figure 3.2f). This observation was further verified by the Paired Samples T-test (P=0.000) (P ≤ 0.05, 2-tailed). It can therefore be assumed that freeze-thawing sperm samples can significantly increase the level of DNA fragmentation. Considering sperm samples are frozen in the absence of a cryoprotectant for laboratory research and clinical analysis (as with the SCSA test), freezing sperm prior to experimental procedure and/or sperm DNA fragmentation detection will most likely give rise to biased/over-estimated results.

Figure 3.2f: Mean percentage difference between sperm DNA fragmentation in 40 semen samples before and after freezing. The difference in the level of DNA fragmentation between the untreated control group and frozen-thawed sperm samples was statistically significant (n=40, P=0.000) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed).

A statistically significant positive correlation existed between the level of sperm DNA fragmentation in fresh and frozen-thawed semen samples (n=40, r=0.442, P=0.004) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.2g). This relatively high correlation suggests that the same underlying quantity (DNA fragmentation) is being measured, but on different scales. Therefore, it can be assumed that sperm freezing increases DNA fragmentation in sperm samples proportionately. Whilst the smallest increment in DNA fragmentation between the fresh and frozen-thawed samples was by a factor of 0.683, the largest increase was by a factor of 26.33. On average, sperm DNA fragmentation increased by a magnitude of 4.44 following freeze-thawing (n=40, SD=5.76).
Figure 3.2g: Relationship between sperm DNA fragmentation in fresh and frozen-thawed sperm samples. The correlation observed was statistically significant (n=40, r=0.442, P=0.004) (Pearson Correlation, P ≤ 0.05, two-tailed).

It was of interest to determine if patient age played a role in the susceptibility of stored sperm to cryo-induced DNA fragmentation. Patient age was compared to the level of DNA fragmentation in frozen-thawed sperm samples. The correlation between patient age and percentage sperm DNA fragmentation in frozen-thawed semen was not statistically significant (n=40, r=0.235, P=0.145) (Pearson Correlation, P ≤ 0.05, two-tailed). Therefore, age is unlikely to have a strong effect on sperm susceptibility to DNA damage.

3.2e Concentration dependent effect of hydrogen peroxide on sperm DNA fragmentation and PS translocation

Twenty semen samples were treated with five different concentrations of H₂O₂ in a dose dependent manner and the effect of H₂O₂ on sperm PS translocation and DNA fragmentation was assessed using Annexin V and TUNEL respectively.

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Table 3.2e: Percentage sperm PS translocation assessed by Annexin V following the incubation of 20 semen samples with different concentrations of hydrogen peroxide.

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Percentage PS translocation at 0mM, 0.1mM, 1mM, 10mM and 100mM H₂O₂ ranged from 2.4-14.14% (n=20, mean=6.47, SD=3.52), 1-17.6% (n=20, mean=7.79, SD=4.04), 3.6-38% (n=20, mean=12.79, SD=8.24), 2.8-47.8% (n=20, mean=27.19, SD=13.54) and 4.4-48.6% (n=20, mean=27.08, SD=12.67) respectively (Table 3.2e).

The difference in the mean level of PS translocation between the untreated group and samples exposed to H₂O₂ concentrations of 1mM (P=0.001), 10mM (P=0.000) and 100mM (P=0.000) was statistically significant (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed). However, mean percentage PS translocation between the untreated cohort and the group exposed to 0.1mM H₂O₂ (P=0.056) was not significantly different (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed) (Figure 3.2h). It can therefore be assumed that 1mM, 10mM and 100mM H₂O₂ significantly increases the level of sperm phosphatidylserine translocation.

This observation was further verified by a paired samples t-test which also presented statistically significant differences between sperm samples treated with 1mM and the control group (n=20, P=0.002), 10mM and the control group (P=0.000), and 100mM and the control group (P=0.000) (Paired Samples T-Test, P ≤ 0.05, 2-tailed). Using this statistical test, a significant difference also resulted between sperm samples treated with 0.1mM H₂O₂ and the control group (P=0.045) (Paired Samples T-Test, P ≤ 0.05, 2-tailed).
Figure 3.2h: Mean percentage sperm PS translocation assessed by Annexin V following the incubation of sperm with different concentrations of hydrogen peroxide. Statistically significant differences were observed between sperm treated with 1mM $H_2O_2$ and the untreated control group (n=20, P=0.001), 10mM $H_2O_2$ compared to the untreated control group (n=20, P=0.000), and 100mM $H_2O_2$ and the untreated control group (n=20, P=0.000) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed).

![Graph showing mean % phosphatidylserine translocation in sperm treated with $H_2O_2$](image)

Sperm DNA fragmentation following $H_2O_2$ treatment

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Table 3.2f: The levels of sperm DNA fragmentation assessed by TUNEL following the incubation of sperm with different concentrations of hydrogen peroxide.

Percentage DNA fragmentation in sperm exposed to 0mM, 0.1mM, 1mM, 10mM and 100mM H$_2$O$_2$ ranged from 0-12.8% (n=20, mean=6.98, SD=3.83), 1.48-17% (n=20, mean=7.24, SD=4.16), 0.6-25.6% (n=20, mean=8.43, SD=5.38), 1.8-29% (n=20, mean=10.25, SD=6.51) and 3.2-70.2% (n=20, mean=19.36, SD=16.70) respectively (Table 3.2f). Mean percentage DNA fragmentation between the control group and samples exposed to 10µM (P=0.022) and 100mM (P=0.000) H$_2$O$_2$ was significantly different (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed). But, the difference in mean percentage DNA fragmentation between the control group and samples exposed to 0.1mM (P=0.717) and 1mM (P=0.270) H$_2$O$_2$ was not statistically significant (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed) (Figure 3.2i).

![Mean % DNA fragmentation in sperm treated with H$_2$O$_2$](image)

Figure 3.2i: Mean percentage sperm DNA fragmentation assessed by TUNEL following the incubation of sperm with different concentrations of hydrogen peroxide. Statistically significant differences resulted between the sperm samples treated with 10mM H$_2$O$_2$ and the untreated control group (n=20, P=0.000), and 100mM H$_2$O$_2$ and the untreated control group (n=20, P=0.000) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed).

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Table 3.2g: Statistical significance observed when studying the dose-dependent effect of hydrogen peroxide on sperm PS translocation and DNA fragmentation in untreated sperm samples.

When the mean levels of PS translocation were compared to DNA fragmentation, a general increase in both parameters was observed (Figure 3.2j). Membrane damage was consistently higher than...
nuclear damage at all concentrations of hydrogen peroxide. There was no significant difference between the percentage mean level of PS translocation and DNA fragmentation at H$_2$O$_2$ concentrations of 0mM (n=20, P=0.704), 0.1mM (n=20, P=0.704), 1mM (n=20, P=0.097) and 100mM (n=20, P=0.157) (T-test, P ≤ 0.05, 2-tailed) (Figure 3.2j). However, there was a statistically significant difference between the two parameters at 10mM H$_2$O$_2$ (n=20, P=0.000) (T-test, P ≤ 0.05, 2-tailed) (Figure 3.2j).

![Relationship between mean % PS translocation and DNA fragmentation at 5 concentrations of H$_2$O$_2$](image)

Figure 3.2j: Comparison between the mean percentages of phosphatidylserine translocation and DNA fragmentation at five different concentrations of H$_2$O$_2$. A significant difference existed between the two parameters at 10mM H$_2$O$_2$ (n=20, P=0.000) (T-test, P ≤ 0.05, 2-tailed).

In spite of the strong positive correlation observed between percentage mean PS translocation and DNA fragmentation at all five concentrations of H$_2$O$_2$, this relationship was not statistically significant (n=20, r=0.774, P=0.125) (Pearson Correlation, P ≤ 0.05, two-tailed) (Figure 3.2k).
Figure 3.2k: Relationship between mean percentage sperm PS translocation and DNA fragmentation at different concentrations of hydrogen peroxide. The result was not statistically significant (n=20, r=0.774, P=0.125) (Pearson Correlation, P ≤ 0.05, two-tailed).

### 3.2f Effect of temperature on sperm DNA fragmentation and PS translocation

Twenty semen samples were incubated at room temperature (25°C), 34°C and 37°C for one hour, and sperm PS translocation and DNA fragmentation was assessed using Annexin V and TUNEL respectively.

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Table 3.2h: The levels of sperm PS translocation assessed by Annexin V following the incubation of sperm at 3 different temperatures for one hour. The level of PS translocation in sperm samples incubated at 37°C for one hour was significantly higher than in samples incubated at 25°C (P=0.019) and 34°C (P=0.027) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed).

To determine the effect of external heat on sperm PS translocation, samples were incubated at 25°C, 34°C and 37°C and assessed by Annexin V. Percentage PS translocation at 25°C, 34°C and 37°C ranged from 0.2-33.6% (n=20, mean=7.9, SD=7.02), 2.4-22.2% (n=20, mean=7.44, SD=4.83) and 1.4-28.6% (n=20, mean = 10.18, SD=6.70) respectively (Table 3.2h).

There was a statistically significant difference in the mean level of PS translocation between the control group (25°C) and samples exposed to 37°C (P=0.019), and between samples exposed to 34°C and those exposed to 37°C (P=0.027) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed) (Figure 3.2l). It can therefore be assumed that the incubation of sperm at 37°C for one hour can significantly increase the levels of phosphatidylserine translocation, compared to sperm stored at room or testicular temperature.

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<th>Temperature</th>
<th>PS Translocation (%)</th>
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<td>25°C</td>
<td>0.2-33.6% (n=20, mean=7.9, SD=7.02)</td>
</tr>
<tr>
<td>34°C</td>
<td>2.4-22.2% (n=20, mean=7.44, SD=4.83)</td>
</tr>
<tr>
<td>37°C</td>
<td>1.4-28.6% (n=20, mean = 10.18, SD=6.70)</td>
</tr>
</tbody>
</table>

Figure 3.2l: Mean percentage sperm PS translocation assessed by Annexin V following the incubation of sperm at 3 different temperatures for one hour. The level of PS translocation in sperm samples incubated at 37°C (n=20) for one hour was significantly higher than in samples incubated at 25°C (n=20, P=0.019) and 34°C (n=20, P=0.027) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed).
To determine the effect of external heat on sperm DNA fragmentation, samples were incubated at 25°C, 34°C and 37°C and assessed by TUNEL. A general increase in DNA damage was observed with increasing temperature. The percentage DNA fragmentation at 25°C, 34°C and 37°C ranged from 0-37.6% (n=20, mean=8.28, SD=10.57), 1.2-25% (n=19, mean=8.34, SD=6.84) and 4.6-22.2% (n=20, mean=11.48, SD=5.17) respectively (Table 3.2i). The semen sample from patient #28 exposed to 34°C could not be analysed due to technical difficulties.

There was a significant difference in the mean level of DNA fragmentation between the control and samples exposed to 37°C (P=0.014), and between samples exposed to 34°C and those exposed to 37°C (P=0.004) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed) (Figure 3.2m). It can therefore be assumed that the incubation of sperm at 37°C for one hour can significantly increase the levels of DNA fragmentation, compared to sperm stored at room or testicular temperature.

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<th>37°C</th>
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Figure 3.2i: The levels of sperm DNA fragmentation assessed by TUNEL following the incubation of sperm at 3 different temperatures for an hour. The semen sample from patient #28 exposed to 34°C could not be analysed due to technical difficulties.
Figure 3.2m: Mean percentage sperm DNA fragmentation assessed by TUNEL following the incubation of sperm at 3 different temperatures for one hour. The level of DNA fragmentation in sperm samples incubated at 37°C (n=20) for one hour was significantly higher than in samples incubated at 25°C (n=20, P=0.014) and 34°C (n=20, P=0.004) (Wilcoxon Ranks Test, P ≤ 0.05, 2-tailed).

Table 3.2j: Statistical significance observed when studying the effect of incubating semen samples at 25°C or 34°C versus 37°C on sperm PS translocation and DNA fragmentation.

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<tr>
<th></th>
<th>PS translocation at 37°C</th>
<th>DNA fragmentation at 37°C</th>
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</thead>
<tbody>
<tr>
<td>PS translocation at 25°C</td>
<td>P=0.019</td>
<td></td>
</tr>
<tr>
<td>PS translocation at 34°C</td>
<td>P=0.027</td>
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<tr>
<td>DNA fragmentation at 25°C</td>
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<td>P=0.014</td>
</tr>
<tr>
<td>DNA fragmentation at 34°C</td>
<td></td>
<td>P=0.004</td>
</tr>
</tbody>
</table>

3.2g Study conclusions

The assessment of potentially detrimental processes involved in ART concluded that:

- Sperm PS translocation and DNA fragmentation are not comparable parameters of sperm apoptosis.
- Patients with sperm concentrations <15M/ml have a greater degree of DNA fragmentation compared to those with sperm concentrations >15M/ml.
- Sperm concentration increases with days of abstinence prior to ejaculation.
- Freeze-thawing sperm without a cryoprotectant significantly increases the degree of DNA fragmentation.
- Hydrogen peroxide has a concentration dependent effect on sperm PS translocation and DNA fragmentation.
• Incubating sperm at 37°C generates a significantly greater degree of DNA fragmentation than at 25°C or 34°C.

3.3 The assessment of cumulus and granulosa cell DNA fragmentation as a biomarker for fertility potential

Refer Aims 1.9c, and Materials and Methods 2.3

Cumulus cells and follicular fluid were obtained from 48 female patients undergoing IVF/ICSI at the Centre for Reproductive and Genetic Health, University College London Hospital. The DeadEnd™ Fluorometric TUNEL System was used to measure the level of DNA fragmentation in these cells.

Image 3.3a: Granulosa cells stained with DAPI following the TUNEL procedure. Magnification: x600.

Image 3.3b: Cumulus cells stained with DAPI following the TUNEL procedure (left). The same cells stained under the filter for Spectrum Green is on the right. The lack of green fluorescent signals from the cumulus cells suggests the lack of DNA fragmentation within the cells in view. These cumulus cells were obtained from a patient undergoing IVF, so sperm cells were also present. A spermatozoa stained with fluorescein-12-dUTP is visible, signifying the presence of DNA fragmentation in the sperm cell. Magnification: x600.
3.3a The assessment of DNA fragmentation in granulosa and cumulus cells from the same patient

Percentage DNA fragmentation in granulosa cells ranged from 0-27.8% (n=31, mean=3.25, SD=5.17), and the levels of cumulus DNA fragmentation ranged from 1.4-45.6% (n=31, mean=17.18, SD=10.4) (Table 3.3a).

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Table 3.3a: Percentage DNA fragmentation in granulosa and cumulus cells following analysis using the TUNEL assay.

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Figure 3.3b: Percentage sperm DNA fragmentation in cumulus and granulosa cells. The degree of DNA fragmentation was almost consistently higher in cumulus rather than granulosa cells.

Both cumulus and granulosa cell samples were obtained from 31 patients. Cumulus cells had a higher level of DNA fragmentation than granulosa cells in 29 of these samples. A statistically significant difference was observed between mean percentage DNA fragmentation in granulosa and cumulus cells (n=31, SD=10.95, P=0.000; Paired samples T-test, P ≤ 0.05, 2-tailed) (Figure 3.3c). The
Wilcoxon Signed Ranks Test was also used to discriminate between the levels of DNA fragmentation in the two cell types, establishing a significant difference; P=0.000 (P ≤ 0.05, 2-tailed).

A weak correlation was observed between the levels of DNA fragmentation in granulosa and cumulus cells (n=31, r=0.139, P=0.455; Paired Samples Correlation, P ≤ 0.05, 2-tailed), but was not statistically significant.

This observation suggests that the level of DNA fragmentation in granulosa and cumulus cells is not comparable, and that cells in closer proximity to the oocyte have a significantly greater level of DNA fragmentation than those further away within the follicle.

### 3.3b The effect of patient age on cumulus and granulosa cell DNA fragmentation

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</table>
The ages of female patients who donated their granulosa (follicular fluid) and cumulus cells ranged from 30-45 years (n=49, mean=37.0, SD=3.31) (Table 3.3b).

Granulosa cells were not available for analysis from 10 patients (patient no. 12, 13, 14, 24, 27, 28, 33, 39, 42 and 45) (Table 3.3b). A positive correlation was observed between patient age (n=39, mean=36.77, SD=3.55) and percentage DNA fragmentation in granulosa cells (n=39, mean=3.51, SD=6.22) but was not statistically significant (n=39, r=0.148, P=0.369) (Pearson Correlation, P ≤ 0.05, two-tailed). The Spearman’s rho correlation further corroborated this (n=39, r=0.025, P=0.881).

Patients who donated follicular fluid were also grouped by age based on those < 37 years and those ≥ 37 years. A statistically significant difference was not observed between mean percentage DNA fragmentation in granulosa cells of patients < 37 years (n=18, mean=2.34, SD=2.59) and those ≥ 37 years (n=21, mean=4.51, SD=8.10) (P=0.259) (Independent Samples Test, P ≤ 0.05, two-tailed) (Figure 3.3d). Equal variances were not assumed because the Levene’s test for equality of variances was P ≤ 0.05 (P=0.048).

Cumulus cells were not available for analysis from 9 patients (patient no. 4, 5, 16, 20, 22, 29, 31, 32 and 41) (Table 3.3b). A very weak correlation, was observed between percentage DNA fragmentation and patient age. However, this correlation was not statistically significant (n=39, r=0.025, P=0.881).
fragmentation in cumulus cells (n=40, mean=16.27, SD=9.61) and patient age (n=40, mean=37.29, SD=3.17) when using both the Pearson Correlation (n=40, r=-0.065, P=0.692) and Spearman’s rho tests (n=40, r=-0.081, P=0.620) (P ≤ 0.05, two-tailed). Neither test demonstrated statistical significance.

A statistically significant difference was not observed between mean percentage DNA fragmentation in cumulus cells of patients < 37 years (n=17, mean=16.92, SD=8.00) and those ≥ 37 years (n=23, mean=15.80, SD=10.80) (P=0.720) (Independent Samples Test, P ≤ 0.05, two-tailed) (Figure 3.3e)

![Relationship between mean % DNA fragmentation in cumulus cells in patients above and below the age of 37 years](image)

Figure 3.3e: Mean percentage cumulus cell DNA fragmentation in patients < 37 years (n=17) and ≥ 37 years (n=23). The difference in the level of mean % cumulus cell DNA fragmentation between the two groups was not statistically significant; (P=0.720; Independent Samples Test, P ≤ 0.05, two-tailed).

These observations suggest that maternal age has no effect on the level of DNA fragmentation in granulosa and cumulus cells.

### 3.3c Effect of ovarian stimulation protocol on percentage DNA fragmentation in cumulus and granulosa cells

Patients were grouped according to the protocol used for ovarian stimulation, prior to undergoing assisted reproduction, and their respective levels of DNA fragmentation in granulosa and cumulus cells assessed. Two patients underwent natural cycles, and the protocol used was not available for two patients.

There was no statistically significant difference between the level of DNA fragmentation in granulosa cells following either one of the 3 protocols; cetrotide (n=19, mean=5.01, SD=8.33), mlp (mid luteal
phase) (n=11, mean=1.55, SD=1.71) and mlp/so (mid luteal phase sub optimal) (n=6, mean=3.50, SD=4.14) (P=0.374; ANOVA) (Figure 3.3f).

Figure 3.3f: There was no significant difference between the mean percentages of granulosa cell DNA fragmentation based on the stimulation protocol; cetrotide (n=19), mlp (n=11), mlp/so (n=6) (P=0.374; ANOVA).

There was also no statistically significant difference between the level of DNA fragmentation in cumulus cells following either one of the 3 protocols; cetrotide (n=20, mean=18.32, SD=10.93), mlp (n=9, mean=12.26, SD=8.85) and mlp/so (n=8, mean=15.04, SD=7.26) (P=0.292; ANOVA) (Figure 3.3g).

Figure 3.3g: There was no significant difference between the mean percentages of granulosa cell DNA fragmentation based on the stimulation protocol; cetrotide (n=20), mlp (n=9), mlp/so (n=9) (P=0.292; ANOVA).
3.3d The assessment of cumulus cell DNA fragmentation following IVF versus ICSI

The percentage DNA fragmentation in cumulus cells from oocytes undergoing ICSI ranged from 8.4-45.6% (n=18, mean=18.26, SD=10.36), whilst those from oocytes undergoing IVF ranged from 1.4-29.6% (n=22, mean=14.65, SD=8.86) (Table 3.3c).

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Table 3.3c: Percentage sperm DNA fragmentation in cumulus cells obtained from oocytes undergoing IVF and ICSI.

Cumulus cells from oocytes subjected to ICSI were incubated at 37°C overnight in the laboratory, and were not exposed to any spermatozoa. Surprisingly, it was these cell samples that appeared to have a higher level of DNA damage than the cumulus cells exposed to sperm, despite the difference not reaching statistical significance (Figure 3.3h).
The Levene’s test for equality of variance was $P=0.722$, therefore equal variances were assumed. An Independent Samples $t$-test resulted in $P=0.241$, suggesting the lack of a significant difference between the level of cumulus DNA fragmentation following IVF and ICSI ($P \leq 0.05$, 2-tailed) (Figure 3.3h). The Mann-Whitney U test confirmed this by also producing a result of $P=0.438$ ($P \leq 0.05$, 2-tailed), which was not statistically significant.

![Mean % DNA fragmentation in cumulus cells following IVF and ICSI](image)

Figure 3.3h: The mean % difference in the level of DNA fragmentation in cumulus cells obtained either IVF ($n=22$) or ICSI ($n=18$) cycles was not statistically significant ($P=0.241$; Independent samples $t$-test).

This result suggests that the overnight incubation of cumulus cells with spermatozoa has no effect on cumulus cell DNA fragmentation.

### 3.3e Relationship between cumulus and granulosa cell DNA fragmentation and fertilization rate

Fertilization rate was determined by calculating the proportion of oocytes that achieved successful fertilization, of the total oocytes inseminated with sperm. Fertilization rate ranged from 25%-100% ($n=48$, mean=71.89%, SD=20.60) (Table 3.3d).

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</table>
A negative correlation of no statistical significance (n=38, r=-0.129, P=0.440) (Pearson Correlation, P ≤ 0.05, two-tailed) was generated between percentage DNA fragmentation in granulosa cells (n=38, mean=3.57, SD= 6.21) and fertilization rate (n=38, mean=72.26, SD= 20.32).

No correlation existed between percentage DNA fragmentation in cumulus cells (n=40, mean=16.27, SD=9.49) and fertilization rate (n=40, mean=69.02, SD=20.61) (r=-0.013, P=0.937) (Pearson Correlation, P ≤ 0.05, two-tailed).

This suggests that the degree of DNA fragmentation in granulosa and cumulus cells have no influence on oocyte fertility potential.

### 3.3f The influence of patient age on fertilization rate

Fertilization rate was determined by calculating the number successful fertilizations (number of oocytes with 2 pronuclei) from the total number of oocytes inseminated following IVF or ICSI. Patient age was compared to fertilization rate following insemination. The rate of fertilization was not available for patient no. 4 (Table 3.3e).

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Figure 3.3e: The relationship between fertilization rate and patient age.

When assessing the relationship between patient age (n=48, mean=37, SD=3.35) and fertilization rate (n=48, mean=71.89, SD=20.81), a weak correlation was observed (n=48, r=0.081, P=0.586) (Pearson Correlation, P ≤ 0.05, two-tailed), but was not statistically significant.
A statistically significant difference was not observed when comparing fertilization rate in patients <37 years (n=21, mean=70.64, SD=19.14) and those ≥ 37 years (n=27, mean=72.87, SD=22.34) (P=0.716) (Independent Samples Test, P ≤ 0.05, two-tailed) (Figure 3.3i).

Figure 3.3i: Relationship between fertilization rate in patients <37 years (n=21) and ≥37 years (n=27). The difference in the rate of fertilization between the two age groups was not statistically significant (P=0.716; Independent Samples Test, P ≤ 0.05, two-tailed).

These observations suggest that maternal age has no influence on the rate of fertilization and that there is no relationship between these two parameters.

3.3g Relationship between the rate of embryos that developed into blastocysts and percentage DNA fragmentation in cumulus and granulosa cells

The rate of embryos that developed into blastocysts was determined by calculating the proportion of total embryos that developed from cleavage stage embryos and were suitable for ART. Blastocyst rate ranged from 9.09%-100% (n=45, mean=53.06, SD=23.72) (Table 3.3f). Data regarding the development of blastocysts was not available for 4 patients (no. 13, 31, 32 and 47) (table 3.3f)

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Figure 3.3f: Relationship between the rate of embryos that developed into blastocysts in each patient and percentage DNA fragmentation in cumulus and granulosa cells.

Granulosa cells were not obtained from 10 patients. A weakly positive correlation of no statistical significance (n=36, r=0.069, P=0.689) (Pearson Correlation, P ≤ 0.05, two-tailed) was observed between percentage DNA fragmentation in granulosa cells (n=36, mean=3.71, SD=6.44) and rate of embryos that developed into blastocysts (n=36, mean=50.80, SD=24.52). Cumulus cells were not obtained from 9 patients. A negative correlation of no statistical significance (n=38, r=-0.130, P=0.437) (Pearson Correlation, P ≤ 0.05, two-tailed) was observed between percentage DNA fragmentation in cumulus cells (n=38, mean=16.65, SD=9.72) and rate of embryos that developed into blastocysts (n=38, mean=51.38, SD=21.26).

These results demonstrate that granulosa and cumulus cell DNA fragmentation have no effect on the rate of embryo development into blastocysts.

3.3h Relationship between cumulus and granulosa cell DNA fragmentation and pregnancy outcome

To observe the effect of DNA fragmentation in cumulus and granulosa cells on pregnancy outcome, patients who achieved single or multiple pregnancies following the transfer of one or more blastocysts following a single assisted reproduction cycle was classified as a ‘positive pregnancy’. Patients who resulted in a singleton intrauterine pregnancy or twin pregnancy were all categorized as having a ‘positive’ pregnancy. Those who failed to get pregnant following the transfer of one or more blastocysts following a single assisted reproduction cycle was classified as a ‘negative pregnancy’. Patient no. 43 achieved a clinical pregnancy but it was followed by a miscarriage. Blastocysts were not transferred in patient no. 46 and 47 as they underwent PGD (for reciprocal and Robertsonian translocations) and the embryos were affected. Information regarding pregnancy outcome was not available for patient no. 4, 12, 13, 18, 29, 31, 32, and 40 (Table 3.3g).

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Table 3.3g: Relationship between cumulus and granulosa cell DNA fragmentation and pregnancy outcome (0=negative pregnancy; 1=positive clinical pregnancy, singleton pregnancy; 2=positive pregnancy, twin pregnancy. Pregnancy outcome results were not available for patient no. 4, 12, 13, 18, 29, 31, 32, and 40.

Surprisingly, the mean level of granulosa cells DNA fragmentation was higher in the group of patients that achieved a positive pregnancy than those who didn’t achieve a pregnancy (Figure 3.3j). The difference in the level of granulosa cell DNA fragmentation in patients who achieved a positive pregnancy (n=21, mean=3.93, SD=6.17) and those who failed to achieve a positive pregnancy (n=10, mean=1.90, SD=2.27) was not statistically significant based on an Independent Samples Test (P=0.324) (P ≤ 0.05, 2-tailed) (Figure 3.3j). The Mann-Whitney U test further confirmed this statistical observation (P=0.385) (P ≤ 0.05, 2-tailed).

The mean level of DNA fragmentation in cumulus cells appeared to be the same in the group of patients that achieved a positive pregnancy and those who did not (Figure 3.3k). The difference in the level of cumulus cell DNA fragmentation in patients who achieved a positive pregnancy (n=24, mean=16.71, SD=10.31) and those who failed to achieve a positive pregnancy (n=10, mean=16.43, SD=6.77) was not statistically significant (n=34, P=0.938, Independent Samples Test, P ≤ 0.05, 2-tailed) (Figure 3.3j). The Mann-Whitney U test further confirmed this statistical observation (P=1.00) (P ≤ 0.05, 2-tailed).

![Relationship between mean % DNA fragmentation in granulosa cells and pregnancy outcome](image)

Figure 3.3j: The difference in the mean level of granulosa cell DNA fragmentation between the group that achieved a positive pregnancy (n=21) and the group that failed to achieve a pregnancy (n=10) was not statistically significant (P=0.324; Independent Samples Test, P ≤ 0.05, 2-tailed).
Figure 3.3k: The difference in the mean level of cumulus cell DNA fragmentation between the group that achieved a positive pregnancy (n=24) and the group that failed to achieve a pregnancy (n=10) was not statistically significant (P=0.938; Independent Samples Test, P ≤ 0.05, 2-tailed).

These results suggest that granulosa and cumulus cell DNA fragmentation have no effect on pregnancy outcome, and that there is no relationship between these parameters.

3.3i Study conclusions

The assessment of DNA fragmentation in granulosa and cumulus cells in relation to fertility potential concluded that:

- The degree of DNA fragmentation in granulosa and cumulus cells is not comparable.
- Maternal age has no effect on the level of DNA fragmentation in granulosa and cumulus cells.
- The stimulation protocol used during IVF and ICSI has no influence over the degree of DNA fragmentation detected in granulosa and cumulus cells.
- The overnight incubation of cumulus cells with spermatozoa has no effect on cumulus cell DNA fragmentation.
- The degree of DNA fragmentation in granulosa and cumulus cells has no influence on oocyte fertility potential.
- Maternal age has no influence on the rate of fertilization.
- The degree of granulosa and cumulus cell DNA fragmentation has no effect on the rate of blastocyst development.
- The degree of granulosa and cumulus cell DNA fragmentation has no effect on pregnancy outcome.
3.4 The assessment of murine embryo DNA fragmentation in response to culture medium containing GM-CSF

Refer Aims 1.9c, and Materials and Methods 2.4

Embryos were cultured in medium containing 5 different concentrations of recombinant mouse GM-CSF in group culture in a standard petri dish and single culture using an EmbryoSlide®. All embryos were cultured in a standard incubator (refer Flow Diagram 2.4a). The number of blastomeres displaying signs of DNA fragmentation was recorded within each embryo, along with the total number of cells displaying DNA fragmentation (in all the embryos) at each concentration of GM-CSF. The total number of embryos with no DNA fragmentation was also compared to those with cells containing DNA fragmentation at each concentration of GM-CSF.

Image 3.4a: Murine blastomeres stained with DAPI following the TUNEL procedure (left). The same cells did not display green fluorescence (right), signifying the lack of DNA fragmentation in the cells in view. Magnification: x600.
3.4b: Murine blastomeres stained with DAPI following the TUNEL procedure (left). Of the 3 cells observed one cell displays green fluorescence (right), signifying the presence of DNA fragmentation within that cell. Magnification: x600.

3.4a The effect of culture medium containing GM-CSF on DNA fragmentation in murine embryos cultured in a standard petri dish

Total percentage DNA fragmentation within each embryo cultured in a standard petri dish ranged from 0-100 % (n=192, mean=43.27, SD=43.47). Percentage DNA fragmentation in embryos belonging to the control group containing 0ng/ml GM-CSF (n=43, mean=50.17%, SD=42.97) and those supplemented with GM-CSF concentrations of 1ng/ml (n=34, mean=44.61, SD=44.60), 2ng/ml (n=38, mean=43.54, SD=45.09), 5ng/ml (n=36, mean=39.68, SD=44.88) and 10ng/ml (n=41, mean=38.34, SD=41.35) (Tables 3.4a and 3.4b).

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Table 3.4a: Degree of DNA fragmentation in each embryo cultured in a standard petri dish with media supplemented with 0 ng/ml and 2 g/ml GM-CSF. Total cells = total number of blastomeres in that embryo, DF=blastomeres displaying signs of DNA fragmentation as analysed by TUNEL.
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Table 3.4b: Degree of DNA fragmentation in each embryo cultured in a standard petri dish with media supplemented with 2 ng/ml and 5 g/ml and 10 ng/ml GM-CSF. Total cells = total number of blastomeres in that embryo, DF=blastomeres displaying signs of DNA fragmentation as analysed by TUNEL.

A one-way between-groups analysis of variance was conducted to examine the effect of GM-CSF on mean percentage embryo DNA fragmentation measured by the TUNEL test. There was no statistically significant difference in mean percentage DNA fragmentation between the 5 groups of embryos (P=0.760; P ≤ 0.05, ANOVA). This observation was further confirmed by a Kruskall-Wallis Test (P=0.745; P ≤ 0.05) (Figure 3.4a).
Figure 3.4a: Mean percentage DNA fragmentation in murine embryos cultured in medium containing 0ng/ml (n=43), 1ng/ml (n=34), 2ng/ml (n=38), 5ng/ml (n=36) and 10ng/ml (n=41) GM-CSF in a standard petri dish. The difference in the level of DNA fragmentation between the groups was not statistically significant. (P=0.760; P ≤ 0.05, ANOVA). Error bars indicate standard error.

Although a steady decrease in mean percentage DNA fragmentation was observed with increasing concentration of GM-CSF, these results were not statistically significant (Figure 3.4a). This may be due to the small sample size. It can perhaps still be assumed that GM-CSF reduces the rate of embryo DNA fragmentation in a concentration dependent manner, when embryos are cultured in a standard incubator.

The number and percentage of blastomeres containing DNA fragmentation was calculated in all embryos cultured in a standard petri dish at 0ng/ml (total blastocysts=43, total blastomeres=2540, blastomeres with DNA fragmentation=1225, total % blastomeres with DNA fragmentation=48.23%), 1ng/ml (total blastocysts=34, total blastomeres=2106, blastomeres with DNA fragmentation=882, total % blastomeres with DNA fragmentation=41.88%), 2ng/ml (total blastocysts=38, total blastomeres=2243, blastomeres with DNA fragmentation=1008, total % blastomeres with DNA fragmentation=44.94%), 5ng/ml (total blastocysts=36, total blastomeres=2106, blastomeres with DNA fragmentation=730, total % blastomeres with DNA fragmentation=34.66%), and 10ng/ml GM-CSF (total blastocysts=41, total blastomeres=2494, blastomeres with DNA fragmentation=871, total % blastomeres with DNA fragmentation=34.92%) (Figure 3.4b). Statistical analysis could not be carried out as the scoring of all blastomeres at each concentration of GM-CSF was not repeated and the average could not be calculated.
Figure 3.4b: Total percentage of blastomeres displaying DNA fragmentation in embryos cultured at each concentration of GM-CSF. Statistical analysis could not be carried out as the scoring of all blastomeres at each concentration was not repeated.

The total number and percentage of embryos containing one or more cells with fragmented DNA at each concentration of GM-CSF was also recorded (Figure 3.4c). Statistical analysis could not be performed on these results as the experiments were not repeated.

Figure 3.4c: Distribution between the total number of embryos containing at least one cell with fragmented DNA and embryos containing cells free from DNA fragmentation. These embryos were cultured in a standard petri dish. Statistical analysis could not be performed on these results as scoring was not repeated.
Unlike when the percentage of cells containing DNA fragmentation within each embryo was recorded, GM-CSF did not appear to have a dose dependent effect on DNA fragmentation when whole embryos were classified as containing or not containing cells with fragmented DNA (Figures 3.4c and 3.4d).

### 3.4b The effect of culture medium containing GM-CSF on DNA fragmentation in murine embryos cultured using an EmbryoSlide®

The total percentage DNA fragmentation within each embryo cultured using an EmbryoSlide® ranged from 0-100 % (n=67, mean=42.73, SD=43.07). The percentage DNA fragmentation in embryos belonging to the control group (0ng/ml) ranged from 0-92.86% (n=10, mean=43.27%, SD=43.07) and those supplemented with 1ng/ml 0-100% (n=12, mean=47.6, SD=49.79), 2ng/ml 0-98.04% (n=16, mean=47.85, SD=44.27), 5ng/ml 0-100 (n=16, mean=34.44, SD=41.08) and 10ng/ml 0-100 (n=13, mean=41.72, SD=42.78) (Tables 3.4c and 3.4d).

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Table 3.4c: Degree of DNA fragmentation in each embryo cultured in an EmbryoSlide® with media supplemented with 0 ng/ml and 1 g/ml GM-CSF. Total cells = total number of blastomeres in that embryo, DF=blastomeres displaying signs of DNA fragmentation as analysed by TUNEL.

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Table 3.4d: Degree of DNA fragmentation in each embryo cultured in an EmbryoSlide® with media supplemented with 2 ng/ml and 5 g/ml and 10 ng/ml GM-CSF. Total cells = total number of blastomeres in that embryo, DF=blastomeres displaying signs of DNA fragmentation as analysed by TUNEL.

A one-way between-groups analysis of variance was conducted to explore the effect of GM-CSF on embryo DNA fragmentation measured by the TUNEL test. There was no statistically significant difference in mean percentage DNA fragmentation between the 5 groups of embryos (P=0.918; One-Way Between-Groups ANOVA, P ≤ 0.05). This observation was confirmed by a Kruskall-Wallis Test (P=0.725; P ≤ 0.05) (Figure 3.4e).
GM-CSF did not appear to have a dose-dependent effect on embryo DNA fragmentation when embryos were cultured using an EmbryoSlide®. The difference in the level of DNA fragmentation between the five different groups was not statistically significant (Figure 3.4e).

The number and percentage of blastomeres containing DNA fragmentation was calculated in all embryos cultured in an EmbryoSlide® at 0ng/ml (total blastocysts=10, total blastomeres=379, blastomeres with DNA fragmentation=139, total % blastomeres with DNA fragmentation=36.68%), 1ng/ml (total blastocysts=12, total blastomeres=432, blastomeres with DNA fragmentation=121, total % blastomeres with DNA fragmentation=28.01%), 2ng/ml (total blastocysts=16, total blastomeres=646, blastomeres with DNA fragmentation=230, total % blastomeres with DNA fragmentation=35.6%), 5ng/ml (total blastocysts=16, total blastomeres=762, blastomeres with DNA fragmentation=248, total % blastomeres with DNA fragmentation=32.55%), and 10ng/ml GM-CSF (total blastocysts=13, total blastomeres=537, blastomeres with DNA fragmentation=248, total % blastomeres with DNA fragmentation=46.18) (Figure 3.4f). Statistical analysis could not be carried out as the scoring of all blastomeres at each concentration of GM-CSF was not repeated.
Figure 3.4f: Total percentage of blastomeres displaying DNA fragmentation in embryos cultured in an EmbryoSlide® in media supplemented with each concentration of GM-CSF. Statistical analysis could not be performed on these results as scoring was not repeated.

GM-CSF also did not have a dosage dependent effect on DNA fragmentation when whole embryos cultured in an EmbryoSlide® were categorised based on whether they contained or did not contain cells with fragmented DNA (Figures 3.4g and 3.4h).

Figure 3.4g: Distribution between the total number of embryos containing at least one cell with fragmented DNA and embryos containing cells free from DNA fragmentation. These embryos were cultured using an EmbryoSlide®. Statistical analysis could not be performed on these results as scoring was not repeated.
3.4c Dose-dependent effect of GM-CSF on embryo DNA fragmentation based on the culture method

The mean percentage DNA fragmentation was compared in embryos cultured in standard petri dishes and those cultured using an EmbryoSlide® to determine if the culture method influenced the effectiveness of GM-CSF at different concentrations (Figure 3.4i).
There was no significant difference between mean percentage DNA fragmentation in embryos cultured in standard petri dishes and those cultured using an EmbryoSlide® at either one of the 5 concentrations of GM-CSF; 0ng/ml (P=0.655), 1ng/ml (P=0.847), 2ng/ml (P=0.748), 5ng/ml (P=0.692) and 10ng/ml (P=0.799) (Independent-samples t-test, P ≤ 0.05, two-tailed) (Figure 3.41). This suggests that the influence of different concentrations of GM-CSF on embryo DNA fragmentation is not determined by the method by which embryos are cultured.

3.4d Study conclusions

The assessment of murine embryo DNA fragmentation in response to culture medium containing GM-CSF concluded that

- GM-CSF reduces the rate of embryo DNA fragmentation in a dose dependent manner, when embryos are cultured in a group using a standard petri dish.
- GM-CSF did not appear to have a dose-dependent effect on embryo DNA fragmentation when embryos were cultured singly using an EmbryoSlide®.
- GM-CSF does not have a dose dependent effect on DNA fragmentation when whole embryos are graded as containing cells with DNA fragmentation versus not containing a single cell with fragmented DNA, regardless of whether they are cultured in a standard petri dish or an EmbryoSlide®.
- The rate of embryo DNA fragmentation does not significantly differ between culturing embryos in a petri dish in a group, or singly in an EmbryoSlide®, in media supplemented with GM-CSF.
- In the absence of GM-CSF, single culture results in a greater degree of embryo DNA fragmentation than group culture.

3.5 The assessment of human embryonic stem cell DNA fragmentation and aneuploidy following exposure to pre-tested toxins

Refer Aims 1.9e, and Materials and Methods 2.5
3.5a The assessment of human embryonic stem cell DNA fragmentation following exposure to pre-tested toxins

Undifferentiated hESCs were cultured in the presence of four toxins of four increasing concentrations and scored for DNA fragmentation using TUNEL. These values were compared to DNA fragmentation in a control group of untreated hESCs.

All the microscope slides containing human embryonic stem cells treated with 4 different concentrations of 5-azacytidine, cadmium chloride, cisplatin and sodium arsenite were processed using the TUNEL assay together. The level of DNA fragmentation detected on each slide was then compared to the untreated control sample of human embryonic stem cells. Approximately 300 cells within the demarcated area to which the probes bound were scored along with the number of cells fluorescing green (TUNEL positivity), and total percentage DNA fragmentation was calculated.

Percentage DNA fragmentation in hESCs exposed to 5'-azacytidine ranged from 0.222%-6.55%. Surprisingly, it was at the lowest concentration (1 Aza = 5'-azacytidine $3.3 \times 10^{-12}$ Molar) that the level of DNA fragmentation was the highest, and greater than in the control (6.55% vs. 4.54%) (Figure 3.5a).

![Figure 3.5a: Dose dependent effect of 5'azacytidine on percentage DNA fragmentation in human embryonic stem cells](image)

Percentage DNA fragmentation ranged from 3.53%-6.18%, with the highest level of damage visible in cells treated with $1 \times 10^{-8}$ Molar cadmium chloride (2 CdCl$_2$) (Figure 3.5b). Except in cells treated with the second highest concentration of cadmium chloride (3 CdCl$_2$=$1 \times 10^{-7}$ Molar), all other groups
exhibited higher levels of DNA damage than the control group. In general, cadmium chloride appeared to have the most detrimental effect of human embryonic stem cell DNA fragmentation.

Figure 3.5b: Dose dependent effect of cadmium chloride on percentage DNA fragmentation in human embryonic stem cells (UC= untreated hESC control, 1 CdCl2=1x10^{-9} Molar cadmium chloride, 2 CdCl2=1x10^{-8} Molar cadmium chloride, 3 CdCl2=1x10^{-7} Molar cadmium chloride, 4 CdCl2=1x10^{-6} Molar cadmium chloride).

Human embryonic stem cells cultured in different doses of cisplatin could not be scored for DNA fragmentation, as all 4 experimental slides demonstrated a complete loss of cells.

Cells cultured in medium containing 4 different concentrations of sodium arsenite all demonstrated relatively low levels of DNA damage (Figure 3.5c). Percentage DNA fragmentation ranged from 0.467%-3.11% and all four experimental groups had lower values than the untreated control group. Once again, it was the lowest dose of sodium arsenite (1 NaAsO_2=3.3x10^{-14} Molar) that appeared to have the greatest effect on DNA fragmentation (3.11%).
I expected to observe a decline in the level of DNA fragmentation as the concentration of toxin decreased, but an irregular pattern was observed in each case. Furthermore, only the lowest concentration of 5-azacytidine (Dose 1=3.3x10^{-12} Molar 5'-azacytidine) (6.55%), Dose 2 of cadmium chloride (1x10^{-8} Molar cadmium chloride) and Dose 4 of cadmium chloride (1x10^{-6} Molar cadmium chloride) (5.87% and 6.18% respectively) showed a higher level of DNA damage than the untreated control group (4.54%). Statistical analysis to determine the significance of these results could not be carried out as the experiments were not repeated.

A line graph with increasing gradients for each toxin, at a DNA fragmentation level higher than that of the line associated with the control group was initially expected, but this was not observed (Figure 3.5d)
3.5b The assessment of human embryonic stem cell aneuploidy following exposure to pre-tested toxins

Undifferentiated hESCs were exposed to four toxins of four increasing concentrations and scored for aneuploidy in chromosomes 13, 15, 16, 18, 21, and 22 using FISH. These values were compared to the level of aneuploidy in a control group of untreated hESCs.

Figure 3.5d: Dose dependent effect of 5-azacytidine, cadmium chloride and cisplatin on percentage DNA fragmentation in undifferentiated human embryonic stem cells.
Image 3.5a: An untreated human embryonic stem cell processed by FISH for chromosomes 13, 18 and 21. This cell appeared normal for the chromosomes analysed. Magnification: x1000.

Image 3.5b: Human embryonic stem cell treated with cadmium chloride processed by FISH for chromosomes 13, 18 and 21. This cell was aneuploid for chromosome 18. Magnification: x1000.

In the analysis of chromosomes 13, 18 and 21 following treatment with 5′-azacytidine, the highest level of aneuploidy (12%) was observed following treatment with 3.3x10^{-10} Molar 5′-azacytidine (3 Aza) (Figure 3.5e).
Figure 3.5e: Dose dependent effect of 5'-azacytidine on percentage aneuploidy in human embryonic stem cells (LC=lymphocyte control, UC=untreated hESC control, 1 Aza=3.3x10^{-12} Molar 5'-azacytidine, 2 Aza=3.3x10^{-11} Molar 5'-azacytidine, 3 Aza=3.3x10^{-10} Molar 5'-azacytidine, 4 Aza=3.3x10^{-9} Molar 5'-azacytidine).

No aneuploidy was observed following treatment with 1x10^{-7} Molar cadmium chloride (3 CdCl₂). In fact, percentage aneuploidy in the lymphocyte and untreated human embryonic stem cell control was higher (2% and 3% respectively) than the level of aneuploidy observed at 1x10^{-7} Molar cadmium chloride (3 CdCl₂) (Figure 3.5f).

Figure 3.5f: Dose dependent effect of cadmium chloride on percentage aneuploidy in human embryonic stem cells (LC=lymphocyte control, UC=untreated hESC control, 1 CdCl₂=1x10^{-9} Molar cadmium chloride, 2 CdCl₂=1x10^{-8} Molar cadmium chloride, 3 CdCl₂=1x10^{-7} Molar cadmium chloride, 4 CdCl₂=1x10^{-6} Molar cadmium chloride).

Cisplatin appeared to have a concentration-dependent effect on aneuploidy for chromosomes 13, 18 and 21. Percentage aneuploidy steadily increased along with the concentration of cisplatin (Figure
Cisplatin did not appear to have any influence on human embryonic stem cell aneuploidy at a concentration of $1 \times 10^{-11}$ (1 Cis) as percentage aneuploidy was the same at this dose and in the untreated human embryonic stem cell control (4.5%) (Figure 3.5g).

![Dose dependent effect of cisplatin on % aneuploidy (chromosomes 13, 18 and 21) in human embryonic stem cells](image)

Figure 3.5g: Dose-dependent effect of cisplatin on percentage aneuploidy in human embryonic stem cells (LC=lymphocyte control, UC=untreated hESC control, 1 Cis=$1 \times 10^{-11}$ Molar cisplatin, 2 Cis=$1 \times 10^{-10}$ Molar cisplatin, 3 Cis=$1 \times 10^{-9}$ Molar cisplatin, 4 Cis=$1 \times 10^{-8}$ Molar cisplatin).

Sodium arsenite did not appear to have a concentration dependent effect on percentage aneuploidy for chromosomes 13, 18 and 21 (Figure 3.5h)

![Dose dependent effect of sodium arsenite on % aneuploidy (chromosomes 13, 18 and 21) in human embryonic stem cells](image)

Figure 3.5h: Dose-dependent effect of sodium arsenite on percentage aneuploidy in human embryonic stem cells (UC=untreated hESC control, 1 NaAsO$_2$=$3.3 \times 10^{-14}$ Molar sodium arsenite, 2 NaAsO$_2$=$3.3 \times 10^{-13}$ Molar sodium arsenite, 3 NaAsO$_2$=$3.3 \times 10^{-12}$ Molar sodium arsenite, 4 NaAsO$_2$=$3.3 \times 10^{-11}$ Molar sodium arsenite).
The highest two concentrations of 5′-azacytidine had the most severe effect on human embryonic stem cell aneuploidy for chromosomes 15, 16 and 22. At 3.3x10^{-10} (3 Aza) and 3.3x10^{-9} Molar 5′-azacytidine (4 Aza), percentage aneuploidy was 28.5% and 26.5% respectively (Figure 3.5i).

Figure 3.5i: Dose dependent effect of 5′-azacytidine on percentage aneuploidy in human embryonic stem cells (LC=lymphocyte control, UC= untreated hESC control, 1 Aza=3.3x10^{-12} Molar 5′-azacytidine, 2 Aza=3.3x10^{-11} Molar 5′-azacytidine, 3 Aza=3.3x10^{-10} Molar 5′-azacytidine, 4 Aza=3.3x10^{-9} Molar 5′-azacytidine).

The highest level of aneuploidy (23.75%) for chromosomes 15, 16 and 22 in cells treated with cisplatin was seen at the highest concentration of this drug (4 Cis=1x10^{-8} Molar cisplatin) (Figure 3.5j).

Figure 3.5j: Dose-dependent effect of cisplatin on percentage aneuploidy in human embryonic stem cells (LC=lymphocyte control, UC= untreated hESC control, 1 Cis=1x10^{-11} Molar cisplatin, 2 Cis=1x10^{-10} Molar cisplatin, 3 Cis=1x10^{-9} Molar cisplatin, 4 Cis=1x10^{-8} Molar cisplatin).
The highest level of aneuploidy (29%) for chromosomes 15, 16 and 22 in cells treated with sodium arsenite was also seen at the highest concentration of this drug (4 \( \text{NaAsO}_2 \)=3.3x10^{-11} \text{ Molar sodium arsenite} ) (Figure 3.5k).

**Figure 3.5k**: Dose-dependent effect of sodium arsenite on percentage aneuploidy in human embryonic stem cells (UC= untreated hESC control, 1 \( \text{NaAsO}_2 \)=3.3x10^{-14} \text{ Molar sodium arsenite}, 2 \( \text{NaAsO}_2 \)=3.3x10^{-13} \text{ Molar sodium arsenite}, 3 \( \text{NaAsO}_2 \)=3.3x10^{-12} \text{ Molar sodium arsenite}, 4 \( \text{NaAsO}_2 \)=3.3x10^{-11} \text{ Molar sodium arsenite}).

FISH analysis for chromosomes 15, 16 and 22 could not be carried out on hES cells treated with \( \text{CdCl}_2 \) as there were insufficient cells to score on the processed slides.

### 3.5c Study conclusions

The assessment of human embryonic stem cell DNA fragmentation and aneuploidy following exposure to pre-tested toxins concluded that:

- Human embryonic stem cells have variable degrees of DNA fragmentation and aneuploidy.
- 5'-azacytidine, cadmium chloride and sodium arsenite do not have a dose dependent effect on human embryonic stem cell DNA fragmentation.
- Whilst cisplatin and sodium arsenite increase the rate of aneuploidy with increasing concentration, cadmium chloride decreases the rate of aneuploidy with increasing concentration.
4. Discussion
4.1 The assessment of DNA fragmentation and aneuploidy in spermatozoa

4.1a Study results

Although the level of DNA fragmentation varied considerably when using the SCSA, SCD, Halosperm® and SCD-FISH assays, the results obtained using Halosperm® was somewhat comparable to those observed using SCSA. Determining whether Halosperm® is a reliable and sufficient test to measure DNA fragmentation however, requires further work. Reproducing the SCD test using first principles generated technical complications. Preparation of the lysing solution and the effect it had on sperm cells proved problematic and the visualization of halos was poor in some cases. This may contribute to the unexpected results generated.

Despite expecting SCD and SCD-FISH to generate similar results considering they were both made using in-house solutions, the significant difference between the degrees of aneuploidy recorded when using the SCD and SCD-FISH tests implicates the influence of post-washes in the inconsistency surrounding halo visualization. A similar observation was noted by Muriel et al., (2007), who attributed the phenomenon to the aggressive conditions of the lysing solution. This issue was apparently rectified when using the Halosperm® kit (Muriel et al., 2007). The halos and FISH signals observed using the Halosperm® kit and reagents prepared in our lab were identical during the optimization process, so the inconsistency in halo preservation requires further investigation.

The statistically significant difference between the levels of aneuploidy observed using FISH and SCD-FISH suggests the results these two tests generate are different to one another. The positive correlation observed suggests that the same underlying quantity (i.e. percentage of cells displaying aneuploidy) is being measured, but not on the same scale. A lower correlation (r<0.2) would imply that we are not measuring the same quantity. Because spermatozoa with fragmented DNA tend to display spotted FISH signals, discriminating between single and multiple signals proved difficult. Muriel et al., (2007) claim that FISH signals in spermatozoa without DNA fragmentation had the propensity, albeit rarely, to spread from the core to the halo in the nucleoids. This can easily cause two FISH signals to overlap, resulting in an underestimation of the aneuploidy rate. However, I observed that the dispersion of FISH signals led to an over-estimation of aneuploidy. A frequent occurrence, this can result in subjectivity when interpreting the signals and therefore errors during the scoring procedure. Based on these results, SCD-FISH is an unsuitable method to assess aneuploidy or DNA fragmentation, either independently or simultaneously.
The lack of a significant positive correlation between the level of aneuploidy and DNA fragmentation in sperm cells analysed by these methods does not necessarily discourage the suggestion of such a correlation, but may be more indicative of the unreliability of the methods themselves. Considering the literature that supports the hypothesis that a positive relationship exists between DNA fragmentation and aneuploidy (Liu et al., 2004; Muriel et al., 2007), the negative values of the correlation coefficient are unexpected. As in the case of many commonly-used statistics, $r$ is not robust and its value can be misleading if outliers are present. The Pearson Correlation in particular is not outlier resistant (Wilcox, 2005).

A significant negative correlation was observed between the sperm concentration and sperm DNA fragmentation when using the Halosperm® test. No other relationships were observed between sperm concentration, total percentage motility, patient age, days of abstinence and sperm DNA fragmentation when using the SCD-FISH, SCD, Halosperm® and SCSA tests, or aneuploidy measured by the FISH and SCD-FISH tests.

A double-blinded study is recommended to determine the level of subjectivity and reliability of these assays in the detection of sperm DNA fragmentation and during the simultaneous assessment of DNA fragmentation and aneuploidy on the same sperm cell. The reliability of our data may be questioned, in terms of scoring accuracy, but this simply further highlights the ineffectual nature of the test considering the time and effort required to familiarize oneself with the scoring procedure. Re-counting the same slides in the future is probably worthwhile, in addition to using a larger sample size.

### 4.1b Chromosome abnormality and FISH

The high precision of conventional FISH aneuploidy has been established in somatic cells, with sensitivities stated to be in the range of 98% to 100% (Shaffer and Bui, 2007). Although it is believed that the accuracy in sperm would be similar to that of somatic cells, despite the gentle decondensation prior to hybridization, the condensed nature of sperm chromatin may have a subtle influence on accuracy. The main limitation of standard FISH analysis of 10 000 – 15 000 sperm cells remains the labour intensive nature of the work. Manual microscopic analysis of a single slide (approximately 500 cells) typically required 3 hours of technician time. Some laboratories have now employed automated FISH in the analysis of sperm, thus reducing the amount of time spent scoring fluorescent signals when performing aneuploidy analysis (Carrell and Emery, 2007). The major advantages offered by the automated analysis system has led to its popularity in some clinical and research laboratories. Whilst the technician time is greatly reduced, the system has the capacity to
store images for subsequent manual analysis at a faster rate than microscopic, manual analysis. The software can also return the microscope to the actual slide location allowing direct observation of the sperm as opposed to the processed image (Carrell, 2008). This is important as it was noted during this project that the signals observed manually through the microscope and those captured on camera appear different to one another. Another advantage is that the automated system provides better archival storage of images and data from each sample analysed. Furthermore, it facilitates the analysis of samples with low cell numbers (Carrell, 2008), which is clearly beneficial in the analysis of semen samples from men with sub-optimal fertility.

Infertile men have an 8- to 10-fold higher prevalence of chromosomal anomalies than fertile men in the absence of other phenotypic features (Chandley, 1998). In a review of 11 studies involving 9766 azo-oligospermic men, the chromosomal anomaly rate was highest in the azoospermic subjects (13.7%), being predominantly numerical or structural defects. However, not all anomalies impact fertility; 0.37% of sperm donors with normal semen parameters possessed chromosomal translocations (Ravel et al., 2006). Whilst the increased rates of chromosomal anomalies in ICSI children (Bonduelle et al., 1996) most likely arise due to sperm aneuploidy, the absolute rate is small (Mitchell, 2002). An unbalanced chromosome complement is usually found in 4-40% (Ogur, 2006; Martin, 2008) of the sperm produced by oligospermic patients as a result of a Robertsonian translocation, resulting in trisomy or uniparental disomy. Although most reciprocal translocation carriers only have a spermatogenic phenotype of meiotic arrest, more than 50% of the sperm produced are chromosomally unbalanced (Estop et al., 1995; Martin, 2008).

The ultimate aim of gamete aneuploidy testing is to translate the relative risk to the embryo (Carrell, 2008). This is difficult to execute considering the various problems that compound aneuploidy studies. Most studies employ 6 chromosome probes, a small proportion of the chromosome complement. This is particularly unhelpful considering certain pathologies are more commonly associated with a certain set of chromosomes. For example, miscarriage is often associated with chromosomes 1, 15, 16, 17 and 22, and not the chromosomes most often analysed (13, 18, 21, X and Y) (Bettio et al., 2008). Chromosome abnormality not only increases risk to offspring but also affects infertility therapy outcomes (Gianaroli et al., 2005). Diagnosis of such elevated risks in chromosome aneuploidy has the potential to reduce risk to the offspring and can also reduce the high financial and emotional expense of recurrent IVF failure (Carrell, 2008). However, the cost-effectiveness of sperm aneuploidy testing is questionable because of the health burdens of failed ART or abnormal conception and live birth, whether screening may fail to detect and prevent adverse outcomes, and cost issues including the public vs. private nature of medicine (McLachlan and O'Bryan, 2010).
Evaluation of sperm for aneuploidies in the prediction of ART outcomes and identification of suitable candidates for preimplantation genetic diagnosis (Carrell, 2008; Martin, 2008) is expensive and complex because of the heterogeneous nature of chromosomal anomalies, preventing reliable detection. Furthermore, the reason aneuploidy screening has not taken off outside specialized centres may be due to the relatively low absolute risk of abnormal sperm, the lack of clinical trial data showing improved outcomes after screening, and the tendency of most couples to proceed with ICSI regardless of screen results (Griffin et al., 2003). Trial data to establish the threshold level at which couples should undertake preimplantation genetic diagnosis, use donor sperm or abandon ART is necessary. The sensitivity of FISH analysis is limited to the chromosome regions for which the probes are used. Approaches like comparative genomic hybridization or microarray analysis will likely result in the detection of more subtle structural defects when screening sperm. This may revolutionise testing for chromosomal abnormalities and provide a basis for observed sperm aneuploidy in 46, XY men (Cheung et al., 2007; Fragouli et al., 2007).

4.1c DNA fragmentation tests: scientific fact or commercial fiction? (Refer to 4.2h)

Many assays have been developed to measure the level of DNA fragmentation in sperm from infertile men and fertile donors. Techniques used to analyse sperm DNA fragmentation in any clinical andrology or assisted reproductive technology laboratory should be simple, reproducible, and ideally without the need for new, complex or expensive technology (De Jonge, 2002). The advantages of the SCD test include the fact that it does not rely on the determination of either colour or fluorescence intensity, as in the case of other semi-quantitative tests. Ideally, the endpoint measured by the SCD test consists of determining the percentage of spermatozoa with non-dispersed or dispersed nuclei, which should theoretically be accomplished by the naked eye. This is unfortunately not always as simple. Whilst the Halosperm® kit measure only ~ 500 sperm cells on a light microscope slide, 5,000 can be analysed using the SCSA test (Evenson and Wixon, 2005). The results generated from a study by Fernandez et al., (2005) bring to light a substantial difference in the numerical values between the results generated from the SCSA and SCD tests. The SCD test displays a significantly higher sensitivity for detecting sperm DNA fragmentation (mean difference of 2.16%). Additionally, they deviate more in samples with higher SCSA DFI levels; a sample with a DFI of 30%-35% can have a SCD result ranging between 20%-50% (Evenson and Wixon, 2005). This pattern of variation and lack of correlation was consistent with the results observed when the DFI levels calculated were compared to those obtained following the SCD-FISH and SCD procedures. Furthermore, the inter-observer error can be as high as 12% (Evenson and Wixon, 2005). Therefore, the real biological value can be
either negligible or nearly double the value stated. There also appeared to be a lack of significance concerning the percentage of sperm cells with fragmented DNA when the samples were observed by different technicians (results not shown). Whilst the promotion of the commercial Halosperm® kit continues, a significant absence of unbiased scientific literature validating the simultaneous examination of DNA fragmentation and aneuploidy brings its authenticity into question.

4.1d Future work

When attempting to validate novel tests for DNA fragmentation and aneuploidy, the experimental slides analysed should be scored in a double blind study to ensure accurate, consistent scoring by different individuals and to ensure that the employment of such tests is not prone to the technician’s interpretation.

A limitation of the present study is the inability to assess the entire chromosome complement in the spermatozoa analysed using FISH. Since only chromosomes X, Y and 18 were assessed, a proportion of the spermatozoa categorised as 'chromosomally normal' may well be aneuploid for the chromosomes that were not investigated using FISH. As a result, the use of CGH, or other developing technologies, is recommended to analyse the entire sperm chromosome complement, thus benefitting studies of sperm aneuploidy and sperm DNA fragmentation by increasing the quality of the data generated and potentially reducing costs. This issue can also be alleviated by increasing the number of chromosomes analysed using FISH. However, studies using array CGH in human PGD embryos reported no change in the overall rates of aneuploidy compared to studies where only two chromosomes were analysed (Delhanty et al., 1993; Fragouli et al., 2010). The lack of a correlation between sperm DNA fragmentation and aneuploidy may in fact be due to this lack of information regarding the chromosome complement in sperm. Additional studies on sub-microscopic copy number variations obtained by CGH may also eventually provide clinically important data regarding its effect on fertility and embryogenesis.

It cannot be stressed enough, that DNA fragmentation tests should be validated and any criteria established before the possible clinical use of such tests. I suggest analysing the level of sperm DNA fragmentation in samples of semen provided by the same individual using the SCD, SCSA, Halosperm®, TUNEL and Comet assays and comparing the observed degree of DNA fragmentation to the corresponding delivery rate following IVF and ICSI.

Whilst it is speculated that patients with spermatogenic problems have different rates of aneuploidy that depend on the degree of infertility, potential associations between sperm DNA fragmentation
and aneuploidy may be concealed in patients with high levels of oxidative damage produced after spermiation. This can lead to sperm DNA fragmentation being produced in the genital tract due to exogenous damaging agents, masking association between sperm DNA fragmentation and aneuploidy. Therefore, further studies are also recommended to assess the incidence of sperm aneuploidies in sperm with intact DNA, along with the assessment of oxidative stress in the semen.

Assays that are deemed suitable for clinical testing should be comparable to one another and provide the same results regardless of the technician using the test. The cost-effectiveness and actual clinical value of sperm DNA fragmentation testing need to be resolved prior to its potentially widespread use. Vitally, if sperm DNA fragmentation testing is to be used in conjunction with ART, threshold levels above which the degree of DNA fragmentation is incompatible with successful IVF outcome should be determined in sperm samples from infertile men. Furthermore, confounding factors such as maternal age, duration of subfertility etc. need to be taken into consideration.

4.2 The assessment of processes involved in ART on sperm DNA damage and PS translocation

4.2a Relationship between PS translocation and DNA fragmentation

A study by Muratori et al., (2003) investigated the relationship between sperm PS translocation and DNA fragmentation. The binding of Annexin V to the membrane of viable sperm during incubation was found to be transient, eventually leading to necrosis. Considering the sharp correlation they observed between sperm DNA fragmentation and necrosis, they showed that basal Annexin V positivity correlated with subsequent de novo DNA fragmentation. Possible reasons as to why our study did not produce a strict correlation between DNA fragmentation and Annexin V positivity is that both parameters were measured at the same time, instead of allowing DNA damage to occur over time. The positive correlation between the externalisation of PS to the sperm outer membrane and sperm DNA damage (Muratori et al., 2003) is reflected in the sub-optimal rates of fertilisation, implantation and pregnancy in ART (Henkel et al., 2004; Sun et al., 1997; Barroso et al., 2006).

4.2b Relationship between sperm concentration, male age and sperm DNA fragmentation

Threshold values for sperm concentration are routinely used to categorise men into different fertility groups (Guzick et al., 2001; Nallella et al., 2006; Murray et al., 2012). Considering the potential relationship between DNA fragmentation and fertility status, I wanted to assess the relationship
between sperm DNA fragmentation and sperm concentration. The percentage of DNA fragmentation between samples with a sperm concentration above and below 15M/ml was significantly different \( p=0.009 \). Sharbatoghli et al., (2012) found a negative correlation between DNA fragmentation and sperm concentration.

Because male ageing can result in reproductive hormonal and cellular changes that can influence semen quality, I wanted to determine its effect on DNA fragmentation. Although patients over the age of 40 showed more sperm DNA fragmentation compared to those below 40 years, this was not statistically significant. A study by Nijs et al., (2011) also did not reveal a significant male age-related increase in sperm DNA fragmentation. A possible explanation for the increase in DNA fragmentation in older men is the age dependent accumulation of DNA damage as well as the less efficient system of apoptotic cell selection (Singh et al., 2003). Age-related damage to genes involved in the apoptotic pathway (Martin & Rademaker, 1987), an aggregate of different environmental and/or physiological factors may also contribute towards age-related DNA damage (Sloter et al., 2004). Interestingly, it has been suggested that male age may actually be a proxy for a ‘cohort effect’, where men of the same birth cohort have experienced a common specific exposure (Sharpe & Skakkebaek, 1993).

The increase in abnormal semen parameters found in older men, coupled with diminished DNA repair and age-related factor in women of advanced maternal age, is likely to have a prominent negative effect on the reproductive capacity of couples. A study by Moskovtsev et al., (2007) demonstrated that prolonged incubation of density gradient selected sperm had a negative impact on sperm survival in older patients and patients with extensive sperm DNA damage. Sperm DNA fragmentation has also been shown to occur continuously, post-ejaculation (Muratori et al., 2003). The linear relationship observed between basal DNA fragmentation and subsequent damage in vitro suggests the influence of some intrinsic sperm feature on the development of further in vitro damage (Muratori et al., 2003). Thus, it has been suggested that semen samples from individuals with abnormal or borderline sperm parameters should be prepared immediately, prior to their use in ART (Moskovtsev et al., 2007). This may help overcome the negative effect of extended incubation currently practiced in IVF units.

4.2c Effect of freeze-thawing on sperm DNA fragmentation

Sperm freezing in the absence of cryoprotectant is a common practice in basic scientific research. It is also performed prior to the clinical assessment of DNA damage when using the ‘gold-standard’ SCSA test. Therefore, I wanted to determine the effect of freeze-thawing sperm on DNA
I did not study the effect of PS translocation following freeze-thawing as this procedure is only performed in the assessment of DNA fragmentation. Furthermore, following our preliminary experiments comparing PS translocation and DNA fragmentation, there did not appear to be a significant correlation between the two parameters. Sperm samples that were frozen-thawed showed a significantly higher level of DNA damage than the control samples of fresh sperm. A significant increase in DNA fragmentation was detected in the frozen-thawed samples compared to the untreated controls (7.63% vs. 16.65%; P=0.000) (Figure 3.2f). The analysis of DNA fragmentation following sperm freezing can lead to an over-estimation in the level of damage, error prone studies and prejudice one's research. This misrepresentation may also bias clinical decisions, which in turn can affect patient treatment.

Said et al., (2010) suggest that DNA damage may not be due to cryopreservation itself, but the subsequent process of thawing. Whilst sperm DNA fragmentation increased with time, the highest rate of damage occurred within 4 hours post-thawing. Thawed sperm samples should therefore be used as quickly as possible in a clinical setting (Gosalvez et al., 2009). Whilst cryoprotectants can be used as a remedial measure, they can themselves have a toxic effect at high concentrations as they induce osmotic changes to which spermatozoa are highly sensitive (Gao et al., 1993; Said et al., 2010).

Several aspects of spermatozoa, including motility, are dependent on ion permeability changes that are regulated by environmental changes (Darszon et al., 2006). ATPase, which maintains the intracytoplasmic ion concentration and cell volume, is highly sensitive to hypothermia and has restricted activity at low temperatures (Suzuki et al., 1998; Tateno et al., 2000). This can hinder cell preservation at low temperatures. A study by Riel et al., (2007) demonstrated that human sperm stored in electrolyte-free solution maintained their motility and viability for approximately 5 weeks. The injection of human sperm into mouse oocytes revealed that sperm storage of two weeks does not negatively affect DNA integrity (Riel et al., 2007). The use of frozen-thawed mouse spermatozoa in ICSI resulted in normal murine blastocysts and viable fetuses. Whilst more research is necessary for this study to bear significance for ART in humans, it may be worth considering in the practice of basic scientific research. Whilst cryopreservation is still more ideal, sperm storage at 4°C in electrolyte-free solution is simpler, cheaper than storing in liquid nitrogen and less time-consuming.

4.2d Effect of oxidative stress on sperm membrane and nuclear damage

Mature sperm may have the ability to scavenge the excess H$_2$O$_2$ generated more effectively (Sahoo et al., 2008) and protect themselves against the harmful effects of oxidative stress (Kumar et al.,
The observation that the highest concentration of H$_2$O$_2$ used in this study failed to increase the level of DNA damage above 20% (Figure 3.2i), less than the 30% thought to be of clinical significance, may be explained by a study which showed that mature spermatozoa have a higher glutathione peroxidase activity compared to immature sperm cells.

The control group showed that the level of sperm DNA fragmentation was very variable within individual samples. The positive correlation between these two parameters with increasing concentration of hydrogen peroxide suggests that the basal level of sperm membrane damage may predict impending DNA fragmentation.

Muratori et al., (2003) eliminated the involvement of sperm endonuclease activity as a cause of spontaneous DNA fragmentation, strengthening the theory that ROS accounts for the phenomenon in ejaculated sperm during *in vitro* incubation. They also showed that the inhibition of an enzymatic ROS scavenger significantly increased the level of DNA damage. However, our study used neat semen. Contamination by immature round germ cells and leukocytes cannot guarantee that DNA damage observed in the control groups is due to ROS derived purely from sperm cells.

It has been well established that oxidative stress induced by ROS impairs the fertilizing ability of spermatozoa. A study by Chi et al., (2008) showed that supplementing sperm preparation medium with antioxidants significantly improved the overall functional parameters of the spermatozoa by reducing ROS levels. Whilst the addition of EDTA significantly improved sperm motility, catalase significantly increased the acrosome reaction rate. Antioxidant supplementation did not, however, reduce lipid peroxidation (Chi et al., 2008).

**4.2e Effect of thermal stress on sperm membrane and nuclear damage**

The significant increase in sperm DNA fragmentation at 37°C after merely 1 hour is important clinically. A study on the effects of different sperm preparation methods and incubation time on sperm DNA fragmentation observed that sperm swim-up increases the level of DNA fragmentation more compared to density gradient centrifugation (Zhang et al., 2011). They suggested that samples prepared by gradient centrifugation may be more stable in terms of DNA fragmentation, than samples prepared by swim-up. Consistent with previous reports (Muratori et al., 2003), the study also demonstrated that sperm DNA continues to degrade following preparation. Muratori et al., (2003) observed that sperm DNA damage could occur *in vitro* under experimental conditions, and that such DNA damage was not caused by internal ROS. As such, our study shows similar results; the occurrence of sperm DNA damage post ejaculation under conditions used during sperm preparation.
4.2f Alternative approaches

As a feature of apoptosis, PS externalisation has been exploited to separate dead and apoptotic spermatozoa using colloidal super-paramagnetic microbeads (Grunewald et al., 2001; Glander et al., 2002; Said et al., 2008). These beads are conjugated with Annexin V and bind to PS, thus separating damaged cells by magnetic-activated cell sorting (MACS). Whilst the cells with externalised PS will bind to the microbeads, those non-apoptotic cells with intact membranes will remain and can be used in ART. In addition to the removal of apoptotic cells, MACS is also thought to result in a fraction of spermatozoa with a greater proportion of normal morphology, undisturbed mitochondrial potential and less DNA damage (Said et al., 2005, 2006).

A study by Said et al., (2008) suggested that the integration of MACS with conventional sperm preparation techniques improves semen quality, cryo-survival rates and oocyte penetration through the elimination of apoptotic sperm. Clinical trials showed better cleavage and pregnancy rates following ICSI in men with oligo-, astheno- and teratozoospermia using MACS as opposed to density-gradient centrifugation (Dirican et al., 2008). The reports of full term pregnancies using this technique deem it safe and reliable for use in ART (Polak et al., 2010; Rawe et al., 2010). Thus, it may be worth considering the use of MACS in the selection of non-apoptotic sperm in ART. However, the advantages will have to be balanced with the relatively high expense of the equipment which may hinder the cost-effectiveness of this method (Henkel, 2012).

It may be of interest to study the dynamic rate at which DNA damage is induced in a sample. Data pertaining to time-related DNA damage in prepared samples is limited. Most studies concentrate on the time limits within which fresh sperm samples must be processed. If not administered promptly, in terms of diminishing motility and ability to penetrate the zona pellucida (Centola et al., 1997), fresh sperm DNA has been shown to degrade rapidly (Gosalvez et al., 2009). Sperm samples prepared by different methods may therefore display different DNA fragmentation dynamics. Thus, the predictive value of DNA fragmentation analysis under different conditions can be determined, and conditions encountered by sperm within the female reproductive tract or when exposed to artificial extenders, can be replicated.

In humans, defective chromatin remodelling increases the susceptibility of sperm to oxidative attack (Aitken & De Iuliis, 2010), particularly in under-protaminated regions of DNA (Aitken et al., 2009; Aitken & De Iuliis, 2010). Thus, the inter-individual histone to protamine ratio of fertile and infertile cohorts, and their susceptibility to DNA damage may possess predictive power as a biomarker for infertility diagnosis/ fertility potential.
4.2g Will ART compromise sperm chromatin integrity?

It is important not to view these results in isolation. The influence that the imbalance between ROS production and total antioxidant capacity in the seminal plasma can have on the generation of OS and sperm dysfunction *in vivo* must be considered, and is a major limitation in our study design. Different sperm preparation methods and incubation times have diverse effects on sperm DNA fragmentation (Zhang *et al.*, 2011). Conventional centrifugation is thought to increase superoxide dismutase activity which in turn converts the superoxide ion into hydrogen peroxide. This conversion also decreases the level of available intracellular superoxide levels in immature and mature spermatozoa when compared to neat, unprocessed samples. If true, processes during ART can increase the level of hydrogen peroxide. Centrifugation processes also cause mitochondrial membrane damage, which in turn derails the electron transfer chain (Yeagle, 1987). This contributes to the increase in DNA damage. Furthermore, when spermatozoa are pelleted during preparation, they come into contact with other sperm cells and leukocytes (Aitken & Clarkson, 1988) which can induce lipid peroxidation and DNA fragmentation (Selley *et al.*, 1991).

Overall, whilst high levels of ROS are not the only cause of male infertility, they also contribute to low live birth rates. This is because, as demonstrated by this study, induced oxidative stress can increase the level of DNA fragmentation. The exact concentrations at which ROS affects sperm function at a pathological level may help clinicians to treat infertility caused by oxidative damage. The detrimental effect of exogenous H$_2$O$_2$ on sperm motility emphasises the importance of minimising ROS production during sperm preparation (du Plessis *et al.*, 2010). Reports suggest that pronucleus formation can be achieved using sperm with oxidatively damaged DNA using ICSI (Duru *et al.*, 2000). Therefore, methods need to be developed to accurately diagnose and prevent DNA damage in spermatozoa designated for ART. If not, the long-term health of children born from IVF/ICSI may be severely compromised. Because sperm preparation is necessary for ART, maintaining sperm integrity is imperative for the success of the procedure.

This study reiterates the importance of minimising the production of exogenous stresses during sperm preparation in ART. Many DNA fragmentation tests have established their use in epidemiological studies that investigate the reproductive impact of environmental and occupational compounds (Delbes *et al.*, 2010). Their predictive value for spontaneous abortions, malformations and chromosomal damage is being actively investigated (Lewis & Agbaje, 2008; Sakkas & Alvarez, 2010). Despite the multiple applications of these tests in various fields and the precautions taken to improve their sensitivity (Mitchell *et al.*, 2011), it appears necessary to conduct further studies to understand their significance, sensitivity and correlation (Barratt *et al.*, 2010). Further research is
required to determine the mechanisms accountable for and preventing DNA damage, including antioxidants therapy.

In conclusion, the present study suggests that sperm preparation techniques in ART that generate oxidative stress, heat and freezing are causes for DNA fragmentation in ejaculated human sperm. Furthermore, increasing male age and sub-optimal sperm concentration were also related to DNA fragmentation. Further investigation is needed to tackle the aetiology of excessive ROS production and its effect on sperm susceptibility to DNA fragmentation in infertile men and subsequent pregnancy outcome following ART.

4.2h The clinical use of DNA fragmentation assays

Originally intended as a complementary test to those already in place prior to routine IVF procedures, it is now customary for patients to request consultations for abnormal sperm DNA damage results. A new parameter in the evaluation of male factor infertility, DNA fragmentation analysis may have the potential to predict ART outcome but there are limitations to what it can accomplish. Concerns over its usefulness as an independent or complementary factor of male infertility and what it actually measures still exist. Whilst the level of damage observed is at a cellular level, it is impossible to determine which sequences of the genome are affected. The age of the female partner and her oocyte quality plays a role in her ability to repair the sperm damage during fertilization. The heterogeneous nature of spermatozoa needs to be examined carefully; it is difficult to determine whether characteristics of sperm quality such as morphology and DNA damage represent an individual gamete or underlying spermatogenic process (Aitken and De Iuliis, 2010). This is particularly important in ICSI. Ironically, because the success rates of ICSI compete with those of IVF, there has been little incentive to develop new sperm selection tests. To accurately interpret the data, it is important to distinguish between genuine DNA damage and artefacts due to lack of reagent access to DNA (Barratt et al., 2010) and compensate for the varying changes in sperm chromatin structure following ejaculation (Kvist et al., 1988).

Until such discrepancies are ironed out, the establishment of sperm DNA fragmentation testing in the routine diagnostic investigations of infertile men should be used with caution. Comparing values obtained from different assays, or even different laboratories using the same technique, due to variations in conditions and protocol that may influence the outcome. Clearly defined reference values to discriminate between fertile and infertile groups have not been established (Simon et al., 2010) and should be used with caution. These issues highlight the fact that predicting fertility on the basis of a single sperm variable is extremely difficult, whether a measure of DNA damage or any
other characteristic of semen quality (Aitken et al., 2008). This makes the validation of DNA fragmentation assays an even more tedious, but necessary, task.

These factors perhaps contribute to unexpected reports where successful pregnancies result despite a high level of sperm DNA damage. Amongst the several factors that influence the predictive value of DNA fragmentation tests are, single vs. double stranded DNA fragmentation (single stranded damage, as measured by the SCD test, is of better prognosis), percentage of spermatozoa with DNA damage, whether it is primary or secondary DNA damage and capacity of the oocyte and embryo to repair DNA damage (Lewis et al., 2008). The type of DNA fragmentation test used is also important, because tests that measure the level of DNA damage under physiological conditions like Comet at neutral pH, or TUNEL, have a higher predictive value than those (SCSA and SCD) that measure DNA damage (or susceptibility to damage) under non-physiological conditions (Lewis et al. 2008).

Amongst the several techniques that have detected a close association between DNA damage and indicators of reproductive health, the Comet, TUNEL assay and SCSA (reviewed by Evenson et al., 2002; Agarwal and Said, 2003) have been the most robust. A recent study using the Comet assay noted a relationship between sperm DNA fragmentation and ART outcomes in IVF cycles but not in ICSI (Simon et al., 2010). However, a study employing the SCSA showed a negative relationship between the rate of continuing pregnancies and implantation rate in ICSI (but not IVF) cycles and DFI (Speyer et al., 2010). Current data suggest that damaged sperm DNA may have the greatest impact on IUI pregnancy rates and on pregnancy loss following IVF and ICSI (Collins et al., 2008; Zini et al., 2008). However, larger, properly designed and controlled prospective studies are necessary to confirm these results (Barratt et al., 2010).

The reason sperm function assays have thus far failed to have a significant impact on the clinical management of infertile couples may be due to the lack of standardised protocols. It would be unfortunate if scepticism about the clinical significance of DNA damage generates complacency amongst infertility specialists. Embryo competency obviously depends on the quality of the gametes involved in fertilization. Analysis of the gametes prior to the initiation of an IVF cycle can identify defects which may alter normal embryogenesis, thus improving the quality of embryos transferred. This provides the clinician with information that can affect clinical decisions, increase pregnancy rates and minimise recurring failure following IVF. Identifying the transmission risk of chromosomal and genetic defects to early embryogenesis is fundamental in enhancing patient care.

DNA damage in the male germ line may result in adverse clinical outcomes and the pathophysiology and clinical consequences of sperm DNA damage are being actively researched. Despite its plausible
appeal and fervour of its supporters, the benefits of widespread DNA testing that only achieve distressing couples with knowledge in the absence of effectual therapeutic strategies is questionable. Commercially, however, it is no doubt lucrative. The clinical and scientific community considers it a matter of urgency to translate the basic science behind how a cell prepares for fertilization into routine clinical practice (reviewed by Barratt, 2010). However, it is equally important, if not more, to allow the science behind such applications to draw level with its practice before its widespread implementation (Harper et al., 2012).

4.2i Future work

As stated previously, the development of oxidative stress and subsequent sperm dysfunction may be due to imbalances between ROS production and antioxidants capacity in the seminal plasma. Since the steps involved in sperm processing during ART result in the removal of protective antioxidants in the seminal plasma, the levels of DNA fragmentation in both neat and processed sperm samples should be recorded. Furthermore, the levels of DNA fragmentation in neat semen, and processed semen, before and after the addition of antioxidants such as catalase, superoxide dismutase and vitamin C should be assessed. Whilst it will be unethical to perform on human semen samples, it may also be interesting to add antioxidants to culture media during ART processing of semen and observe the effect on seminal ROS levels, subsequent fertilization, blastocyst development and fertilization rates in animal models. In the case of human semen samples, particularly those with high seminal ROS levels, the effect of adding antioxidants to the culture media during ART processing of semen should be investigated on a research basis. Seminal ROS levels can be analysed by chemiluminescence using luminol, which measures both intracellular and extracellular ROS in the semen.

Further studies should be performed to measure other types of ROS and exogenous ROS to better understand the relationship between ROS production and the introduction of exogenous ROS. Since O$_2^-$ may be more lethal that H$_2$O$_2$, it may be of interest to observe its effect on sperm DNA fragmentation and PS translocation. It may also be worth analysing specific concentrations at which ROS begin to compromise sperm function, as such information may assist clinicians in the treatment of infertility based specific circumstances such as increased levels of ROS, but low physiological defence.

If the relationship between smoking and sperm parameters is to be assessed in the future, biochemical tests such as serum or salivary cotinine levels can be used to verify the reported number of cigarettes smoked per day by patients, and the duration of smoking in years. It is difficult to verify
the potential relationship between semen parameters and cigarette smoking in a dose dependent manner because of the subjective nature of the smoking history, and other confounding variables such as alcohol, drugs, and abnormalities of genital examination. A large-scale study can be carried out to investigate the effects of smoking amongst infertile men whilst they continue to be active smokers and then again at different time intervals after they quit smoking. This may be a better approach to address the issue of a causal relationship between smoking and male infertility.

4.3 The assessment of cumulus and granulosa cell DNA fragmentation as a biomarker for fertility potential

The detection of apoptosis in both oocytes and cumulus cells of primordial follicles establishes that the communication axis between germ and somatic cells is not one-directional but bi-directional (Albertini et al., 2001; Nakahara et al., 1997). As a consequence of this mutual dependence, it is likely that apoptosis related processes affecting the cumulus cells will have an impact on the oocyte itself. Furthermore, a high degree of apoptosis in cumulus cells may diminish oocyte quality and embryo developmental competence.

4.3a Difference in apoptosis between cumulus and granulosa cells

The observation that cumulus cells had a higher degree of DNA fragmentation than granulosa cells was in disagreement with Nakahara et al., (1997), who described that granulosa cells had a higher incidence of apoptosis than cumulus cells. They suggested that the cumulus portion which is closer proximity to the oocyte is healthier than the mural portion in terms of the occurrence of apoptosis. They believed this to be a reasonable phenomenon in keeping with the healthy status of the oocyte.

Differences in the degree of apoptosis between granulosa cells and cumulus cells have also been recorded in other studies (Depalo et al., 2003). Cells from the follicular fluid may therefore not reflect the actual quality of the respective oocyte or embryo (Corn et al., 2005). Moreover, as with this study, the pooling of follicular fluid will not allow for the accurate connections of apoptotic features to the oocyte.

4.3b Patient age

Patient age did not have any influence on the degree of DNA fragmentation in cumulus or granulosa cells. Similarly, several other studies (Diaz-Fontdevila et al., 2009; Raman et al., 2001; Host et al., 2002; Nakahara et al., 1997; Abu-Hassan et al., 2006) agree that there is no obvious relationship between patient age and the level of cumulus and granulosa cell DNA fragmentation. In contrast,
Lee *et al.*, (2001) observed that patients over 40 years old had significantly higher levels of cumulus cells apoptosis, and decreased rates of fertilization. Apoptotic gene expression profiling also differed between patients less than or older than 38 years (Moffatt *et al.*, 2002).

### 4.3c Fertilization rate after IVF/ICSI

The results of this study suggested that the degree of DNA fragmentation in either granulosa or cumulus cells has no influence on fertilization rate regardless of whether IVF or ICSI is performed. Numerous studies have been carried out on the consequences of cumulus and granulosa cell apoptosis during *in vitro* fertilization, but with conflicting results (Nakahara *et al.*, 1997; Bencomo *et al.*, 2006; Jancar *et al.*, 2007). Similar to this study, others concluded that cumulus apoptosis had no effect on ICSI outcome (Abu Hassan *et al.*, 2006 Corn *et al.*, 2005; Diaz-Fontdevila *et al.*, 2009). The studies by Corn *et al.*, (2005) and Diaz-Fontdevila *et al.*, (2009) both used the TUNEL assay to detect DNA fragmentation. Furthermore, studies suggest that whilst human cumulus cells contain a mechanism to undergo apoptosis, exposure to sperm may alter their profile (Moffat *et al.*, 2002; Fontdevila *et al.*, 2008). Basing the selection of oocytes for ICSI on cumulus cell DNA fragmentation, to increase the chances of a positive outcome needs further work.

Host *et al.*, (2000, 2002) observed that apoptosis in cumulus cells impaired the fertilization rate of metaphase II oocytes after ICSI. Similarly, Nakahara *et al.*, (1997) observed that oocytes which actually fertilized were derived from follicles with granulosa cells containing the lowest incidence of apoptotic bodies. However, most of their research analysing apoptosis were conducted by detecting apoptotic bodies in cumulus and granulosa cells revealed as cytoplasmic fragments containing condensed chromatin or fragments of condensed chromatin when examined by fluorescence microscopy. Evidence also suggests that the rate of fertilization on day 1 of preimplantation embryo development is higher in oocytes originating from follicles with no or miniscule levels of apoptosis (Lee *et al.*, 2001; Nakahara *et al.*, 1997; Host *et al.*, 2000; Host *et al.*, 2002). A study of bovine cumulus DNA fragmentation using TUNEL also demonstrated the significantly higher level of cumulus cell apoptosis from unfertilized oocytes compared to those fertilized (Li *et al.*, 2009). In contrast, Raman *et al.*, (2001) demonstrated that fertilization rate of oocytes after ICSI was positively correlated with the cumulus cell DNA fragmentation using the Comet assay.

Several studies suggest an indirect relationship between cumulus DNA fragmentation and fertilizing potential of the corresponding oocyte (Diaz-Fontdevila *et al.*, 2009; Raman *et al.*, 2001; Hakuno *et al.*, 1996). It was suggested that the falling away of cumulus cells from the oocyte due to apoptosis may be a sign that the oocyte has achieved competence for fertilization. Research on porcine and
bovine studies suggest that the cumulus cells may play a role in easing oxidative stress on spermatozoa during fertilization, thus increasing their rate of apoptosis (Tatemo et al., 2000; Van Soom et al., 2002; Tanghe et al., 2003).

Apoptosis is thought to play a role in ovarian function (Tilly, 1996). The presence of apoptotic markers in cumulus cells may be indicative of the need for cumulus cells to undergo apoptosis at some stage of oocyte development (Moffatt et al., 2001). A high degree of apoptosis in the cumulus cell population may debilitate its supportive capacity, possibly leading to poor oocyte development. Loss of the surrounding cumulus cells has been shown to reduce the rates of oocyte maturation, fertilization and further in vitro development of the embryo (Zhang et al., 1995). This highlights the importance of the oocyte–cumulus association.

4.3d Exposure of cumulus cells to sperm during IVF versus the removal of cumulus cells during ICSI

The results suggested that the incubation of cumulus oocyte complexes with spermatozoa have no impact on the level of cumulus DNA fragmentation. In contrast, Diaz-Fontdevila et al., (2009) recorded an increase in cumulus cell apoptosis in cumulus-oocyte complexes incubated with spermatozoa, but no such increase was observed in cumulus cells incubated in culture medium without spermatozoa for 18 hours. Another study claimed that the length of time in in vitro culture influenced the rate of apoptosis. They reported an increase in cumulus cell apoptosis from 7%-32% following a 24 hour incubation in culture medium without spermatozoa, an observation which correlated with a decline in Bcl-2 expression (Jose de los Santos et al., 2000). Several studies observed an increase in cumulus cell DNA fragmentation following insemination (Jose de los Santos et al., 2000; Moffat et al., 2002). Moffatt et al., (2002) also observed that overnight co-incubation of human cumulus cells with spermatozoa significantly increased apoptosis rates compared to cumulus cells incubated in culture medium without spermatozoa. This was in line with other animal studies (Yuan et al., 2005).

These outcomes may be due to the deleterious effect of spermatozoa on cumulus cells. It is thought that large amounts of reactive oxygen species are produced when high numbers of spermatozoa are incubated with oocytes for long period of time (Aitken et al., 1998). As with this study, other control studies demonstrated that the increase in TUNEL positivity was due to the presence of spermatozoa as opposed to merely incubating cumulus cells alone overnight (Moffat et al., 2002). Several studies have also shown that the incubation of spermatozoa and oocytes for shorter time periods can result in fertilization and embryos of better quality (Gianaroli et al., 1996; Menezo et al., 2010).
4.3e Embryo development

In agreement with this study, several others found no correlation between cumulus cell apoptosis and embryo quality (Diaz-Fontdevila et al., 2009; Corn et al., 2005; Host et al., 2002). Corn et al., (2005) stated that cumulus cells associated with oocytes that developed into normal blastocysts, a significantly lower rate of apoptosis was detected at day 5 of embryo cleavage.

Host et al., (2002) suggest that cumulus complexes undergo a general decay or controlled cell destruction prior to ovum pick up, and that cumulus DNA fragmentation influences embryo development. Assuming the reason to be the transfer of apoptotic signals from the cumulus cells to the oocyte, the timing of gap junction closure between these two cell types and the initiation of apoptosis may have a crucial effect on oocyte and embryo development (Host et al., 2002). As a result, LH induction of the final maturation phase of the immature oocyte may be compromised by apoptosis (Host et al., 2002).

Whilst some studies have observed a correlation between granulosa cell apoptosis and embryo quality (Nakahara et al., 1997), others have found no difference in the incidence of cumulus cell apoptosis according to embryo quality (Lee et al., 2001). Lee et al., (2001) suggest that although oocytes with a high degree of cumulus cell DNA fragmentation may fail to fertilize, cumulus cell apoptosis has no impact on embryo quality and development.

4.3f Pregnancy outcome

A problem when studying the impact of granulosa and cumulus cell DNA fragmentation on pregnancy outcome is that these cells are usually pooled from all follicles prior to analysis, and is not collected on a per follicle basis. As indicated by a study by Corn et al., (2005) it is possible that there is substantial variance in the level of apoptotic cumulus cells within each follicle of the same patient. As a result, the study would be more accurate if the level of apoptosis was only considered in the cumulus cells from follicles from which corresponding embryos or blastocysts had been transferred. Even this may be difficult in the case of singleton pregnancies following multiple embryo transfer as it is not possible for embryologists to determine which embryo in particular implanted. Furthermore, oocytes and embryos will also need to be cultured singly. To obtain the most accurate results, only transfers that resulted in a 100% implantation rate should be considered, and compared to patients with failed implantation (Corn et al., 2005). A drawback of this approach will be the probably small patient numbers/sample size, preventing statistical significance (Corn et al., 2005).
In support of this study, several others failed to observe a statistically significant difference in cumulus and granulosa cell apoptotic levels between pregnant and non-pregnant patients (Diaz-Fontdevila et al., 2009; Nakahara et al., 1997; Lee et al., 2001; Raman et al., 2001).

It has been reported that high basal serum FSH levels in women undergoing IVF can increase apoptosis in granulosa cells (Seifer et al., 1996). A study by Oosterhuis et al., (1998) disputed this theory by describing how women with a lower percentage of apoptotic granulosa cells became pregnant than those who did not, despite having normal FSH level. They observed a cut-off level of 13%, where patients with ≤ 13% apoptosis became pregnant, but those with higher levels did not.

4.3g Factors that may affect cumulus and granulosa apoptosis and discrepancies between results in different studies

In addition to the contradictory reports associating levels of cumulus and granulosa cell apoptosis with maternal age, fertilization rate, pregnancy rate, embryo quality and causes for infertility, the mean levels of apoptotic cumulus cells observed in mature cumulus-oocyte complexes also varies greatly. Mean levels of apoptosis ranging from approximately 0.43%-34% have been recorded (Lee et al., 2001; Bencomo et al., 2006; Ruvolo et al., 2007; Abu-Hassan et al., 2006; Corn et al., 2005; Moffat et al., 2002; Host et al., 2002)

Several factors can affect cumulus and granulosa cell apoptosis, and contribute towards the discrepancies observed in different studies. For example, cumulus cell DNA fragmentation was correlated to the maturation stage of the corresponding oocyte where immature oocytes were associated with a higher proportion of apoptotic cumulus cells (Corn et al., 2005; Host et al., 2002; Host et al., 2000). No such relationship was observed by Moffatt et al., (2002), but they evaluated different, immunohistochemical markers to assess cellular apoptosis.

Reports associating the levels of apoptosis in granulosa and cumulus cells with respect to age, fertilization rate, embryo quality and pregnancy rate vary within different study groups. The contradictions observed amongst different investigations may be due to three main factors (Diaz-Fontdevila et al., 2009). The first involves the stimulation protocol. It is thought that hormones such as E₂, FSH, LH, and hCG exert an inhibitory effect on granulosa cell apoptosis, but other hormones like androgens, GnRH, or GnRH analogues increase the apoptotic rate compared with cycles without stimulation (Malamitsi-Puchner et al., 2004; Kaneko et al., 2000; Ruvolo et al., 2007). The second reason for observed discrepancies is due to the different methods used to detect apoptosis. The Comet assay, flow cytometry, Hoeestcht, Apodkit staining, SCSA or TUNEL assay may have been used.
The final reason is related to the origin of cumulus cells. Cells used for analysis may have been derived from individual follicles (where the fate of the corresponding oocyte can be traced) or from a pool of aspirated follicles (as was the case in this study). In the latter scenario, it is impossible to link cumulus cell apoptosis from one follicle to a specific oocyte.

Other factors that may lead to discrepancies amongst the results observed may be related to whether cumulus cells are obtained from oocytes undergoing IVF or ICSI, and the time lapse between the derivation of such cells and DNA fragmentation analysis (Diaz-Fontdevila et al., 2009). The most pathological conditions that require IVF-ET may also influence the relationship between the level of cumulus cell apoptosis and IVF outcome. For example, the basal level of apoptosis was similar for a majority of the infertilities including male factor infertility, tubal factor infertility and unexplained infertility (Diaz-Fontdevila et al., 2009). However, a significantly higher level of apoptosis was detected in cumulus cells from patients with endometriosis (Diaz-Fontdevila et al., 2009; Nakahara et al., 1998; Nakahara et al., 1997; Agarwal et al., 2006). Patients with endometriosis were found to have a higher 8-OHdG index than patients with other forms of infertility (Seino et al., 2002). The increased frequency of apoptosis in granulosa cells was thought to be due to the high level of oxidative stress produced by endometriosis lesions (Toya et al., 2000). Studies have also suggested that the anti-apoptotic effect of hyaluronic acid is decreased in patients with endometriosis (Underhill, 1992; Turley et al., 1991; McCourt et al., 1994).

### 4.3h Is cumulus apoptosis a predictor of IVF-ET outcome?

In spite of the hypothesis that the DNA status of cumulus and granulosa cells may predict pregnancy outcome following IVF-ET, this study did not observe such a relationship. These results were supported by those of Host et al., (2000) who demonstrated that the degree of apoptosis in cumulus cells of the oocyte cannot be compared between oocytes of varying fertilization states (no fertilization, normal fertilization and abnormal fertilization). They concluded that a correlation did not exist between the degree of cumulus cell apoptosis and consequent fertilization of the corresponding oocyte.

In contrast, Lee et al., (2001) stated that increased levels of cumulus cell apoptosis is linked to ovarian hyperstimulation response, fertilization outcome, and pregnancy following IVF-ET. They claimed that the occurrence of apoptosis in cumulus cells can be used to predict oocyte quality, outcomes of IVF–ET, and age-related decline in fertility (Lee et al., 2001).
Raman et al., (2001) noted a positive relationship between oocyte competence and cumulus cell DNA status. They observed that oocytes that successfully fertilized following ICSI were associated with cumulus cells containing high levels of DNA fragmentation. Raman et al., (2001) postulated that increased levels of apoptosis in cumulus cells suggest increased maturity of the corresponding oocyte. They claimed that a positive relationship between increased apoptosis and oocyte maturity resulted in better fertilization. However, Host et al., (2000) proposed a different theory regarding cumulus cell apoptosis and oocyte maturity. They suggested that decreased frequencies of apoptosis in individual follicles result in better outcomes with regards to the oocyte because apoptosis inhibits maturation, and that increased apoptosis leads to decreased maturity and subsequently decreased fertilization (Host et al., 2000). Still, a detrimental threshold for the incidence of apoptosis has not yet been established, and so the consequences on embryo quality are difficult to determine (Diaz-Fontdevila et al., 2009).

4.3i Future work

In future studies assessing the levels of DNA fragmentation in granulosa and cumulus cells in relation to IVF-ET outcome, cumulus cells should be used from follicles from which the corresponding embryo or blastocyst has been transferred following single culture and elective single embryo transfer (refer 4.3f). If multiple embryos are transferred, only transfers that result in a 100% implantation rate should be considered, and compared to patients with failed implantation.

Microarray platforms and RT-PCR may also help identify genomic biomarkers expressed in cumulus cells that can reliably and non-invasively predict oocyte developmental competence to reinforce morphological criteria.

Considering ROS and antioxidants are known to intervene in reproductive physiology and pathology, their presence and effects in the follicular fluid and the cumulus-oocyte complex should be studied. Cells obtained from ART patients can be investigated for antioxidants such as superoxide dismutase and compared to parameters such as female age, types of infertility and IVF-ET outcome. A luminometer can be used to determine if an upper ROS cut-off level exists in the follicular fluid of women undergoing IVF, above which oocyte quality is diminished and/or embryo development is hampered. A potential threshold value can also then be compared to types of female infertility.

It may be beneficial to use a metabolic approach to assess biological markers such as hormones, growth factors, ROS, anti-apoptotic factors, proteins, peptides, amino acids and sugars in the follicular fluid. If these criteria are integrated with other parameters including oocyte morphology
and gene expression may assist embryologists select optimal oocytes and determine fertility potential.

4.4 The assessment of murine embryo DNA fragmentation in response to culture medium containing GM-CSF

4.4a Study results

Embryos were cultured in medium containing 5 different concentrations of recombinant mouse GM-CSF in group culture in a standard petri dish. When the effect of culture medium containing GM-CSF on DNA fragmentation in murine embryos cultured in a standard petri dish was examined, the highest percentage of mean DNA fragmentation was observed in the control group treated with 0ng/ml GM-CSF. Whilst there appeared to be a general decline in percentage DNA fragmentation as GM-CSF concentration increased, these observations were not statistically significant. This lack of significance is probably due to the small sample size of embryos analysed. When the percentage of embryos containing one or more blastomeres with fragmented DNA was assessed, a GM-CSF concentration dependent effect was not observed. Still, it was interesting to note that the lowest percentage of embryos containing blastomeres with fragmented DNA was in embryos cultured in medium supplemented with 2ng/ml GM-CSF. Yet again, the differences between the five groups were not statistically significant.

Embryos were also cultured in medium containing 5 different concentrations of recombinant mouse GM-CSF in group culture using an EmbryoSlide®. Unexpectedly, the highest percentage of mean DNA fragmentation was observed in the group of embryos cultured in medium supplemented with treated with 2ng/ml GM-CSF. The lowest mean percentage DNA fragmentation was observed in embryos treated with 5ng/ml GM-CSF. A GM-CSF concentration dependent effect was not observed in this study and the differences between the five groups was not statistically significant. When the percentage of embryos containing one or more blastomeres with fragmented DNA was assessed, the highest percentage of embryos containing blastomeres with fragmented DNA was observed in embryos treated with 2ng/ml GM-CSF whilst the lowest was at 5ng/ml GM-CSF. Yet again, the differences between the five groups were not statistically significant.

There was no significant difference between mean percentage DNA fragmentation in embryos cultured in standard petri dishes and those cultured using an EmbryoSlide® at either one of the 5 concentrations of GM-CSF, suggesting that the influence of different concentrations of GM-CSF on embryo DNA fragmentation is not determined by the method by which embryos are cultured.
4.4b Concentration gradient dependent effects

A study by Robertson et al., (2001) using genetically deficient GM-CSF mice observed a lack of GM-CSF expression in the embryo itself. They suggested that the developing embryo is regulated by maternal GM-CSF emanating from the uterine and oviductal epithelium in a paracrine manner as it travels through the tract during early pregnancy. Optimal effects of GM-CSF were obtained at a concentration of 2ng/ml (77pM) (Robertson et al., 2001). This concentration is thought to be of the same order of magnitude as the concentrations that stimulate biological effects in other cell lineages in vitro, and is similar to the GM-CSF content of the uterine luminal fluid derived during the preimplantation period (~60-80pM) (Robertson et al., 1992).

A study by Elaimi et al., (2012) reported that the addition of GM-CSF to culture medium did not have a positive influence on blastocyst development, and that in fact, GM-CSF had a negative effect on the rate of blastulation and cell number at higher concentrations (5ng/ml and 10ng/ml). A study by Papayannis et al., (2007) also described that the addition of 2ng/ml GM-CSF prior to embryo freezing failed to increase the percentage of embryos reaching the blastocyst stage or the rate of re-expansion. Behr et al., (2005) demonstrated that a GM-CSF concentration of 0.125ng/ml promoted preimplantation development in the mouse embryo. Several repeated experiments by their group showed that increasing the concentration of GM-CSF from 0.25ng/ml to 2ng/ml in the culture medium failed to further promote embryo development to the blastocyst stage. Behr et al., (2005) demonstrated that low concentrations of GM-CSF (1ng/ml and 2ng/ml) did not improve blastocyst development, and that embryonic development was impeded at a GM-CSF concentration of 20ng/ml.

The unresponsiveness and resistance of embryos to higher concentrations of GM-CSF is comparable to observations in other target cells. This reaction, or rather lack of, may be due to receptor down-regulation or other negative feedback mechanisms (Cannistra et al., 1990). The low affinity α subunit of the GM-CSF receptor is thought to be present in blastocyst stage embryos (Robertson et al., 2001), limiting the binding capacity of the ligand to the GM-CSF receptor (Behr et al., 2005). GM-CSF is also though to down-regulate it receptor, and this form of negative regulation may explain why increased concentrations of GM-CSF do not promote embryonic development in vitro (Behr et al., 2005).
4.4c GM-CSF in embryo culture medium and its importance in human IVF

GM-CSF is thought to play a significant role in embryo development as it promotes embryonic growth and viability by wielding a positive control over genetic paths that include cell proliferation, progression to blastocyst, zona pellucida hatching, and embryo implantation in the endometrium (Sjoblom et al., 1999; Robertson et al., 2001). The survival- promoting effects of GM-CSF on the inner cell mass of embryos cultured in vivo and in vitro illustrate its embryotrophic properties (Sjoblom et al., 1999; Robertson et al., 1999) required for early embryo development, just prior to and after implantation. The embryotrophic effects of GM-CSF are thought to be more significant in human embryos (Sjoblom et al., 1999) where high rates of embryo development are relatively difficult to achieve in vitro (Robertson, 2007). It is claimed that adding cytokines to culture medium can double the proportion of IVF embryos that develop into the blastocysts (Sjoblom et al., 1999) and improve their developmental competence (Robertson, 2007). Embryos cultured in GM-CSF were found to reach the blastocyst stage 14 hours faster on average, and contain approximately 35% more cells (Sjoblom et al., 1999), mainly due to an increase in the size of the inner cell mass and a 50% decrease in apoptotic nuclei (Sjoblom et al., 2002). The amount of GM-CSF secreted into culture medium by autologous endometrial cells co-cultured with human IVF embryos was found to correlate with the probability of a successful pregnancy following embryo transfer (Spandorfer et al., 1998).

Its incorporation into clinical research has yielded promising results with regard to outcome parameters in women undergoing IVF (reviewed by Siristatidis et al., 2013; Sjoblom et al., 1999; Kim et al., 2001; Sjoblom et al., 2001; Shapiro et al., 2003; Agerholm et al., 2010; Origio Website, 2011).

A study by Sjoblom et al., (1999) assessed the effect of supplementary GM-CSF on in vitro embryo development. They observed a rise in the percentage of embryos that reached blastocyst stage (75.5% vs. 30%, P<0.001), improved hatching (78% vs. 47%, P<0.001), and increased number of cells in the blastocyst by 35% (P<0.003). The latter effect appeared to be more obvious in the inner cell mass. These authors supported the use of GM-CSF supplementation in culture media to promote derivation of implantation competent blastocysts, regardless of the quality of embryos and culture media used. The same study group (Sjoblom et al., 2001) used the TUNEL assay on thawed 2-4 cell embryos from women undergoing IVF/ICSI, to demonstrate that cell death was more abundant when GM-CSF was not added to the culture medium containing blastocysts (mean number of apoptotic cells 5.6 ± 3.0 versus 2.8 ± 1.4, P < 0.05). They concluded that the addition of GM-CSF was crucial in the promotion of cell viability during early embryo development.
The impact of GM-CSF signalling in early embryos is not limited to short-term survival, but also affects long-term developmental competence (Robertson, 2007). Murine embryo culture was known to have adverse effects on the fetus that manifested as growth restriction in utero, rapid compensatory growth soon after birth, and greater body mass with increased fat deposits in adults (Thompson et al., 2002). The exposure of embryos to GM-CSF prior to transfer was found to restore embryo implantation rate, correct deficiencies in placental structure and fetal growth trajectory and improve the long-term adverse effects of embryo culture on postnatal growth to a certain degree (Sjoblom et al., 2005).

Kim et al., (2001) detected a significantly improved rate of clinical pregnancy in the GM-CSF enhanced media compared to the control groups (46.1% vs. 30.8%, \( P < 0.05 \)) in both IVF and ICSI cycles. The difference was even more apparent when only IVF was used (66.7% vs. 37.3%, \( P < 0.05 \)). They too stressed the benefits of GM-CSF as a culture medium supplement.

A randomized control study (Shapiro et al., 2003) investigated embryo progression from 2PN oocyte to blastocyst in medium supplemented with GM-CSF compared to a control group devoid of the cytokine. Despite an increase in the number of cleavage stage cells per embryo (6.1 versus 5.8, \( P=0.047 \)) and a 50% increase in the proportion of blastocysts that reached the expanded blastocyst stage (1.6 versus 1.1, \( P = 0.001 \)), there was no change in the pregnancy (7% versus 50%) and implantation rates (32% versus 25), regardless of the addition of GM-CSF. They concluded that GM-CSF enhanced embryo growth throughout preimplantation development.

Agerholm et al., (2010) conducted a multicentre randomized control study on the effect of ploidy rate in human oocytes following the addition of 2ng/ml GM-CSF to culture medium. Their study took place from fertilization to day 3 and involved the morphological evaluation of embryos and FISH analysis for chromosomes 13, 16, 18, 21, 22, X and Y. There appeared to be no difference in the aforementioned parameters following the supplementation of GM-CSF and the use of a placebo. They reported a lack of a significant difference between the control group of embryos (65.2%) and those exposed to 2ng/ml GM-CSF (66.75). They also did not observe any significant increases in fertilization rate, cleavage rate or the total number of embryos that developed normally.

The largest multicentre randomized control study was conducted by ORIGIO (2011). Their investigators reported a statistically significant improvement of 44% of the on-going implantation rate and a significant improvement of 28% in the live birth rate (in terms of per embryo transferred and per transfer cycle) in women with a history of miscarriages undergoing IVF, following the addition of GM-CSF to the culture media. One particular study administered GM-CSF directly to
women with recurrent abortions, and resulted in favourable outcomes (Scarpellini and Sbracia, 2003). Whilst the beneficial nature of GM-CSF in terms of clinical pregnancy rate is yet to be established, there is good evidence for women who have suffered >1 miscarriage in their personal history (Siristatidis et al., 2013). It is interesting to note, however, that clinicians and researchers are still very hesitant to use GM-CSF in culture media.

Improvements in sequential culture systems for human IVF during the last decades have also allowed extended culture of early human embryos to the blastocyst stage (Kawamura et al., 2012), and most of studies described above assessed the effect of GM-CSF on embryos at the blastocyst stage. The stage of blastocyst culture allows for the selection of the most developmentally competent embryos for transfer, and achieving a better synchronization between the embryo stage and uterine development (Aplin and Kimber, 2004). Therefore, the transfer of optimal blastocysts to the uterus may result in optimization of the two main parameters that determine improved live birth rates when embryo transfer is performed at the blastocyst stage (as opposed to that at day 2 or 3) following IVF (Kawamura et al., 2012; Glujovsky et al., 2012); better implantation rates and the reduction in multiple births (Sjoblom et al., 1999). Selective single embryo transfer in patients with good prognosis has shown to be effective in reducing multiple pregnancies, without compromising pregnancy rate (Thurin et al., 2004).

Whilst most study groups agreed that GM-CSF enhancement benefited preimplantation embryo development, some differing results were produced regarding its effect on implantation and clinical pregnancy rate. These findings are important as blastocysts formation can be indicative of normal embryo development potential and the outcome of embryo transfer. Whilst implantation and clinical pregnancy rates were not significantly improved in some of the studies reviewed, significant improvements in both implantation and pregnancy rates were reported in a large study carried out by the manufacturers of the commercial product containing GM-CSF (ORIGIO, 2011). They assert the potential role of GM-CSF when used to supplement the culture media of preimplantation embryos during IVF.

The merits of supplementary growth factors are numerous. Increased fertilization rates, decreased degeneration and apoptosis, increased cell numbers, improved blastocysts development, increased hatching, better rates of implantation and successful pregnancy all contribute to the efficacy of ART, furthermore, earlier blastocyst development may improve implantation rates of fresh transfers by promoting synchrony with the short window of endometrial receptivity (Richter et al., 2006; Richter, 2008; Shapiro et al., 2008). Increased cell numbers may also improve cry-tolerance (Richter, 2008). In PGS studies for aneuploidy, it was noted that 32-66% of seemingly normal, euploid embryos failed
develop into blastocysts *in vitro* (Magli *et al*., 2001; Sandalinas *et al*., 2001; Rubio *et al*., 2007). The supplementation of culture media with appropriate growth factors may succeed is ‘rescuing’ these embryos (Richter, 2008).

The supplementation of culture medium with growth factors may also offset the weakening of intercellular connections observed *in vitro*, thus reducing the risk of monozygotic twinning that in associated with extended embryo culture (Behr *et al*., 2000; Milki *et al*., 2003; Wright *et al*., 2004). Interestingly, no cases of monozygotic twinning were observed in over 800 deliveries following co-cultured blastocyst transfers (Menezo and Sakkas, 2002).

Any existing contradictory views on the cytokine effect beyond embryo transfer are probably due to confounding effects such as maternal age, type of subfertility, dose and duration of protocol drugs, socio-economic index and parity (Siristatidis *et al*., 2013). It is thought that the target group of these studies should include subgroups of subfertile women (women with unexplained subfertility and repeated implantation failures or recurrent miscarriage) and those of advanced maternal age. Importantly, the possible manifestations of childhood or adult genetic and/or chromosomal abnormalities incurred by the addition of GM-CSF needs to be addressed (Siristatidis *et al*., 2013). Unfortunately, it may be a while until epidemiological studies and their proper systematic reviews and meta-analyses are able to fully address the long-term effects of GM-CSF supplementation in human embryo culture medium (Siristatidis *et al*., 2013).

**4.4d Mechanisms of GM-CSF**

**4.4di Stress response**

The promotion of blastocyst cell accumulation by GM-CSF in cultured murine embryos is also thought to be associated with the down-regulation of the cellular stress response (Chin *et al*., 2009). Murine blastocysts were cultured *in vitro* from zygote stage with and without recombinant mouse GM-CSF (rmGM-CSF), and *in vivo* developed blastocysts were derived from Csf2 null mutant and wild-type mice. Csf2 mRNA encodes GM-CSF, whilst Csf2ra encodes the GM-CSF receptor α (Robertson *et al*., 2001; Sjoblom *et al*., 2002). The study group assessed the effect of GM-CSF on blastocyst expression of response and apoptosis genes by microarray, qPCR and immunochemistry. Blastocysts exposed to rmGM-CSF *in vitro* were found to have suppressed stress response and apoptosis gene pathways, as demonstrated by microarray analysis of the gene transcription profile (Chin *et al*., 2009). qPCR analysis confirmed that the expression of heat shock protein (HSP) and apoptosis pathway genes Cbl, Hspa5, Hsp90aa1, Hsp90ab1 and Gas5 was inhibited by rmGM-CSF in
in vitro blastocysts (Chin et al., 2009). When embryos were cultured with rmGM-CSF, immunocytochemistry analysis demonstrated a decrease in the level of HSPA1A/1B and BCL2 (Chin et al., 2009). Although BAX and TRP53 were unaltered at the protein level, Bax mRNA expression was reduced following blastocyst exposure to GM-CSF (Chin et al., 2009). In Csf2 null mutation from in vivo developed blastocysts, elevated expression was limited to Hsph1, and not to any other stress response genes (Chin et al., 2009). In other cell lineages, GM-CSF is thought to inhibit apoptosis induced via the extrinsic pathway by the cytokine tumour necrosis factor-α (Quentmeier et al., 2003), a known inducer of apoptosis in embryos (Wuu et al., 1999). This suggests a possible pathway by which GM-CSF may act on the embryo’s stress response. The authors (Chin et al., 2009) concluded that the cellular stress response and apoptosis pathways are inhibited by GM-CSF, in an attempt to facilitate embryo growth and survival. Whilst other cytokines can compensate for the absence of GM-CSF and the lesser degree of environmental stress in vivo than in vitro, the protective effects of this cytokine are particularly apparent in in vitro culture medium (Chin et al., 2009).

4.4dii Glucose uptake

In murine embryos, a wave of apoptosis occurs in the 60-110-cells stage of blastocyst formation, and is prevalent in the inner cell mass (Hardy, 1997). Death in the inner cell mass significantly contributes to retardation of blastocyst development, particularly when sub-optimal concentrations of metabolic substrates are provided in the culture environment (Brison and Schultz, 1997). The inner cell mass is thought to be favourably maintained by insulin, IGF-1 and TGFα (Harvey and Kaye, 1990; Harvey and Kaye, 1992; Brison and Schultz, 1997). A study by Roberston et al., (2001) demonstrated that murine preimplantation embryos express GM-CSF receptors and that GM-CSF promotes cell proliferation and viability in blastocyst stage embryos. The most evident observation in their study was the increase in the total number of blastomeres, presumably involving a mechanism which accelerates cell division or diminishes cell death. This increase in cell number is thought to be due to enhanced blastomere metabolic activity, since the promotion of glucose uptake by GM-CSF was identified using the non-metabolisable glucose analogue 3-OMG. Glucose is a primary energy source in embryos from the moment of morullae compaction. A clear relationship is thought to exist between the exposure of embryos to other growth factors that promote glucose metabolism (particularly insulin and IGF-1) and blastocyst cell number (Harvey and Kaye, 1992; Pantaleon et al., 1997; Beebe and Kaye, 1991; Pampfer et al., 1990). Therefore, the effect of GM-CSF on blastomere number may be related to a reduced incidence of apoptosis, possibly as a consequence of altered metabolic substrate availability (Robertson et al., 2001).
However, according to the ‘quiet embryo’ hypothesis by Henry Leese (Leese, 2002), increased metabolism may be indicative of poor embryonic health. Leese stated that embryos with a ‘quiet’ metabolism had higher viability rates than those with ‘active’ metabolism (Leese, 2002). Furthermore, Lane and Gardner (1996) reported that the rate of glucose uptake is determined by blastocyst quality, where poor quality blastocysts have greater glycolic activity.

Based on the observation that blastocysts cultured in the presence of GM-CSF contained more cells than control blastocysts, Robertson et al., (2001) suggested that GM-CSF can somewhat restore delayed development characteristics of embryos cultured in vitro (Bowman and McLaren, 1970; Harlow and Quinn, 1982). The degree of this increase in cell numbers is similar to that in blastocysts cultured in vitro in the presence of insulin and the IGF family (Harvey and Kaye, 1990; Harvey and Kaye, 1992), CSF-1 (Bhatnagar et al., 1995), and autocrine grown factors including TGF-α (Hoversland and Weitlauf, 1981). The experiments by Robertson et al., (2001) showed that whilst retarded development in the absence of GM-CSF was associated with delays in hatching and implantation in vitro, it did not affect the hatching kinetics in vivo in GM-CSF deficient mice. This supports the theory that the principal force in the timing of hatching and implantation in vivo involves hormonally regulated maternal tract factors (Hoversland and Weitlauf, 1981). Still, GM-CSF may exert a direct or indirect effect in promoting expression of the trophectoderm-derived proteases and attachment molecules involved in the hatching and implantation processes (Robertson et al., 2001).

Furthermore, embryos cultured with 0.125ng/ml GM-CSF were found to contain significantly higher transcripts of Bcl-2, the anti-apoptotic factor, compared to the control group (Behr et al., 2005). The mRNA level of Bax, the pro-apoptotic factor, was similar in both groups. Thus, GM-CSF may influence the process of cellular apoptosis by altering the expression of Bcl-2 in preimplantation embryos (Behr et al., 2005).

**4.4e The use of GM-CSF as an in vitro growth supplement**

The steadily impressive benefits observed in association with a range of different co-culture systems and individual growth factor supplements suggest that further advances can be achieved with many different growth factors or a combination of such factors (Richter, 2008). Whilst most studies have investigated individual factors, it is possible that a complex mixture will reproduce in vivo conditions more accurately (Desai et al., 2000; Desai et al., 2007; Sjoblom et al., 2005; Roudebush et al., 2004; Richter, 2008). Optimal results and marginal scope for developmental disruption will likely be realized with a concoction that accurately reflects the natural environment. The most rational factors to incorporate in culture media include those present in vivo and for which embryos have
receptors, such as, activin, CSF, EGF, GM-CSF, HB-EGF, IGF-1, IGF-2, LIF, PDGF, TGF-α, FGF, M-CSF, VEGF, fibronectin and inhibin (Richter, 2008). More recent methodologies such as proteomic analysis may allow characterization of biological fluids such as follicular, tubal and endometrial fluid, providing a complete detailing of growth factor composition in the in-vivo environment (Huang et al., 2001; Soulet and Rivest, 2002; Angelucci et al., 2006; Shaw et al., 2007; Hanrieder et al., 2008).

Apoptosis has the potential to eliminate viable cells at the earliest stages of embryo development, leading to the death of the organism (Brison and Schultz, 1998). Therefore, the frequency of apoptotic cell death is likely to be indicative of embryo viability and developmental competence (Kamjoo et al., 2002). On the other hand, the protective nature of GM-CSF is still debatable as apoptosis is supposed to be the default pathway of cell fate in embryos as well as other cell types, and elimination of unwanted cells is essential for development. An unresolved issue is the interference with the normal physiological role of apoptosis that selectively removes abnormal blastomeres (Hardy, 1999). Whilst this is quite possible, it was found that human embryos cultured with GM-CSF did not have increased levels of aneuploidy (Agerholm et al., 2010). This suggests that the ‘rescue’ of abnormal embryos via the anti-apoptotic, protective nature of GM-CSF is improbable.

Although the precise cause of apoptosis in preimplantation development is unverified, it is clear that cell death can be regulated by the addition of low doses of GM-CSF to the embryo culture medium whilst accepting the existence of other apoptosis modulators (Behr et al., 2005). The discovery that GM-CSF improves cell number and prevents apoptosis in the IVF preimplantation embryo, and that embryos cultured in medium supplemented with GM-CSF more closely mimic in vivo embryos reinforces the potential clinical application of GM-CSF (Behr et al., 2005). However, before routine clinical application of any growth media supplement, long-term clinical trials need to be conducted to demonstrate its safety and efficacy in human IVF (Behr et al., 2005).

This study was carried out in conjunction with Aisha Elaimi in our laboratory as a part of her PhD study evaluating the Embryoscope™ in the assessment of embryo development. The levels of aneuploidy and DNA fragmentation in embryos were analysed in murine embryos cultured in medium containing 5 different concentrations of GM-CSF in (a) standard petri dishes placed in a standard incubator, (b) EmbryoSlide® culture dishes placed in a standard incubator and (c) EmbryoSlide® culture dishes placed in an Embryoscope™. The level of DNA fragmentation was also compared in embryos grouped according to the type of cell culture (in vitro, in vivo and 2-cell), and the rate of embryo development. The study also involved incubating murine embryos at different concentrations of GM-CSF before recording the rate of blastocyst development using time-lapse imaging with the Embryoscope™, and comparing it to the corresponding levels of DNA
fragmentation in each embryo. The levels of aneuploidy and DNA fragmentation were analysed in all the embryos using FISH and TUNEL respectively. I processed all the embryos (approximately 516 embryos) being analysed for DNA fragmentation using the TUNEL assay. I analysed all the embryos used in my PhD study. A double blind study of percentage DNA fragmentation was carried out on a majority of the embryos by me and Aisha Elaimi, and nearly the exact same level of DNA fragmentation was observed by both (data not shown).

I also carried out the statistical analysis for the following studies; (i) the relationship between the aforementioned culture methods (above a, b, c) and percentage DNA fragmentation at all 5 concentrations of GM-CSF, (ii) the relationship between percentage DNA fragmentation and in vitro, in vivo and 2-cell embryos cultured in medium containing 0ng/ml and 2ng/ml GM-CSF, and (iii) the relationship between percentage DNA fragmentation and the rate of cell division (hours taken to divide from 2-3 cells, 3-4 cells, and 4-5 cells) at all five concentrations of GM-CSF. I did not use any of the results regarding the Embryoscope™ as it was not specifically associated with the subject of my thesis.

4.4f Future work

This study should be repeated using a larger number of murine embryos, and testing them for DNA fragmentation and aneuploidy following treatment with different concentration of GM-CF. The rate of blastocyst development can also be analysed using time-lapse imaging. It may also be interesting to assess the expression levels of genes involved in the apoptotic process and stress response, and the metabolic rate of embryos cultured with GM-CSF.

Although the study conducted by Origio® in 2011 showed an improvement in the live birth rate, this effect was only observed in patients with previous miscarriages. Furthermore, considering published data present varying results and contradicting views on the effect of GM-CSF beyond embryo transfer, it is essential to conduct properly regulated randomized control trials by research groups with no vested interest in the ‘product’ being assessed. It is also vital to explore potential genetic or chromosomal abnormalities encountered as a result of media supplementation with GM-CSF that can manifest in childhood or adult life. Unfortunately, it may be a while before the proper systematic reviews and meta-analysis of epidemiological studies are able to assess the long-term effects of GM-CSF on human preimplantation development.

Infertile women should be treated with the same baseline characteristics and causes of subfertility as the reference group to provide an unambiguous view of the effects adding GM-CSF to the culture
medium will have. Furthermore, variables such as female age, type of subfertility, doses and duration of protocol drugs, socioeconomic index and parity should be considered. I would also advise investigating the cost effectiveness of supplementing media with GM-CSF.

It is incredibly important to further analyse why such an effect was only observed in a subgroup of patients, and study the effect of culturing embryos in medium containing GM-CSF on the long-term health during childhood and adulthood. Further studies should also be carried out on the effect of different concentrations of GM-CSF on the rate of blastulation, rate of aneuploidy, and the rate of metabolism in embryos. Since it is considered unethical to use human embryos, human embryonic stem cells may be a suitable model to determine the effect of GM-CSF on aneuploidy. Furthermore, when analysing the levels of aneuploidy in either embryos or human embryonic stem cells, the use of FISH may be a limiting factor due to its assessment of 3 chromosomes at a time. Instead, array CGH may provide a more accurate picture of the chromosome complement and help determine if in fact GM-CSF has a detrimental level on the rate of aneuploidy. It is also possible to use human embryonic stem cells to determine the effect of GM-CSF on aneuploidy using a dose response study.

4.5 The assessment of human embryonic stem cell DNA fragmentation and aneuploidy following exposure to pre-tested toxins

I expected the degrees of DNA damage and aneuploidy for chromosome 13, 15, 16, 18, 21 and 22 to increase with the concentration of each toxin, but this was not the case. An irregular pattern was observed, where in some instances, the control group of untreated hESCs displayed a higher level of DNA fragmentation and aneuploidy than the treated samples. A control study using lymphocytes was used out to eliminate the possibility of hybridisation error and background noise when using FISH. Because only a small sample of embryonic stem cells was tested for DNA fragmentation and aneuploidy following toxic stress, the observed results cannot really be scrutinized.

Although the recapitulation of whole organs is not yet possible by differentiating hESCs, it has been accepted that hESCs can at best, provide an incomplete portrait of a toxicant response, and no other single model has such potential. Recognizing their main limitation as the absence of organogenesis in vitro, the pluripotent nature of hESCs still provides a useful surrogate assay to evaluate toxicant risk, particularly since ethical reasons prevent humans and human embryos being used to test toxicants.

Despite the evidence that supports the use of hESCs to study developmental toxicity, it must be accepted that in vitro systems cannot fully recapitulate or justify events that disrupt normal human
development due to exogenous chemicals. In vitro systems are incapable of replicating absorption, distribution, metabolism and excretion, making it difficult to envisage how a compound of unknown toxicity will operate in vivo. Furthermore, hESC models cannot imitate toxicity in utero which is influenced by fetal-maternal interactions and the process of organogenesis. However, considering the physiological significance of hESCs to human development, the use of cells derived from human embryos may provide in vitro prediction endpoints more analogous to human development in vivo, than when compared to the use of animal models, other in vitro non-human assays, the EST, and whole embryo culture.

4.5a Opportunities and challenges associated with hESCs as a model for toxicity screening

Human pluripotent stem cells hold great promise in research and medicine due to their virtually unlimited proliferative capacity and multi-lineage potential. Required throughout animal development, they are able to differentiate into several specialized cell types required for research, such as cardiomyocytes, hepatocytes, neurons, and muscle cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007; Park et al., 2009; Nakagawa et al., 2008; Selvaraj et al., 2012; Liu et al., 2011). However, practical applications of hESCs have been hindered by the technical challenges and ethical concerns related to its use (Liu et al., 2013). Furthermore, traditional methods of generating hESCs using surplus IVF embryos are not compatible when designing genetically matched, patient- or disease-specific hESC lines. However, the induction of human somatic cells to revert back to display properties of authentic hESCs has avoided this issue. These induced pluripotent stem cells (iPCs) have similar developmental potential as hESCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008; Nakagawa et al., 2008). It is believed that iPSC technology may prove useful in the generation of disease models, drug discovery, toxicity testing and cell based-therapeutics (Selvaraj et al., 2012; Liu et al., 2011; Selvaraj et al., 2010; Deng, 2010).

When human cells are taken directly from an affected patient and grown in the laboratory, they have a limited lifespan which restricts the types of studies for which they can be used (Liu et al., 2013). However, when cells have been modified to make them immortal, these alterations change their physiology and can cast doubt on subsequent study results (Liu et al., 2013).

The main goal in predictive toxicology is to create in vitro model systems as proxies to monitor the diversity of chemical responses that occur within the human population (Liu et al., 2013). If variations in chemical responses are to be predicted in humans, pluripotent stem cell lines should
ideally be designed from diverse individuals of various genders, races, ages and disease history (Liu et al., 2013). More powerful models of predictive toxicology may be achieved by utilizing patient-specific iPSCs obtained from individuals with known predispositions to specific diseases or resistances to specific drugs (Liu et al., 2013). The use of in vitro toxicity assays based on hESC/iPSCs in preclinical testing can lead to the removal of potentially toxic compounds an earlier stage of preimplantation development. This can save labour, time and decrease the propensity for adverse human outcomes (Liu et al., 2013). However, cell lines established from specialised somatic cells are not identical to germ stem cells and do not recapitulate their differentiation processes (Krtolica and Giritharan, 2010).

Animal experiments have provided information regarding the detrimental effects of environmental toxins towards the development of gametes, but this form of testing has proven problematic due to several species-specific variations, complexity and higher costs associated with animal experiments (Krtolica and Giritharan, 2010). Although animal models have been instrumental in the identification of drug candidates and the assessment of detrimental effects of chemicals on mammalian development, they are limited in their ability to predict toxic outcomes in humans (Liu et al., 2013). Because the sensitivity of human embryos is thought to be higher than that verified in rodents, certain chemicals thought to be safe following animal studies can induce birth defects and serious toxicity in human. For example, thalidomide was found to have no effect on prenatal development in rodents, but caused severe developmental defects in human children whose mothers had ingested it during pregnancy to alleviate morning sickness (Kumar et al., 2012). In 2006, a clinical trial of the TGN1412 antibody to treat multiple sclerosis, rheumatoid arthritis, and leukemia caused devastating systemic organ failure in all six human volunteers who took part in the trial, despite previous animal studies showing no adverse effects (Ponce, 2011). And so, newer models with better predictive ability and insight into human development are necessary.

Increased public attention to environmental health concerns has highlighted the potential relationship between human exposure to health hazards, and the risk of cancer, autism, developmental defects and other unfavourable health outcomes (Liu et al., 2013). In general, the use of in vitro systems based on stem cells reduces the need for animal testing in toxicity screening, allows detailed analysis of the mechanisms of action of specific toxins, has better physiological relevance and translational potential, and can provide detailed dose-response data directly from human models.
4.5b Effects of the experimental toxins on human development

An unavoidable part of modern life, exposure to chemicals, drugs, and physical or biological insults can unfortunately contribute to increasing rates of infertility (Krtolica and Giritharan, 2010). Causes of infertility include poor gamete quality, recurrent failed fertilization, sub-optimal development of the preimplantation embryo and failed implantation. Mounting evidence suggests that environmental pollutants affect gamete development at various stages (Clementi et al., 2008; Hauser and Sokol 2008; Swan, 2006). For example, fungicides are thought to affect normal germ cell development and function leading to infertility (Anway et al., 2005). Human exposure to lead and cadmium can affect spermatogenesis in terms of sperm count, motility and morphology (Telisman et al., 2000), and exposure to endocrine disrupting chemicals can affect the development of early germ cells and predispose individuals to infertility in adulthood (Skakkebaek et al., 2001). Phtalates promote apoptosis in cultured human fetal testicular cells (Habert et al., 2009) and sulfasalazine, a drug often used for the treatment of inflammatory bowel disease, can cause abnormal spermatogenesis and male infertility (Toovey et al., 1981). Furthermore, aberrant primordial germ cell and gonocyte development may encourage testicular cancer (Rajpert-De Meyts et al., 2003).

Once these toxicology models are well established, the screening for reproductive toxicity may become more widespread and used to analyse a range of xenobiotics found in everyday objects, from food additives to clothing and plastics (Krtolica and Giritharan, 2010). Hopefully, the accumulation of such data will help identify potential toxic compounds that contribute to the increased rates of human infertility (Krtolica and Giritharan, 2010).

At best, the hESC model will provide an incomplete picture of toxicant response, since factors such as maternal or hepatic metabolism and bioavailability cannot be considered (West et al., 2010). In vitro systems cannot account for processes that significantly contribute towards the disruption of normal human development due to exogenous compounds. It is difficult to predict how a specific substance will react in vivo, as absorption, distribution, metabolisms and excretion cannot be properly evaluated in an in vitro system (West et al., 2010). Furthermore, a hESC model cannot mimic teratogenicity in utero, which results from maternal-fetal interactions and the complex process of organogenesis (West et al., 2010). For ethical reasons, potential toxicants cannot be tested on humans or human embryos. Recognizing the main limitations of hESCs, the absence or organogenesis in vitro, the pluripotent nature of hESCs can still prove to be a valuable alternative to evaluate toxicant risk (West et al., 2010). Considering the physiological relevance of hESCs to human development, toxicology testing on cells derived from human embryos will likely provide a more
accurate portrayal of study endpoints than those available through animal models, non-human in-vitro assays or whole embryo culture (West et al., 2010).

A study by Unis et al., (2009) evaluated the toxicity of arsenite on 8-cell stage hamster embryos. They found that arsenite exposure resulted in total embryo lethality, major deformities, failure to undergo zona lysis, and significantly higher number of cells with fragmented DNA in embryos at the highest concentrations of arsenite. Their study highlights the sensitivity of preimplantation embryos to relatively small doses or arsenic in the luminal fluid. Their results were consistent with previous observations of neonatal death (Lugo et al., 1969), decreased birth weight, and increased spontaneous abortions in the female partners of men working or living close to smelters (Nordstrom et al., 1979), all due to arsenic exposure (Unis et al., 2009). Chronic exposure of pregnant females to arsenic has also been associated with conceptus mortality and postnatal growth retardation in a concentration-dependent manner (Golub et al., 1998).

Due to its widespread nature, a measurable amount of cadmium is present in almost everything we eat, drink and breathe (WHO, 1992). The ‘safe’ level for human ingestion of Cd has been estimated at 500µg/week (WHO, 1992). In areas that are not polluted by cadmium, human intake of this compound is mainly due to cigarette smoking, with approximately 0.2-1µg of cadmium assimilated with each cigarette smoked (Satarug and Moore, 2004; Nandi et al., 1969; Lewis et al., 1972; Friberg et al., 1974; Elinder et al., 1983). Cigarette smoking has been linked to sub-fertility (Shiverick and Salafia, 1999; Gray and Wu, 2000; Wilks and Hay, 2004; Neal et al., 2005) and ectopic pregnancies (Stillman et al., 1986; Karaer et al., 2006) in several studies. The concentration of Cd in the follicular fluid of female smokers undergoing IVF was reported at 7.93±0.16ng/ml (Zenzes et al., 1995) compared to 6.73±0.31ng/ml in non-smokers. Bioavailability of inhaled cadmium is high, with 10% deposited in lung tissues and 30-40% absorbed into the systemic blood circulation of smokers (Satarug and Moore, 2004). The long biological half-life of cadmium increases the risk of its cumulative effects (Lewis et al., 1972; Jarup et al., 1983; Matsuno et al., 1991).

Cadmium exposure can affect reproduction and development at every stage of the reproductive process. It can disrupt the blood-testis barrier by disturbing cell adhesion, causing oxidative stress and necrosis at higher experimental concentrations (reviewed by Thompson and Bannigan, 2008). It has also been found to incorporate itself into the chromatin of developing spermatozoa (Aoyagi et al., 2002), and alter the morphology of human granulosa cells (reviewed by Thompson and Bannigan, 2008). Cadmium concentration in the ovaries is thought to increase with age and inhibits oocyte development (reviewed by Thompson and Bannigan, 2008) and cumulus expansion (Mlynarcikova et al., 2005). It has been shown to accumulate in embryos from the four-cell stage
onwards, and is thought to inhibit blastocyst development thorough apoptosis and break-down in cell adhesion. Trophoblast formation and invasion are also susceptible to cadmium toxicity (Lin et al., 1997; Goyer et al., 1992; Breen et al., 1994). Animal experiments have demonstrated that cadmium exposure following embryo implantation can lead to various abnormalities, and its effects on embryo development are stage and dose-dependent.

The success of cancer treatment with regimens that are toxic to testicular function has made infertility a major problem in male children and young men, as chemotherapy and radiotherapy can cause long-term or permanent gonadal toxicity in male patients. Whilst endocrine dysfunction can occur (Shalet, 1989), the most concerning manifestation is the continuous reduction in sperm count to the point of azoospermia. Less severe damage to sperm function include the temporary loss of motility and morphological abnormalities which are restored if spermatozoa are produced post-treatment (Meistrich et al., 1992; Gandini et al., 2006). If the duration of azoospermia is long following cytotoxic therapy, abnormal sperm morphology may not revert to normal and sperm counts can plateau in the severe oligospermic range (Anserini et al., 2002), in a manner incompatible with fertility.

The administration of cisplatin to cancer patients during their chemotherapy regimen was found to detrimentally affect the gonads and reproductive function (Laverdière et al., 2005; Wallace et al., 1989). Two doses of 4.5 or 6mg/kg cisplatin reduced the number of healthy antral follicle numbers, decreased circulating AMH and AMH-positive follicles, and increased the number of follicles with apoptosis in rats (Yeh et al., 2006). Single doses of 5mg/kg cisplatin in rats also triggered significant decreases in the number of primordial follicles (Yucebilgin et al., 2004; Borovskaya et al., 2004). These reports confirm that cisplatin has detrimental effects on both growing and primordial ovarian follicles, leading to depletion of the ovarian reserve. A study by Akbal et al., (2009) also described how increasing concentrations of cisplatin significantly decrease mRNA expression levels of the Y-linked gene for testes-specific-protein (TSPY). It was stated that decreased TSPY expression following cisplatin exposure may be another mechanism for male infertility.

The methylation of cytosine residues in mammalian DNA occurs during gametogenesis and embryogenesis, and plays an important role in gene regulation, embryonic development, and genomic imprinting. The drug 5-azacytidine incorporates itself into DNA and blocks DNA methylation. A study exposing male germ cells to 5-azacytidine found that this drug induced dose-dependent reductions in testis and epididymal weights and sperm counts (Doerksen and Trasler, 1996). Dosages of 2.5 and 5mg/kg caused significant preimplantation loss, and the higher dose resulted in decreased fertility. A dose-dependent increase in the average number of abnormal
embryos per litter, sired by males treated with 5-azacytidine, was also reported (Doerksen and Trasler, 1996). Paternal administration of 5-azacytidine interfered with normal male germ cell development and altered fertilization and embryo development, possibly via alterations in germ cell methylation patterns (Doerksen and Trasler, 1996).

A study by Lee et al., (2012) reported that 5-azacytidine inhibited murine development from morula to blastocyst in a dose-dependent manner, whilst half the embryos arrested at the morula stage. Male mice treated with the cytidine analogue 5-aza-2'-deoxycytidine were demonstrated dose-dependent decreased testicular weight, increased histological abnormalities and decreased sperm counts, but no change in androgen status (Kelly et al., 2003). The mating of drug-exposed males led to significantly reduced pregnancy rates accompanied by increased preimplantation losses in the females (Kelly et al., 2003). The study also reported that lowering levels of the predominant DNA methyltransferase, DNMT1, led to the partial protection of the seminiferous epithelium from this deleterious drug (Kelly et al., 2003).

4.5c Future work

Continuing with the current work to examine the level of DNA fragmentation and aneuploidy in human embryonic stem cells treated with a range of known toxins is recommended. It may be valuable to analyse a higher number of chromosomes in the same cell, either through FISH or array CGH.

Human embryonic stem cells can be used to study the in vitro development of undifferentiated pluripotent cells to terminally differentiated types of cells, enacting processes of early embryonic development. The use of such an in vitro model can be enhanced by analysing tissue-specific genes via reporter gene expression, automated high-throughput screening for gene and protein expression pattern changes using microchip arrays for transcriptome and proteome analyses. Human embryonic stem cell lines carrying reporter genes such as green fluorescent protein (GFP) or LacZ under the control of tissue-specific promoters can be used to analyse genes specifically involved in human preimplantation development. This may be valuable in the detection of gene expression levels triggered by embryotoxic substances.

If a high level of DNA damage or aneuploidy is observed, it may be interesting to investigate the ability of these hESCs to differentiate into trophoblast cells, and their level of DNA damage and aneuploidy following exposure to the relevant toxic compounds. Trophoblast cells are important in
the process of implantation. Following the assessment of DNA fragmentation and aneuploidy, the correlation between these two parameters can be analysed.

4.6 Conclusion

The aim of this thesis was to examine the effects of DNA fragmentation, aneuploidy and phosphatidylserine translocation at various stages of gametogenesis and preimplantation embryo development. In summary, the results of this study suggest that sperm DNA fragmentation tests should not be used in clinical practice yet, as their efficacy is not guaranteed. Different clinics and research labs use different tests and therefore the data generated is not comparable. The SCD-FISH test is unsuitable to measure both DNA fragmentation and aneuploidy in the same sperm cell and is impractical for clinical purpose. There is no relationship between sperm DNA fragmentation and aneuploidy. Sperm should not be exposed to procedures that increase the level of oxidative stress, or be incubated at 37°C for even one hour, as they cause sperm DNA fragmentation and phosphatidylserine translocation. Sperm should not be frozen without a cryo-protectant when used for research or when processed for DNA fragmentation analysis by the SCSA test as freeze-thawing also significantly increases the level of DNA fragmentation. Sperm DNA fragmentation is not an adequate parameter to use to predict fertility potential. Granulosa and cumulus cells display variable levels of DNA fragmentation, the cells further away for the oocyte (granulosa) show consistently lower levels of DNA fragmentation. DNA fragmentation in granulosa and cumulus cells cannot be used as a non-invasive biomarker to predict preimplantation development or fertility potential. GM-CSF reduces the level of DNA fragmentation/apoptosis in murine embryos. Considering the purpose of apoptosis in development is to eliminate anomalous cells, those genetically abnormal and those in the wrong place, the elimination of this pathway may result in the accumulation of damage. However, more work is required on human embryos and long-term outcome studies before it is recommended as a growth factor supplement in IVF. Human embryonic stem cells display DNA fragmentation and aneuploidy, and are sensitive to various toxins of different concentrations. The analysis of DNA integrity was proposed as a potentially useful adjunct to existing markers of fertility (Evenson and Darzynkiewicz, 1990), but as the results of the studies described in this thesis show, significant controversy exists over its clinical relevance. Different DNA fragmentation tests were used in these studies, and still, the most appropriate method for the assessment of DNA integrity was difficult to verify, as was interpretation of the clinical data.

Assisted conception methods are thought to be responsible for the birth of over five million babies around the world. Despite monumental advances in equipment, techniques, procedures, supplies and control over environmental factors involved in ART, there is escalating concern regarding the
validity, applicability and implementation of such systems. Ideally, all such procedures relevant to
the human body should be considered experimental until proven safe and effective through
reproducible studies and results by more than one research group (Harper et al., 2012; Brown and
Harper, 2012). New technologies, tests and reagents should always be assessed for efficacy, safety
and cost-effectiveness. It is worth reiterating that standardization of existing and proposed clinical
assays and the need for high quality comparative clinical data is vital.

Basic research leading to clinical application in IVF should be hypothesis driven, where research on
new technologies should ideally be performed on human gametes and embryos donated for
research (Harper et al., 2012). An unbiased assessment of technology and outcomes and the gold
standard of evidence based medicine, clinical randomized control trials will help decide whether the
cause of an effect (negative or positive) is in fact due to a certain process/technology (Harper et al.,
2012). If these tests are eventually sanctioned, their purpose in the patient pathway still needs to be
determined.

Whilst contributing to the significant progression in our knowledge of human embryology, IVF has
also burgeoned into a lucrative business with a vast commercial market (Harper et al., 2013). The
pressure on IVF clinics to ensure the safe delivery of a healthy baby whilst topping league table of
success rates has led to their use of the latest technology, unfortunately most of which have not
been validated. Developments in ART are often fiscally and patient-driven, deferring necessary
research until the procedure is already introduced to the clinical setting. Furthermore, unpredicted
and detrimental consequences may not be reported in children until several years down the line.
The failure to ensure the safety and clinical aspect of such technologies is worrying, particularly as
newer products emerge consistently and fast become a part of routine practice.
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