Developing methods to detect and remedy DNA damage in sperm.

A thesis submitted to the University of Kent for the degree of

PhD in Biochemistry at the Division of Natural Sciences

2023

Marie Claire Aquilina

School of Biosciences

I Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification at the University of Kent or any other University or institute of learning.

The data presented in Chapter 4 was obtained by The Doctors Laboratory (TDL) for use in my thesis. I acknowledge TDL for providing the data but TDL did not have any role in the analysis or interpretation of this data. The research presented in Chapter 5 was partially funded by LogixX Fertility, who provided financial support for procurement of the flow cytometer used in this study. LogixX Fertility played a role in the progression and development of the sperm DNA damage screening method but did not have a role in the final study design, data collection and analysis.

Marie Claire Aquilina

May 2023

II Acknowledgements

I would like to acknowledge first and foremost my primary supervisor, Dr Peter Ellis. It would not have been the same journey without him! I have learned so much from our scientific discussions (mostly over coffee) and I greatly appreciate the patience and guidance he has provided me with through this long PhD journey. I would also like to express my gratitude to my co-supervisor Prof Darren Griffin, for his support and guidance. I thoroughly appreciate all your help.

An immense thank you goes to Prof Sheryl Homa, for teaching me all about clinical andrology, and for always being willing to engage in scientific conversations with the aim of providing greater awareness to the field of andrology.

It has been an amazing experience, and it would not have been the same without the support I received from my lab mates. I have enjoyed annoying all my lab mates, especially (Dr) Stranger, Dr Maria and Izzy! I also owe a big thanks to Giuseppe(eeee), Carla and Lisa for listening to all my struggles and for providing chocolate when needed.

A massive thank you to the support of my family and flat mate, Nathan, for always being so supportive and willing to help every step of the way. And to Marilena, who has been there for me every step of the way on this challenging and rewarding journey of my PhD, I am immensely grateful.

Table of Contents

IV Abbreviations

ART	Assisted reproduction technology
Aal spermatogonia	A-aligned spermatogonia
AO	Acridine orange
AOFT	Acridine orange flow cytometric test
AOT	Acridine orange test
AP site	Apyrimidine site
APE 1	Apurinic/apyrimidinic endonuclease
Apr spermatogonia	A-paired spermatogonia
As spermatogonia	A-single spermatogonia
BER	Base-excision repair
BMP	Bone Morphogenetic Proteins
CASA	Computer assisted semen analysis
cfDNA	Cell-free DNA
DFI	DNA fragmentation index
DGC	Density gradient centrifugation
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand breaks
dUTP	2´-Deoxyuridine, 5´-triphosphate
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
HDS	High DNA stainability

ICSI	Intracytoplasmic sperm injection
IUI	Intra-uterine insemination
IVF	In-vitro fertilisation
LH	Luteinising hormone
LPO	Lipid peroxidation
MAR	Matrix attachment regions
MCSS	Microfluidic chip-based sperm separation
mV	Millivolts
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate + hydrogen
OCT	Optical coherence tomography
OGG1	8-Oxoguanine glycosylase
ORP	Oxidation reduction potential
PUFA	Polyunsaturated fatty acids
QC	Quality control
RLU	Relative light units
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
SCD	Sperm chromatin dispersion
SCSA	Sperm chromatin structure assay
SDF	Sperm DNA fragmentation
SSC	Spermatogonial stem cell
SSOCT	Swept Source Optical Coherence Tomography
TdT	Terminal deoxynucleotidyl transferase

TLS	Time-lapse systems
TP	Transition proteins
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
ULT	Ultra-low temperature
VAP	Velocity average path
VCL	Curvilinear velocity
VSL	Velocity straight path
WHO	World Health Organization
XRCC1	X-ray repair cross-complementing protein 1

V Abstract

Infertility affects millions of couples worldwide, with male-related factors playing a significant role. While basic semen analysis is a standard method for evaluating male infertility, advanced sperm testing can enhance its accuracy. To gain insights into urologists' perspectives on advanced semen analysis tests, a survey was conducted among urologists from different geographical regions. The survey revealed a consensus on clinically relevant parameters in basic semen analysis. Among the various advanced sperm tests offered by urologists, sperm DNA damage testing was the most common. Challenges such as lack of familiarity, test availability, and cost were cited as reasons for not offering advanced testing. Analyzing the underlying causes of increased sperm DNA damage is crucial for guiding patient treatment. Oxidative stress is recognized as a significant contributor to sperm DNA damage, and assessing oxidative stress levels provides valuable information. Seminal oxidative stress can be assessed by determining oxidation-reduction potential (ORP) or reactive oxygen species (ROS) levels. High ORP and/or ROS levels were associated with poorer sperm parameters, and higher levels of sperm DNA damage. To address the need for cost-effective and readily available tests, an in-house flow cytometric sperm DNA damage testing service was validated. Data obtained from the validation of the acridine orange-based flow cytometric test (AOFT) demonstrated its reliability and consistency. Lifestyle modifications and innovative sperm preparation techniques, such as microfluidic-based sperm preparation, can help mitigate sperm DNA damage. Microfluidic devices enable the natural selection of sperm based on various parameters, and an investigative study has shown the microfluidic sperm separation device to produce comparable results to the gold standard method, density gradient centrifugation.

1. Chapter 1: Introduction

1.1. Male Factor infertility

Infertility refers to the inability to achieve pregnancy after having regular unprotected sexual intercourse for 12 months (Zegers-Hochschild et al., 2017). It can be caused by male factors, female factors, or a combination of both. Therefore, a thorough investigation and management of both partners is crucial. Worldwide, at least 48 million couples are affected by infertility (Health, 2022), with male-related factors accounting for up to 50% of these cases (Kumar & Singh, 2015). Common causes of male infertility include endocrine imbalance, genetic abnormalities, urogenital tract infection, varicocele (an enlargement of the veins within the scrotum), and immunological conditions (Punab et al., 2017). To diagnose male fertility status, a semen analysis along with a physical examination is typically performed.

A semen analysis allows for the sperm quality to be assessed and involves the investigation of the following basic semen parameters: total semen volume, pH, total sperm number, sperm morphology, vitality, progressive motility, total motility, sperm agglutination and viscosity (World Health Organization, 2021). The classification of semen parameters, in a semen analysis report, as fertile or infertile is based on the lower 5th centile as reference ranges (**Table 1**). 3589 fertile men, whose partners achieved a time to pregnancy of ≤ 12 months, were used by the 6th Edition WHO laboratory manual for the examination and processing of human semen, for generating the lower reference ranges. However, the 6th Edition makes it explicit, that, relying on the 5th centile values to assess the results of a semen analysis is insufficient for diagnosing male infertility

(Boitrelle et al., 2021; World Health Organization, 2021). Therefore, additional tests including sperm DNA damage and seminal oxidative stress testing, referred to as advanced sperm testing, could help improve the accuracy of assessing the male fertility potential (Wang & Swerdloff, 2014). Advanced sperm testing can be offered in conjunction to conventional semen analysis to help guide patient management. However, to understand the clinical benefits of advanced sperm testing, it is beneficial to understand the fundamentals of spermatogenesis, as discussed below.

Table 1. **Lower reference values of the fertile man** (Campbell et al., 2021). The table shows the distribution of semen parameters from men in couples having a time to pregnancy of ≤ 12 months. The 5th centile provides the lower reference value for classification of semen parameters as fertile or infertile.

		Centiles		
	N	**2.5th**	**5th**	**(95% CI)**
Semen volume (ml)	3586	1.0	1.4	(1.3-1.5)
Sperm concentration (10^6 per ml)	3587	11	16	(15-18)
Total sperm number (10^6 per ejaculate)	3584	29	39	(35-40)
Total motility (PR + NP, %)	3488	35	42	(40-43)
Progressive motility (PR, %)	3389	24	30	(29-31)
Non-progressive motility (NP, %)	3387	1	1	(1-1)
Immotile spermatozoa (IM, %)	2800	15	20	(19-20)
Vitality (%)	1337	45	54	(50-56)
Normal forms (%)	3335	3	4	(3.9-4.0)

PR – progressive motility; NP – non progressive motility; IM – immotile.

1.2. Spermatogenesis

Spermatogenesis is the complete process by which spermatozoa are produced from primordial germ cells (**Figure 1**). Spermatogenesis involves 3 subdivisions: (1) proliferation and differentiation of spermatogonia, (2) meiosis and (3) spermiogenesis (the cytodifferentiation of spermatids).

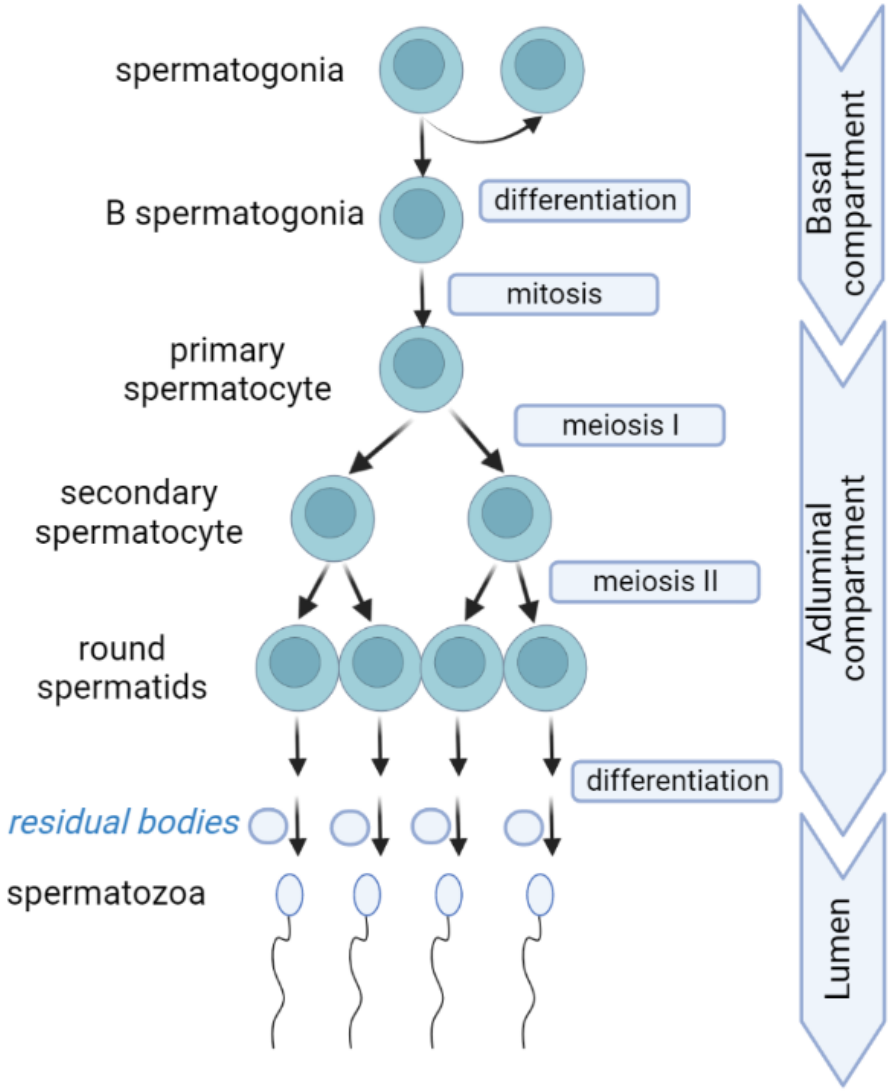


Figure 1. **Schematic representation of the spermatogenesis cycle**. Spermatogenesis is a complex process, in the seminiferous tubules of the testis, that involves the

transformation of diploid spermatogonial stem cells into haploid spermatozoa. Sertoli cells attach to the basement membrane, where spermatogonia also adhere to. The spermatogonia (type A) undergo division and develop into spermatogonia type B, which then differentiate into primary spermatocytes. In meiosis I, these primary spermatocytes separate homologous pairs of chromosomes to form haploid secondary spermatocytes. The secondary spermatocytes undergo meiosis II, to produce four spermatids. The spermatids then migrate towards the lumen and at the end of spermiogenesis, spermatozoa are released into the lumen.

1.2.1. Proliferation and Differentiation of Spermatogonia

The first phase of spermatogenesis involves cellular division of spermatogonial stem cells (SSCs). SSCs reside in the basal compartment of the seminiferous tubules in the testes, and they undergo an exponential increase in numbers prior to meiosis stage. This stage is crucial since it determines the number of spermatozoa that are produced at the end of a spermatogenic cycle (Griswold, 2016).

A-single (A_s) spermatogonia upon mitosis can differentiate into either A-paired spermatogonia (A_{pr}), which are early spermatozoa progenitors, or produce A-single spermatogonia (A_s) to maintain a continuous store of A_s spermatogonia (**Figure 2**) (Abid et al., 2014; Rooij, 2001). A_{pr} spermatogonia undergo incomplete spermatogonial cytokinesis such that the daughter cells remain interconnected by cytoplasmic bridges. This allows for the connected spermatogonia to mature at same rate and through synchronous divisions, chains consisting of four A-aligned (A_{al}) spermatogonia are formed.

A_{al} spermatogonia can further divide into chains of 8, 16 and 32 cells (although only rarely reaching the 32 cell stage) resulting in a syncytium of cells, a distinguishing trait

of the early spermatozoa progenitors (Haglund et al., 2011). The cells can then undergo active proliferation and differentiate into type A_1 spermatogonia (Rooij, 2001). The type A_1 cells give rise to a series of differential spermatogonial types: A_1 divides to form an A_2 spermatogonium, A_2 spermatogonia divide to produce A_3 spermatogonia which then undergo another division to produce A_4. An A_4 spermatogonium can divide to produce either another A_4 spermatogonium or commit and differentiate into an intermediate spermatogonium. Intermediate spermatogonia then further divide to produce type B spermatogonia. The differentiation from type A spermatogonia into type B spermatogonia requires the action of gonadotropins including follicle-stimulating hormone (FSH) and luteinising hormone (LH) (Khanehzad et al., 2021). These are secreted by the anterior pituitary gland under the control of the central hypothalamic signal gonadotropin-releasing hormone (GnRH).

The type B spermatogonia are committed progenitor cells, that will eventually differentiate into primary spermatocytes, in the next stage of spermatogenesis. The ratio of self-renewal and differentiation has to remain constant at around 1:1 to prevent germ cell tumours or depletion of spermatogonial stem cells (Meng et al., 2000; Zhou & Griswold, 2008).

During mitosis, the chromosomes are particularly susceptible to damage and DNA repair processes are ceased to prevent the fusion of telomeres (Orthwein et al., 2014). Due to the sequential differentiation and meiotic processes that begin with the initial division of a stem cell, a total of 4096 spermatozoa (2^{12}) can theoretically be produced from a single stem cell (Russell et al., 1993). However, it is estimated that around 75% of

potential number of mature sperm cells undergo apoptosis and degeneration (Dunkel et al., 1997).

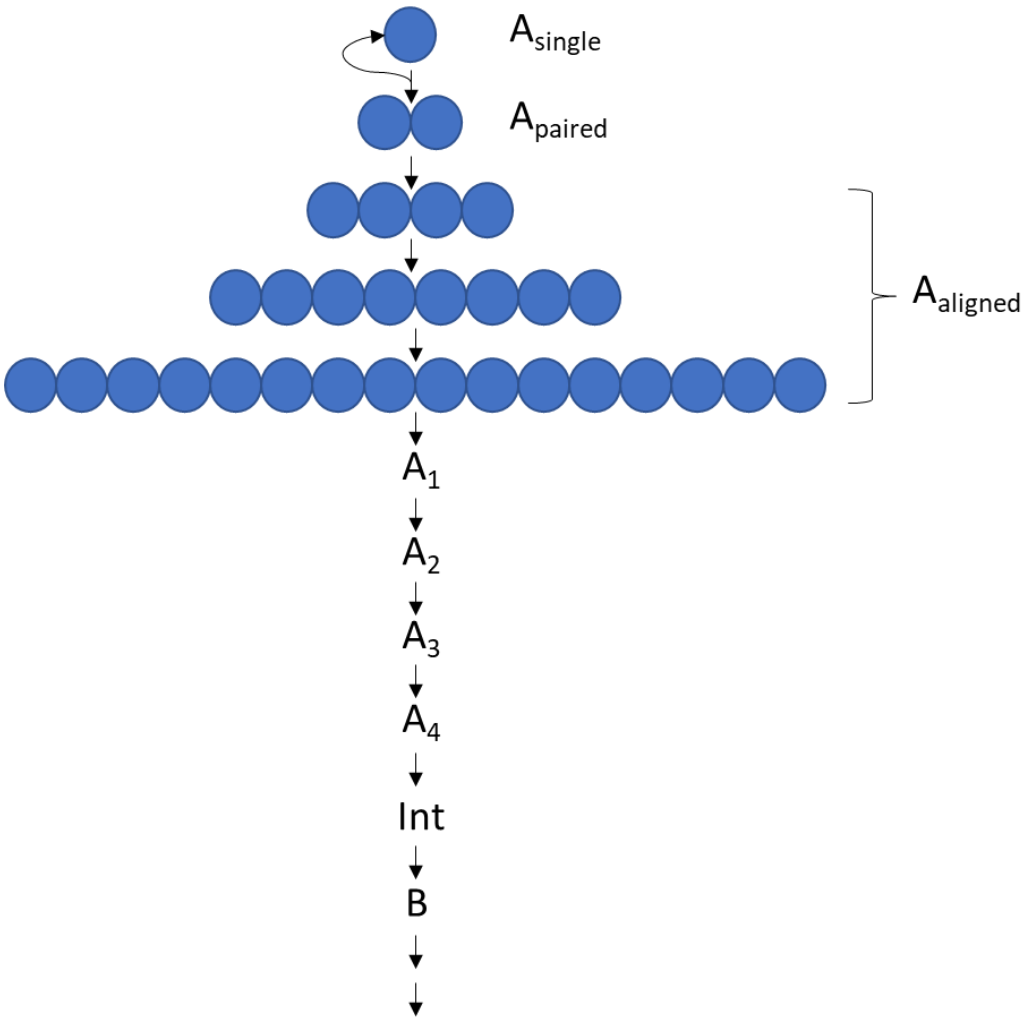


Figure 2. **Schematic representation of the first stage of spermatogenesis**. Following mitosis, A-single (A_s) spermatogonia can differentiate into A-paired spermatogonia (A_{pr}), which are early progenitors of spermatozoa, or produce more A-single spermatogonia (A_s) to maintain a continuous supply of them. A_{pr} spermatogonia are connected by cytoplasmic bridges and divide synchronously to form chains of four A-aligned (A_{al}) spermatogonia. During spermatogonial cytokinesis, the daughter cells remain

interconnected by cytoplasmic bridges, resulting in a syncytium of cells that matures synchronously, a unique characteristic of early spermatozoa progenitors.

1.2.2. Meiosis

Meiotic entry is triggered by retinoic acid (RA) signaling. Unlike female germ cells (which receive RA signals during fetal life and enter meiosis during early development), male germ cells are insulated from RA signaling prior to puberty. Thus, meiosis in males initiates at the onset of puberty in response to RA produced by the Sertoli cells. This in turn regulates the synthesis of growth factors, particularly Bone Morphogenetic Proteins (BMPs) 4 and 8 (Wu et al., 2017), enabling meiotic entry of the spermatogonia (Kostereva & Hofmann, 2008).

The process of meiosis involves two divisions: meiosis I and meiosis II. Meiosis I initiates when the primary spermatocytes, located in the basal compartment of the germinal epithelium, enter the leptotene stage of prophase (De Kretser, 1989). This is followed by migration of the spermatocytes, through the blood-testis barrier as formed by Sertoli cells (**Figure 3**). Upon the spermatocytes reaching the adluminal compartment, the further stages of prophase can proceed. In prophase I, homologous chromosomes pair up and during synapsis, crossing over of genetic material can occur. This establishes new genetic combinations on each chromosome (Gerton & Hawley, 2005). Double-strand breaks (DSBs) made by the topoisomerase type II-like protein, Spo11, allows for the accurate homologue pairing to occur for crossover recombination (Bergerat et al., 1997; Keeney et al., 1997).

The blood-testis barrier establishes an immune-privileged site for the secondary spermatocytes produced at the end of Meiosis I to undergo a second meiotic division. The barrier is formed by the co-existence of tight junctions and adhesion protein complexes including desmosomes and gap junctions (Cheng et al., 2011). The gap junctions permit the diffusion of cell signaling molecules to create the highly specialized immune-privileged microenvironment necessary for meiosis to proceed. The two meiotic divisions of each individual spermatocyte results in a total of four haploid spermatids that are located at the border of the lumen (Holstein et al., 2003). Defects occurring during meiosis can often result in aneuploidy, which can then contribute to embryonic death or developmental abnormalities, if the aneuploid cells are not eliminated by apoptosis, during post-meiotic development (Handel & Schimenti, 2010).

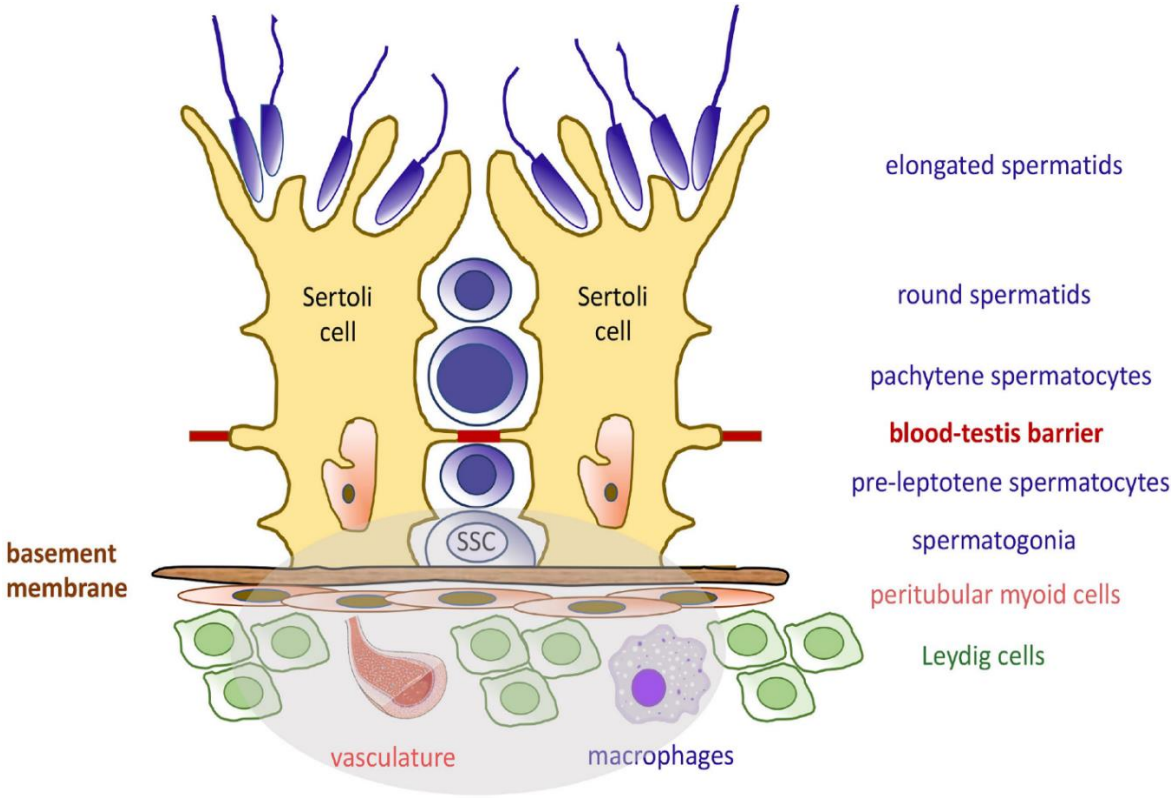


Figure 3. **Schematic representation of spermatogenesis including the presence of the blood-testis barrier** (Hofmann & McBeath, 2022). The blood-testis barrier provides an immuno-privileged microenvironment for the completion of meiosis, separating diploid germ cells from more mature cells. Like Sertoli cells, spermatogonial stem cells (SSCs) are attached to the basement membrane and depend on specific growth factors for self-renewal and pool maintenance. These growth factors are produced by various cells, including Sertoli cells, peritubular myoid cells, Leydig cells, macrophages, and the vasculature. The components of the SSC niche are indicated in the grey area.

1.2.3. Spermiogenesis

Spermiogenesis is the process during which haploid spermatids cytodifferentiate into streamlined spermatozoa. The initial stages of spermiogenesis involves the round spermatids assembling the acrosome and axoneme (**Figure 4**) (O'Donnell, 2015). This is followed by polarization of the nucleus and the acrosome to one side of the cell,

indicating the start of the elongation phase. As the spermatids elongate, the transient microtubular platform delivers proteins to the developing tail. The sperm tail accessory structures, necessary for flagella function, surround the axoneme in the principal and mid piece of the sperm tail (Lehti & Sironen, 2017). The mitochondria then assemble in a helical manner around the outer dense fibers of the sperm tail.

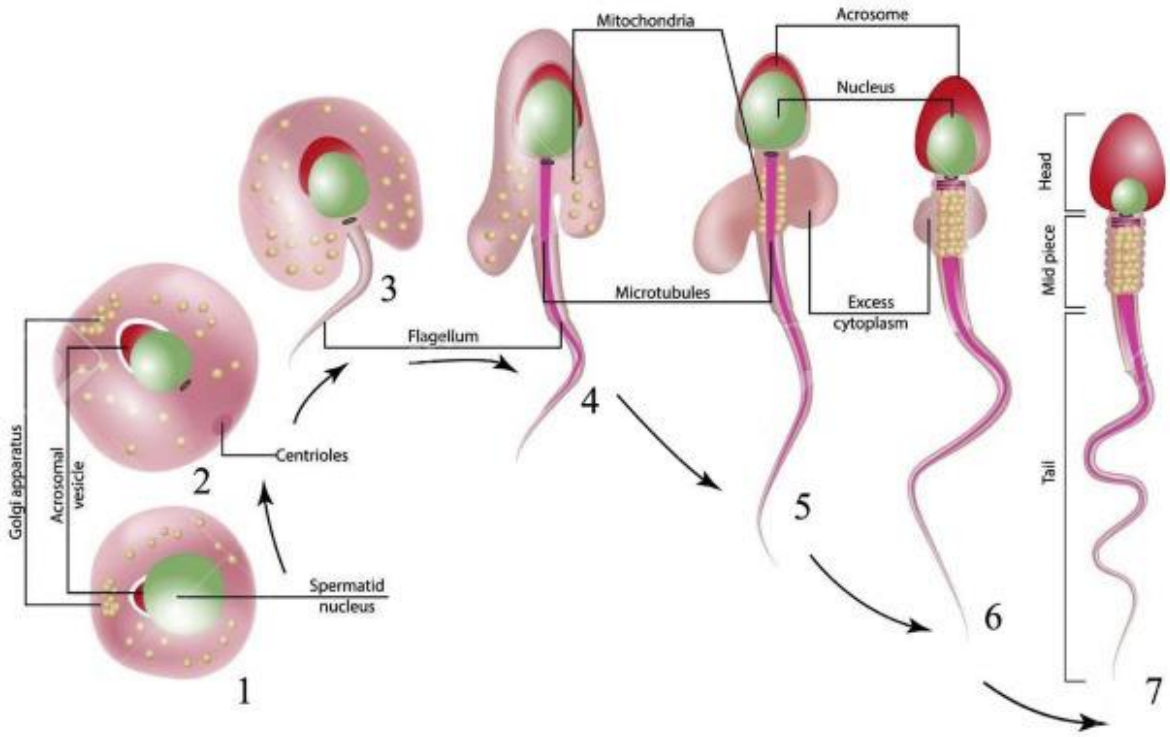


Figure 4. **Schematic representation of spermiogenesis** (Oehninger & Kruger, 2021). During spermiogenesis, the Sertoli and germ cell cytoskeletal network play a critical role in the morphologic changes. (1) Acrosome biogenesis begins with proacrosomal vesicles trafficking to the nucleus, (2) followed by Golgi clustering and nucleus condensation. (3, 4) Spermatid elongation follows, which involves the head-to-tail coupling apparatus and nuclear shaping. Mitochondria are organized in preparation for loading onto the outer dense fibers. (5, 6) The elongated spermatids show a well-defined acrosome, condensed nucleus, and cytoplasmic droplet. (7) Residual cytoplasm is phagocytosed by the Sertoli cell and mature spermatozoa are released into the lumen.

The final stage of spermiogenesis is spermiation and is the process by which spermatids undergo their final remodeling and are released from the Sertoli cells into the seminiferous epithelium (O'Donnell et al., 2011). To achieve the streamlined shape of a spermatozoon most of the cytoplasmic components are gathered into a "waste bag", known as a residual body, which is then phagocytosed by Sertoli cells.

1.3. Sperm genome organization

During spermatogenesis, the sperm genome undergoes extensive remodeling to obtain a final DNA state of an almost crystalline state arranged within a hexagonal lattice (Moritz & Hammoud, 2022). Such tight compaction protects the DNA during the transit occurring prior to fertilization and allows the sperm to adopt its characteristic hydrodynamic head shape, permitting faster swimming through the female reproductive tract (Miller et al., 2010).

The tight compaction is facilitated by the histone-to-protamine exchange. During this remodeling process, histones are replaced by histone variants, many of which are testis specific. Histone variants are then replaced by transition proteins (TPs) and finally by protamines (**Figure 5**) (Moritz & Hammoud, 2022). The incorporation of the histone variants induces nucleosome destabilization by weakening histone association and can promote chromatin accessibility (Martire & Banaszynski, 2020). The histone-to-protamine exchange compacts around 85% of the sperm DNA in toroidal structures that stack together side to side in a very efficient space saving form (Brewer, 2011). These toroids are a result of the positively charged arginine-rich protamines (P1 and P2) that neutralize the negative phosphodiester DNA backbone (Balhorn, 2007). The

incorporation of cysteine in protamines help further stabilize the chromatin through disulfide bonds (Vilfan et al., 2004).

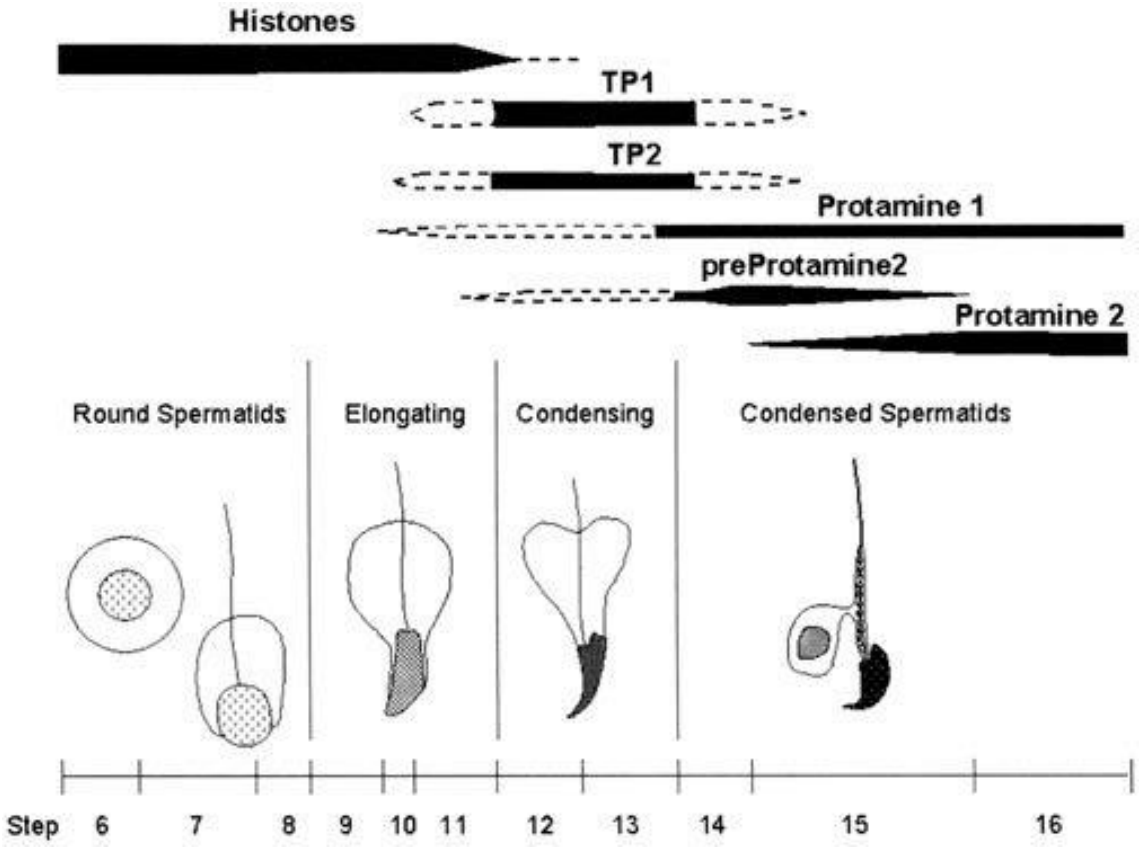


Figure 5. **Key chromatin remodeling events during spermiogenesis in mice** (Meistrich et al., 2003). The sequence of appearance of histones, transition proteins (TPs), and protamines (P1 and P2) is indicated. The steps of spermatid development are shown at the bottom.

The toroid structures in the sperm chromatin contain 50 kbps of DNA and are made of loops of DNA that are attached to a nuclear matrix (**Figure 6**) (Brewer, 2011). Nuclear matrix attachment regions (MARs) are located between each protamine toroid and anchors each toroid in place. MARs function as a checkpoint for sperm DNA integrity

following fertilisation (Ward, 2010) and may be involved in the activation of paternal genome during the early stages of embryogenesis (Ward et al., 1999).

Despite the almost complete transition of histones by protamines, around 10% of human genome remain associated to histone-specific nucleosomes (Yamaguchi et al., 2018). It has been suggested that retained histones and their subsequent post-translational modifications may have a role in the transmission of epigenetic memory from the spermatozoon to the embryo. Consistent with this, research in mice has indicated that the location of retained histones, on gene promoters having high content of unmethylated CpG regions, suggests their role in the transcriptional regulation of these genes and overall organization of the genome following fertilization (Erkek et al., 2013; Torres-Flores & Hernández-Hernández, 2020).

However, analysis of histone retention patterns in sperm remains contested. A recent preprint (Yin et al., 2022) showed that the majority of the signal in ChIP-Seq studies from semen samples is not derived from sperm at all, but is instead due to circulating cell-free DNA (cfDNA) in the seminal plasma. More extensive research will be needed to control for this confounding factor and reveal the true localization of retained histones in sperm from various species including humans.

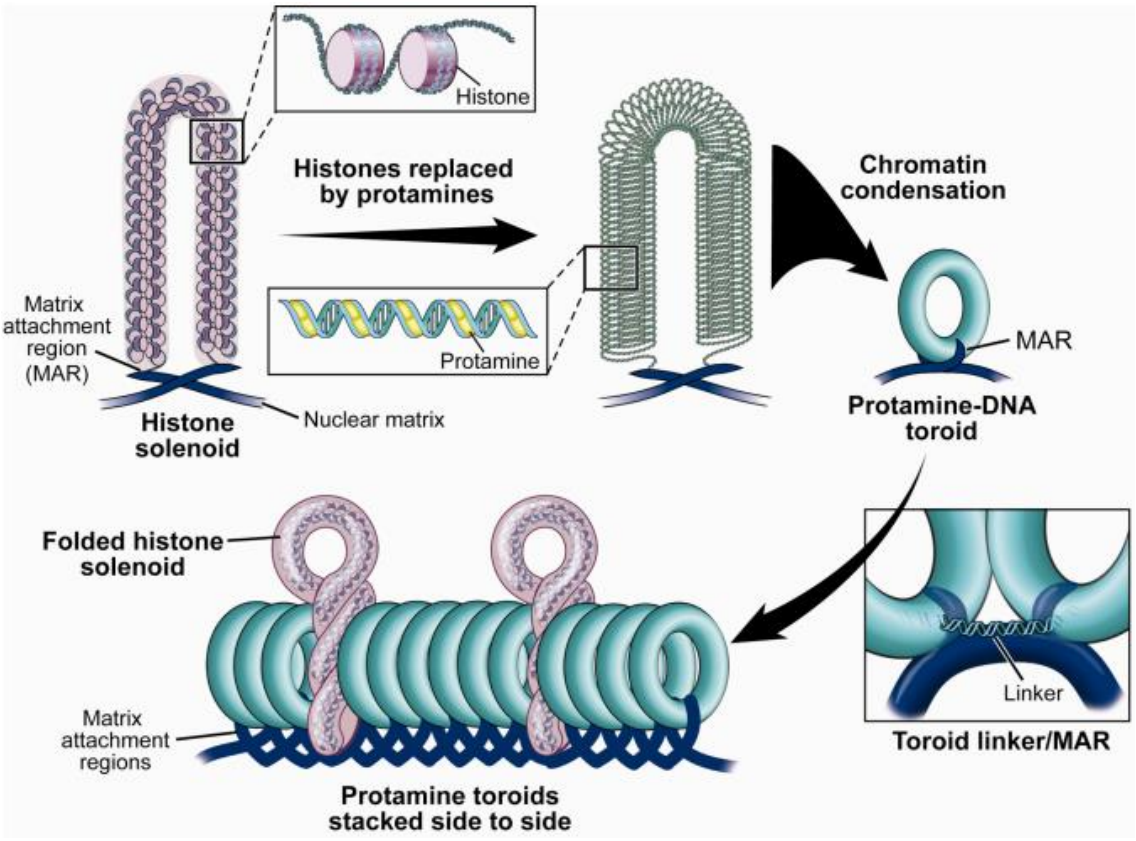


Figure 6. **Sperm Chromatin Remodelling** (Ward, 2010). The bulk of the sperm DNA is arranged into toroid structures that are tightly coiled and stacked side-by-side. The stability of this toroid structure is maintained by protamines, which counteract the repulsion between the phosphodiester backbones. A smaller portion of the DNA is linked with histones in the sperm cells, while the rest is anchored to the nuclear matrix through Matrix Attachment Regions (MARs).

1.4. Sperm DNA Damage

1.4.1. Physiological DNA strand breaks

Spermatogenesis involves a series of events, including meiosis and cell differentiation. This is unique to the mammalian system, as it produces a final cell type having a transcriptionally inactive nucleus, with major parts of the sperm cell stripped off (Miller et al., 2010). Mature spermatozoa are particularly susceptible to the accumulation of

DNA damage owing to the lack of DNA repair responses. This is a direct result of significant chromatin compaction and reduction of cytoplasmic content (González-Marín et al., 2012). A critical event that occurs during spermatogenesis is the formation of double strand breaks (DSBs) during prophase I to enable recombination of homologous chromosomes (Murakami & Keeney, 2008). The free ends of the double strand breaks are repaired through the process of homologous recombination-mediated repair of DSBs, resulting in the characteristic chiasma formation (Guiraldelli et al., 2018).

DNA breaks also occur during the process of histone to protamine replacement. DNA break generation allows for the necessary swivel effect to reduce torsional stress associated with the super helical tension, facilitating histone exchange (Marcon & Boissonneault, 2004). Topoisomerases could be generating the DNA breaks (single and double strand breaks) and could also be involved in sealing the DNA nicks (Marcon & Boissonneault, 2004). Topoisomerases can be trapped through covalent linkages with the DNA breaks, creating protein-associated DNA strand breaks, a significant portion of which are DNA double-strand breaks (Sun et al., 2020). Transition proteins can stimulate the repair of transient DNA nicks (Lévesque et al., 1998) and together with protamines (Sheflin et al., 1991), enhance the ligation of short DNA fragments (Marcon & Boissonneault, 2004). Therefore, as expected, abnormalities in the histone to protamine have been associated with increased levels of DNA breaks owing to failure in post-meiotic DNA repair (Bianchi et al., 1993; Gorczyca et al., 1993). Consistent with this observation, recent work shows that DSBs occurring during mouse spermatid elongation are associated with specific sequence contexts (particularly $(CA)_n$ dinucleotide repeats) that are predicted to fold into a Z-DNA structure. These may be a substrate for topoisomerase activity and/or may act to “buffer” torsional changes during remodelling

(Burden et al., 2023). These spermatid DSB-prone regions are then also subsequently associated with “hotspots” of oxidative DNA damage in mature sperm (see following section – **Section 1.4.2.**).

Such elaborate events that occur during spermatogenesis are necessary for extreme DNA compaction and to enable the sperm to carry the male genetic information to the oocyte. However, all these complex and dynamic processes result in a number of ejaculated spermatozoa possessing varying degrees of abnormalities. In fact, it is estimated that around 45% of the spermatozoa produced are non-viable (Griswold, 2016; Johnson et al., 1983). These findings are of concerns for ICSI procedures that make use of spermatozoa extracted from testicular tissue, since such spermatozoa can be harboring transient DNA strand breaks that have not yet undergone DNA repair (Johnson et al., 1999; Marcon & Boissonneault, 2004).

Additionally, there is emerging evidence suggesting that the levels of DNA damage in ejaculated spermatozoa cannot be completely explained by the processes of abortive apoptosis and defective chromatin packaging mechanism. A number of studies have shown that there is increased levels of DNA damage in ejaculated sperm when compared to DNA damage levels of testicular sperm, suggesting that DNA damage also occurs during epididymal transit (Esteves et al., 2015; Mehta et al., 2015; Suganuma et al., 2005). The increased level of DNA damage during epididymal transit could arise from abortive apoptosis, aberrant protamination and oxidative stress. Antioxidants (including vitamins C and E, superoxide dismutase and glutathione) present within the seminal plasma help protect the spermatozoa against oxidative attack following ejaculation. However, during epididymal storage and transit, the sperm are not protected by seminal

antioxidants and rely on the intrinsic spermatozoon antioxidant capacity for protection against oxidative attack (Tremellen, 2008).

1.4.2. Oxidative induced damage

Oxidative stress occurs when there is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the biological system`s antioxidant defences. Reactive oxygen species are highly reactive chemical species, that are generated as by-products of metabolism. At moderate concentrations, ROS like superoxide anion and hydrogen peroxide play important signaling roles in sperm maturation, capacitation, acrosome reaction, and fertilization (O'Flaherty & Matsushita-Fournier, 2017). Sperm capacitation involves a series of physiological changes in the female reproductive tract that allow sperm to fertilize an oocyte (Ickowicz et al., 2012). This process activates sperm motility and prepares them for the acrosome reaction. The acrosome reaction involves fusion and vesiculation of the outer acrosomal membrane, exposing hydrolytic enzymes needed for sperm to penetrate the zona pellucida of the oocyte. Controlled amounts of ROS help regulate sperm motility by modulating axonemal protein phosphorylation and are also involved in signaling pathways that prepare sperm for fertilization, such as tyrosine phosphorylation during capacitation (Aitken, 2017; Naz & Rajesh, 2004).

Spermatozoa themselves produce ROS via two main sources: (1) the NADPH oxidase system at the sperm plasma membrane and (2) the mitochondrial NADH-dependent oxidoreductase reaction, which is the main source of ROS production in spermatozoa.

ROS can also originate from other endogenous (including leukocytes and immature spermatozoa) and exogenous (including smoking and radiation) sources (**Figure 7**).

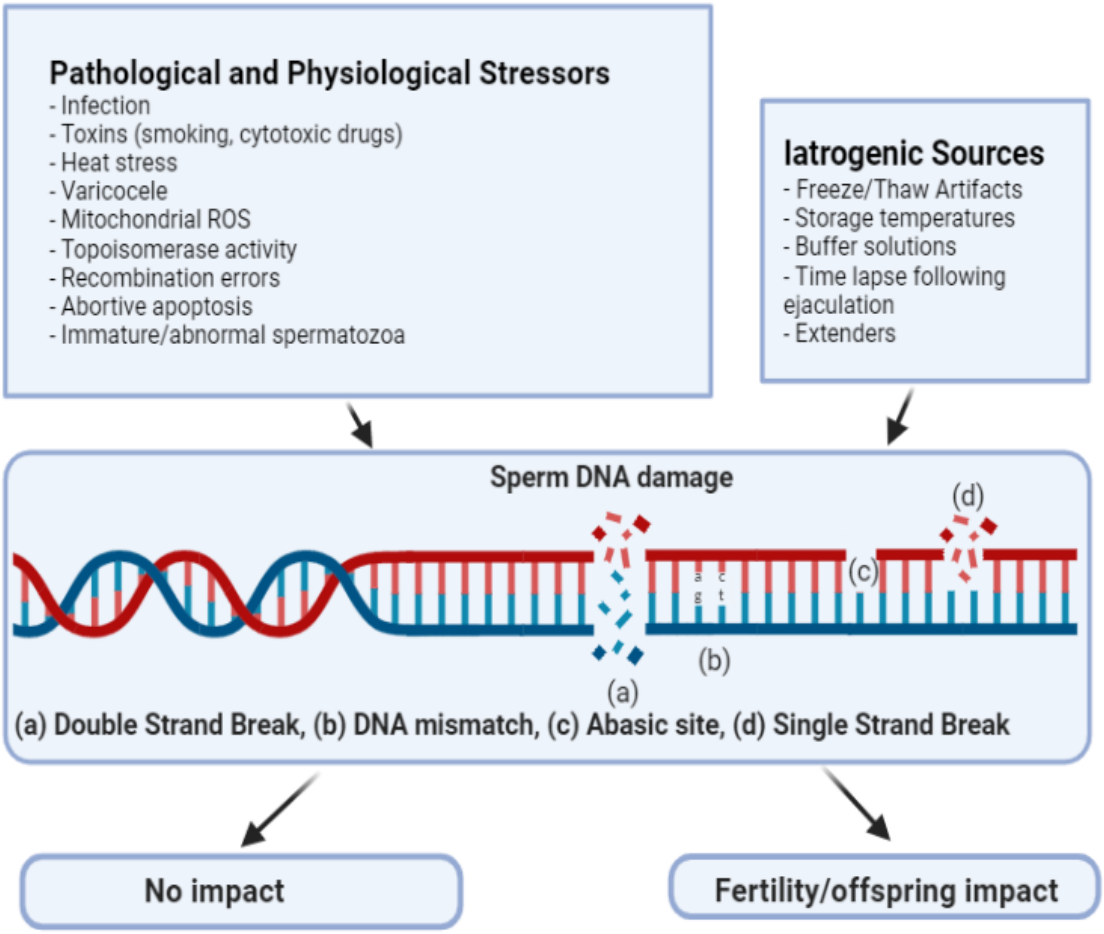


Figure 7. Summary of the main causes of sperm DNA damage (new figure based on data from González-Marín et al., 2012)**.**

Spermatozoa are particularly susceptible to oxidative attack due to the high levels of polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid, in the sperm plasma membrane (Agarwal et al., 2014). PUFAs contain unconjugated double bonds that are separated by methylene grouping. This configuration renders the methyl carbon-hydrogen bond susceptible to abstraction, resulting in unpaired electron on the

carbon atom. The carbon radical seeks stabilisation by combining with an oxygen atom to form a peroxyl radical, which can in turn abstract another hydrogen atom from another methyl carbon-hydrogen bond, resulting in the start of a lipid peroxidation chain reaction (Repetto et al., 2012). High levels of ROS can trigger this cascade of reactions, decreasing sperm viability and sperm motility. Additionally, ROS induces sperm DNA damage, in fact, oxidation of bases is considered to be the major source of DNA damage that occurs post spermiation (Agarwal et al., 2014). Guanine is the most susceptible base to oxidation due to its low redox potential (Seidel et al., 1996). ROS attack on guanine bases generates oxidised guanine residues with a major common oxidation product being 8-OH-deoxyguanosine (8-OHdG). A strong correlation between the presence of 8-OHDdG and DNA strand breaks exists (De Iuliis et al., 2009), and 8-OHdG has been commonly used as a biomarker of oxidative damage to DNA (Shen et al., 1999; Shen & Ong, 2000).

The quasi-silent nature of spermatozoa results in a limited ability to repair oxidised bases. The base-excision repair (BER) pathway, in somatic cells, utilises the enzyme oxoguanine glycosylase for the removal of oxidised guanine (Krokan & Bjørås, 2013) (**Figure 8**). This action generates an open apyrimidine site (AP site), which is then filled with an unaltered residue. However, spermatozoa have a truncated BER ability, meaning that the presence of oxoguanine glycosylase allows oxidised guanine to be cut off, but lack the necessary enzymes to fill the open AP site (T. B. Smith et al., 2013).

Oxidative damage, and the resulting DNA strand breaks, are particularly prominent in the context of “abortive apoptosis” in sperm (Aitken & Koppers, 2011). Owing to their limited cellular resources, sperm are unable to activate the full machinery of a typical

apoptotic cascade. However, they can still trigger mitochondrial permeabilization and release of ROS in response to pro-apoptotic stresses. Consequentially, this leads to widespread oxidative damage throughout the sperm cell and its nucleus. A damaged sperm cell therefore typically exhibits high levels of oxidative DNA damage and DNA fragmentation, and the boundary between “damaged but surviving” and “dying” becomes hard to define.

This difference may in part depend on the repair capacity of the female partner. Since sperm cannot themselves repair oxidative damage, they rely on the oocyte following fertilisation to complete the repair. However, high levels of oxidised bases can overwhelm the oocytes repair capacity (Rashki Ghaleno et al., 2021). Additionally, oocytes from older women, or stressed oocytes following ART procedures can have a weakened repair capacity of oxidised guanine, potentially resulting in de novo mutations (Horta et al., 2020). Additions or deletions of necessary bases in the DNA sequence leads to modifications/frameshift mutations and can affect the entire protein produced. In addition, oxidation of bases can result in the de-amination of bases, causing the replication of altered bases, thereby, generating wrong opposing bases (Krokan & Bjørås, 2013). Such mutations can cause genomic instability in early embryonic development and can lead to rare congenital disorders when embryos escape developmental arrest (Middelkamp et al., 2020).

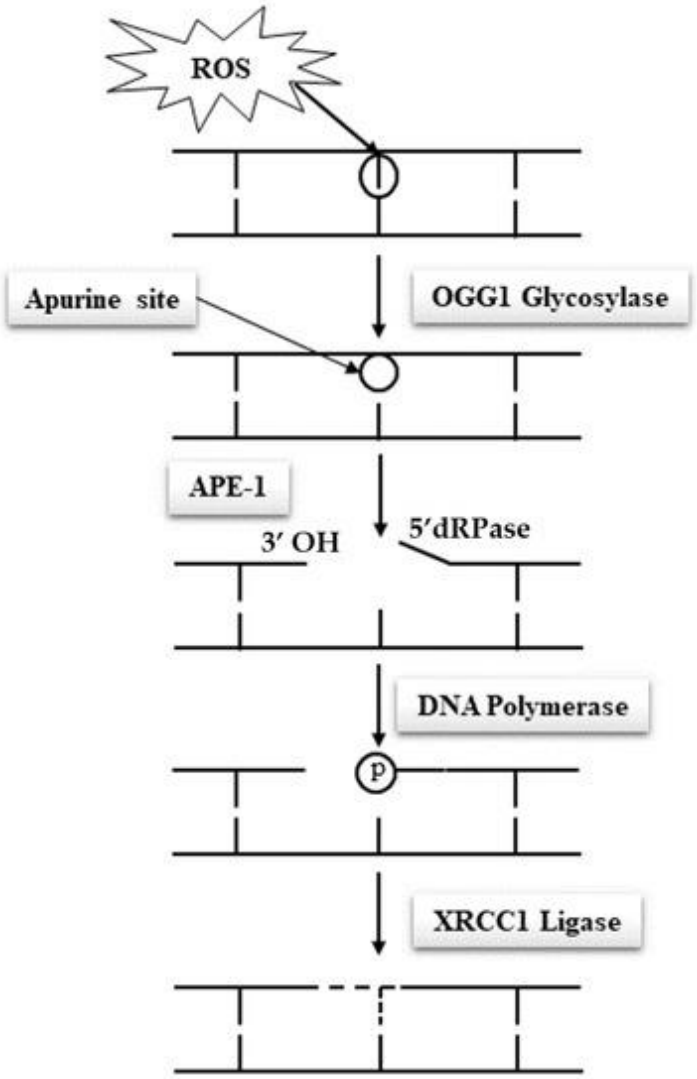


Figure 8. **Diagram of the base excision repair (BER) pathway** (Rashki Ghaleno et al., 2021). One of the key steps in the BER pathway is the recognition and removal of oxidised bases by a glycosylase enzyme called OGG1. OGG1 recognizes and excises the oxidised base from the DNA strand. After the damaged base is removed, a recombinase complex composed of APE1 and XRCC1 proteins is recruited to the site of the damage. APE1 cleaves the DNA backbone at the 5' end of the damaged base, creating a nick that can be repaired by DNA polymerase and ligase enzymes. XRCC1 acts as a scaffold protein that facilitates the assembly and coordination of the other repair proteins involved in the process. Spermatozoa possess only the first part of the BER pathway – up to the removal of the damaged based by OGG1 Glycosylase but lack the activities of APE1 and XRCC1 Ligase.

1.5. Function of sperm DNA integrity in embryonic development

The integrity of sperm DNA is key to reproductive success. Several studies have highlighted the role sperm chromatin has in sperm function and subsequent embryo development (Kim et al., 2019; Kumar et al., 2013; Ribas-Maynou et al., 2022; Ward, 2010). Spermatozoa accumulates DNA damage from three main sources: defective chromatin compaction during spermiogenesis, abortive apoptosis and oxidative stress (Tamburrino et al., 2012). The extent of each of these mechanisms is dependent on various parameters including paternal age (Sharma et al., 2015), environmental toxin exposure (Krzastek et al., 2020), pathological conditions (Panner Selvam et al., 2021) and male abstinence period (Comar et al., 2017).

Parts of the sperm chromatin (the histone-bound chromatin and the MARs of the sperm cells) are inherited unchanged by the embryo – i.e. do not undergo immediate deprotamination and remodelling following fertilization – and are required for proper embryonic development (**Figure 9**) (Ward, 2010). However, defects in chromatin compaction can change the tertiary chromatin structure and can prevent the zygote from properly accessing the paternal genome (de Macedo et al., 2021). Embryonic development does not proceed beyond the first cell cycle without proper organization of DNA into loop domains at the MARs (Shaman et al., 2007). At later embryonic stages, intact histone-bound sequences are required to allow for normal embryonic development. Repeated IVF failures, that result from developmental arrest at the pronuclear stage, are linked to defects in chromatin compaction (Menezo et al., 2017).

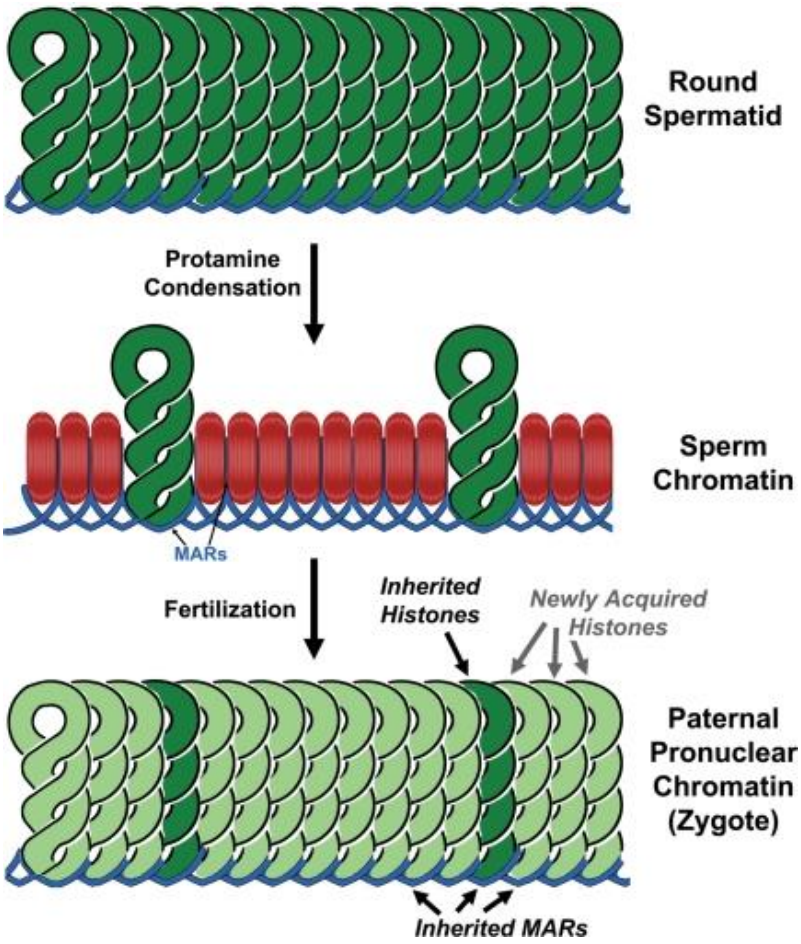


Figure 9. **Inheritance of sperm chromatin structures by the embryo** (Ward, 2010). Following fertilisation, protamines are replaced by histones supplied by the oocyte. Some spermatozoon histones are retained in the paternal pronuclear chromatin of the zygote.

Oxidative stress can promote nuclear decondensation by destabilizing the DNA structures. This renders the sperm chromatin susceptible to ROS attack, promoting single and double DNA strand breaks (Ribas-Maynou & Benet, 2019). Double DNA strand breaks are lethal alterations in the zygote and result in a delay in the cell cycle (Derijck et al., 2008). Spermatozoa harboring double strand breaks that were used for fertilisation exhibited chromosomal alterations in the paternal genome of the embryo and a delay in embryo kinetics (Genescà et al., 1992; Tusell et al., 2004). A study by

Gawecka et al., 2013, demonstrated that zygotes respond to sperm DNA damage through a non-apoptotic mechanism, delaying the initiation of paternal DNA replication by up to twelve hours, but not affecting the maternal DNA replication. This resulted in a large proportion of the embryos being arrested at the 1 or 2 cell stage. Double strand breaks in mice sperm were also correlated to reduced offspring survivability (Fernández-Gonzalez et al., 2008).

When analyzing sperm DNA integrity and the relationship with embryonic development, the oocyte quality should also be taken into account. The oocyte has the capacity to repair sperm DNA damage levels (Ahmadi & Ng, 1999), however, the extent of repair depends on oocyte quality and level of sperm DNA damage. DNA misrepair by the oocyte can result in lower embryonic development and can lead to higher miscarriage rates. This is termed as paternal “late effect” (García-Rodríguez et al., 2018). DNA repair by the oocyte is largely dependent on the age and ovarian environment, however, the oocyte repair capacity is still largely a mystery (Stringer et al., 2018). This could explain the disparity between studies analyzing the influence of sperm DNA damage and reproductive success, since unlike sperm DNA integrity, the oocyte repair capacity cannot be directly measured (Meseguer et al., 2011).

1.6. Paternal Age and sperm DNA quality

Due to various socio-economic factors, the average paternal age has increased by 3.5 years over a 44 year period, from 27.4 to 30.9 years (Bray & Gunnell, 2006; Khandwala et al., 2017). Several studies have associated increased paternal age with lower sperm DNA quality. A study by Moskovtsev et al., 2009, divided patients into four age groups: <30, 30 – 40, 41-50, and >50, and the data showed sperm DNA damage to be highest for men aged >50 and lowest for men aged less than 30 (**Figure 10**).

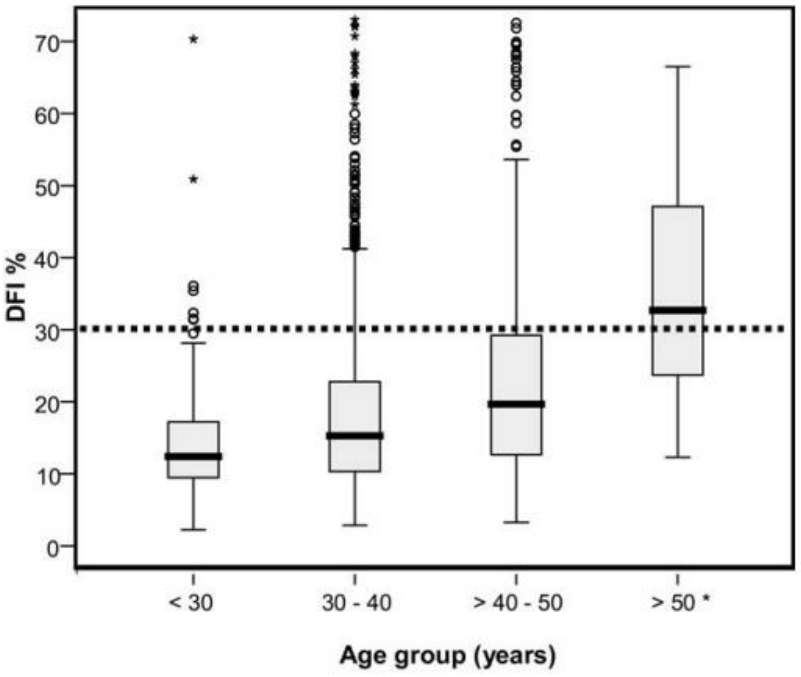


Figure 10. **Boxplots showing % DNA Fragmentation Index (DFI) for four different age groups** (Moskovtsev et al., 2009). Relationship between different age groups and sperm DNA damage levels as represented by %DNA fragmentation Index (DFI) (see **section 1.7.2.**). The sperm chromatin structure assay was used for determining % DFI levels. The % DFI was significantly different among all the age groups.

It is thought that advanced paternal age results in the accumulation of chromosomal aberrations and mutations, and this can have an adverse effect on reproductive

outcomes. A prospective study by Slama et al., 2005, on 5121 women, indicated that when the paternal age was >35, there was a higher risk of spontaneous abortion, with a stronger association for first trimester loss. Around half of spontaneous abortions occur due to chromosomal anomalies in the zygote, which can arise from errors in oogenesis, spermatogenesis, fertilisation or embryogenesis (Boué et al., 1975). Therefore, as male age increases, there is increased risk of errors in spermatogenesis, which may induce spontaneous abortion. A study by Nybo Andersen et al., 2004, recruited 23,821 women to analyse the effect of paternal age on fetal death. After adjustment for maternal age, the study found a higher risk of late fetal death than early fetal death for men aged 50 or more, at time of conception. It has also been suggested that increased paternal age can increase the risk of low birth weight and preterm birth (Tough et al., 2003), and can also shorten offspring life span. A study by Gavrilov & Gavrilova, 2001 indicated that adult daughters (>30 years) born to older fathers (45 to 55 years) lived shorter lives, however, this was not observed with sons born to older fathers. The study postulated that the genes affecting longevity and sensitivity to mutation accumulation might be more concentrated in the X chromosome. However, other studies have reported contradictory results (Hubbard et al., 2009; Robine et al., 2003).

1.7. Detection of sperm DNA Damage

Some studies have reported associations between higher sperm DNA damage and reduced natural pregnancy rates, longer time to pregnancy, and increased risk of miscarriage (Lewis & Simon, 2010; Ribas-Maynou & Benet, 2019; Zini et al., 2008). Sperm DNA damage testing, using various techniques like TUNEL, SCSA, and SCD, can quantify

DNA fragmentation in sperm. Zini et al. (2008) performed a systematic review and meta-analysis of 11 studies examining links between sperm DNA damage and pregnancy outcomes using different tests. Despite heterogeneity, they found increased miscarriage risk with higher DNA damage, with an odds ratio of 2.48 (95% CI 1.52, 4.04, $p<0.0001$). A meta-analysis by Simon et al. (2017) also found associations between sperm DNA damage and reduced clinical pregnancy rates in 41 IVF/ICSI studies, though with methodological limitations.

While these analyses indicate sperm DNA integrity may affect reproductive success, heterogeneity across studies underscores the need for larger, high-quality studies using consistent methods. Key studies have used varying assays and thresholds to assess DNA damage (**Table 2**) and although associations have been reported between higher sperm DNA fragmentation and adverse IVF/ICSI outcomes, additional robust research is required to definitively characterize the potential utility for sperm DNA integrity evaluation in thoroughly assessing male fertility.

Table 2. Characteristics of studies on sperm DNA damage and reproductive outcomes following Assisted Reproductive Technology procedures.

Author	ART	Assay	Association with ART outcome	n	Study design
Benchaib et al., 2003	IVF/ICSI	TUNEL	Fertilisation Rate	104	Prospective
Larson-Cook et al., 2003	IVF/ICSI	SCSA	Clinical Pregnancy Rate	89	Retrospective
Benchaib et al., 2007	IVF/ICSI	TUNEL	Fertilisation Rate, Miscarriage Rate, Pregnancy Rate	322	Prospective
Sedó et al., 2017	ICSI	TUNEL	Embryo Quality	82	Not mentioned
Yang et al., 2019	IUI/IVF/ ICSI	SCSA	Miscarriage Rate (IUI)	2622	Retrospective
Zarén et al., 2019	IVF/ICSI	SCSA	Embryo Quality	352	Retrospective
Chen et al., 2020	ICSI	SCSA	No association	713	Retrospective
Cheng et al., 2020	IUI/IVF/ ICSI	SCSA	Pregnancy Rate	3000	Retrospective
Tang et al., 2021	IVF	SCD	Fertilisation Rate	523	Retrospective
Ten et al., 2022	IVF/ICSI	TUNEL	No association	331	Prospective Study

The existing sperm DNA damage tests can be grouped according to whether they use enzymatic reactions to label DNA breaks (TUNEL assay) or whether they rely on controlled DNA denaturation to reveal DNA breaks (SCSA, comet assay, SCD assay) (Esteves et al., 2021).

1.7.1. TUNEL assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labeling (TUNEL) assay allows for the measurement of single and double stranded DNA breaks. The TUNEL assay uses the DNA polymerase, TdT, to catalyse the addition of labelled dUTPs to the 3'-hydroxyl free ends of single and double stranded DNA (Sharma et al., 2016). The more DNA breaks present, the more dUTP is added, which can then be visualised using fluorescence microscopy or flow cytometry.

The clinical thresholds for sperm DNA fragmentation varies between different labs/ flow cytometers/methodologies used, ranging from 12% to 35% (Domínguez-Fandos et al., 2007; Duran et al., 2002; Sergerie et al., 2005). The established thresholds for sperm DNA fragmentation for the BD Accuri C6 and Accuri C6 Plus flow cytometers is set at 16.8%. This cut-off value had a reported high specificity of 91.6% and a positive predictive value of 91.4%, allowing the discrimination between fertile and subfertile men (**Figure 11**) (Sharma et al., 2016).

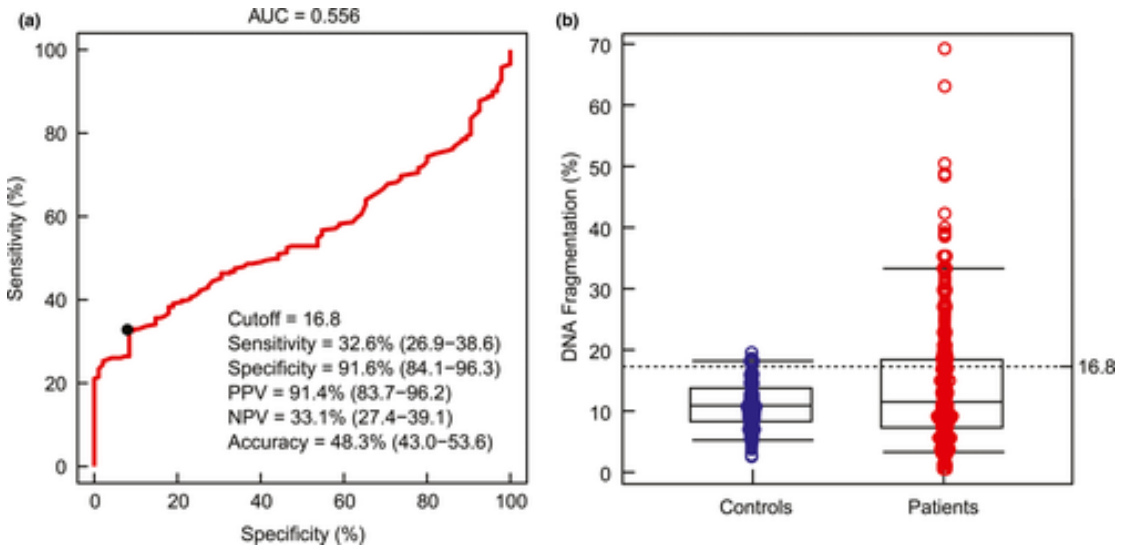


Figure 11: (**a) ROC curve and (b) %DNA fragmentation differences between controls and patients** (Sharma et al., 2016). (a) Receiver operator characteristic (ROC) curve showing TUNEL assay cut-off value at 16.8%. Values within the parenthesis represents the 95% confidence intervals. (b) shows the distribution of TUNEL test values between the controls (proven and unproven fertility) and patients (subfertile – confirmed male factor infertility by a male infertility specialist) men.

1.7.2. Sperm Chromatin Structure Assay (SCSA)

The sperm chromatin structure assay (SCSA) determines the percentage of DNA fragmentation Index (DFI) in sperm, as well as the number of immature spermatozoa (High DNA stainability - HDS) having high histone retention (Evenson, 2016). The test uses acridine orange (AO), a nucleic acid-selective cationic fluorescent dye, that penetrates dense sperm chromatin structure and intercalates into the double stranded DNA, resulting in green fluorescence (515-530 nm). The AO molecule stacks on single stranded DNA resulting in a metachromatic shift from green to red fluorescence (>630nm), that represents the number of sperm having DNA damage. Additionally, the SCSA allows for the measurement of spermatozoa that retain excess nuclear histones

since they fluoresce stronger than the rest of the stained spermatozoa. This allows for the estimation of the number of immature spermatozoa (HDS).

There are four thresholds levels for % DFI established for SCSA that are related to pregnancy outcomes (when there is no female infertility factors) (1) < 15 % DFI – excellent to good pregnancy outcomes, (2) > 15 % to < 25 % DFI – good to fair pregnancy outcomes, (3) > 25 % to < 40 % DFI – fair to poor pregnancy outcomes and (4) > 40 % DFI – very poor pregnancy outcomes (Evenson et al., 2002). Above 25 % HDS may also reduce the odds for good pregnancy outcomes. The established clinical threshold for SCSA is set at 25 % (**Figure 12**) for placing a man into a statistical probability of poorer reproductive outcomes including longer time to natural pregnancy, recurrent pregnancy loss and lower chances of IUI pregnancies (Evenson, 2013; Zhu et al., 2019).

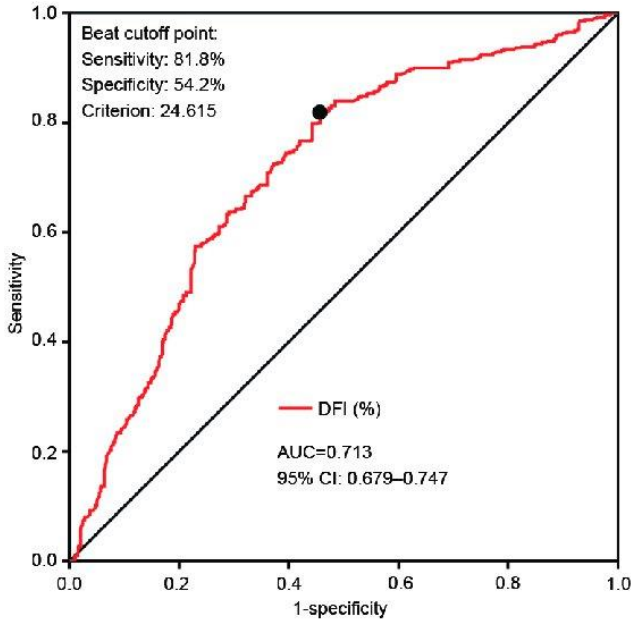


Figure 12. **ROC curve of % DFI for determining clinically relevant threshold** (Zhu et al., 2019). Receiver operator curve analysis of %DNA Fragmentation Index (DFI) in recurrent

pregnancy loss and control group (couples with clinical pregnancy following IVF). A threshold value of 24.6 % was obtained to discriminate from control group.

1.7.3. Acridine Orange Test

The acridine orange test (AOT) is a cytochemical test which utilises the metachromatic properties of acridine orange to differentiate between double stranded DNA and single stranded DNA. Following exposure to acid and acridine orange staining, the acridine orange molecules intercalates into double stranded DNA and emits green fluorescence following exposure to blue light (Ajina et al., 2017; Tejada et al., 1984). Aggregation of acridine orange molecule on single stranded DNA results in the emission of red fluorescence under blue light. The AOT is based on similar principles of the SCSA, however, it utilises fluorescence microscopy instead of flow cytometry to quantify sperm DNA damage levels. Therefore, due to the different methodology/equipment used between the AOT and the SCSA, the clinically relevant thresholds vary between the two assays. A study by Hoshi et al., 1996 revealed that spermatozoa have significantly lower fertilisation rates when there is <50% green AO fluorescence, suggesting that 50% could be the clinically relevant threshold for AOT. A study by Eggert-Kruse et al., 1996 found no correlation between AO testing and sperm motility, however, when assessing sperm morphology found a weak but significant Pearson correlation ($r = 0.15$) between the percentage of normal forms and percentage of green-fluorescing sperm.

1.7.4. Sperm Chromatin Dispersion Test

The sperm chromatin dispersion test (SCD)/Halosperm test is based on the ability of intact DNA to create loops around the nuclear matrix. These correspond to relaxed DNA loops attached to the residual nuclear matrix (Fernández et al., 2003). During SCD, the sperm are suspended in agarose matrix and treated with an acid solution, as a DNA denaturant, followed by a lysing solution. The lysing solution enables the removal of the nuclear protamines resulting in a spread of the DNA loops in the microgel (Fernández et al., 2018). After staining with bright-field dyes or fluorochromes, DNA fragmentation is assessed using microscopy.

The degree of DNA fragmentation is measured by evaluating the size and shape of the dispersed DNA fragments (**Figure 13**). If the sperm DNA is non fragmented, that is, without significant DNA denaturation following acid treatment, large halos of dispersed DNA will show. If the sperm DNA has fragmented DNA, the DNA will not disperse, or have limited dispersal, resulting in a lack of halo or a small halo.

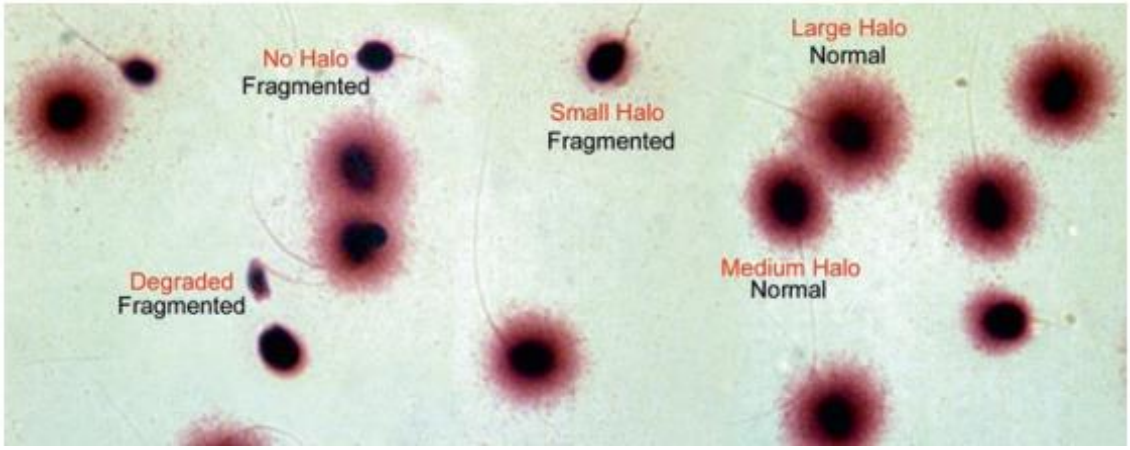


Figure 13. Images of sperm processed by the sperm chromatin dispersion test under bright-field microscopy (Fernández et al., 2018).

Different cut off values for SCD have been reported to be clinically relevant. A study by Gosalvez, Jaime et al., 2015 reported that a cut off value of 16%, having a sensitivity of 85% and specificity of 75%, allowed for the discrimination between sperm donors and male patients attending an infertility clinic (**Figure 14**). Another study, reported a predictive cut off for pregnancy at 25.5%, having a negative predictive value of 72.7% (López et al., 2013).

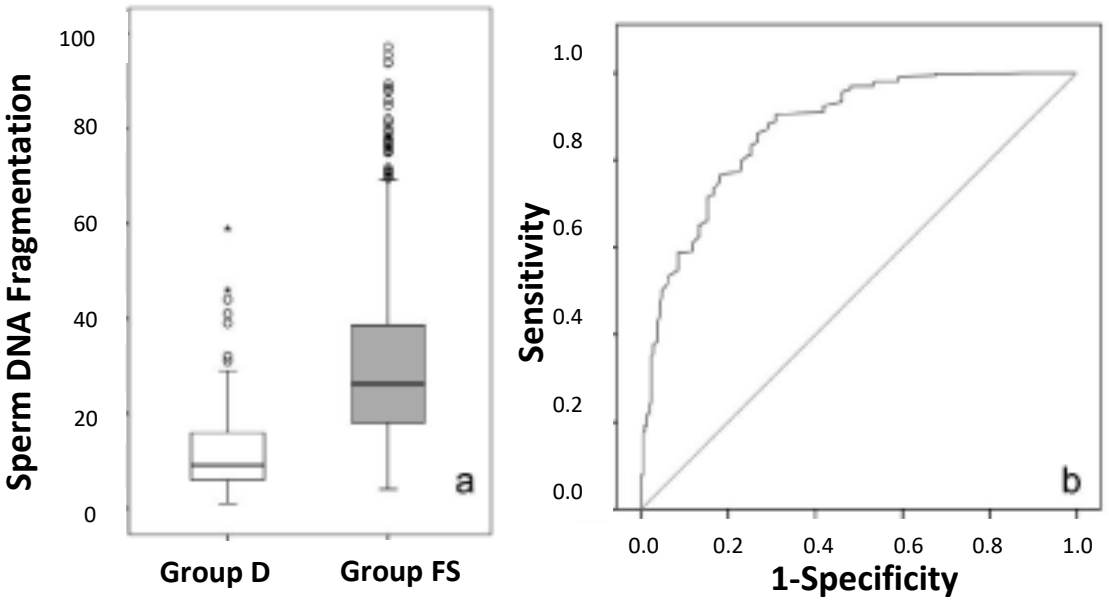


Figure 14. **Difference between between sperm donors and male patients attending an infertility clinic** (Gosalvez et al., 2015). (a) Box-whisker plot showing the distribution of sperm chromatin dispersion assay between group D (Donors) and group FS (First Semen analysis). (b) Receiver operator characteristic (ROC) curve using group D as a discriminative characteristic (at 16% cut off value).

1.7.5. Comet

Single cell gel electrophoresis (comet assay) unlike the SCSA, TUNEL and SCD assays, can distinguish between single strand breaks and double strand breaks. The comet assay

uses an electric field to separate DNA fragments, based on charge and size of strands (Simon & Carrell, 2013). Sperm exhibiting high levels of fragmented DNA will show an intense comet tail and comet length when visualised using a fluorescent dye. Non-fragmented DNA is retained in the nucleus. The comet assay can be performed in neutral buffer solutions (pH 7.5) or in alkaline solutions, pH >13 (**Figure 15**). In a neutral buffer, DNA is preserved as a double helix structure, resulting in double strand breaks to migrate during exposure to an electric field (Afanasieva et al., 2009). Whereas in alkaline denaturing conditions, single and double strand breaks are revealed and detected.

Multiple thresholds have been published for the comet assay. A study by Nicopoullos et al. found that sperm having less than 15% average alkaline comet score achieved the highest chance of live birth (Nicopoullos et al., 2019). The study advised, that if average comet score is greater than 29%, ICSI should be performed since the live birth remains steady even if sperm DNA damage is high. Another study indicated that a threshold of 26% for the alkaline comet assay is highly predictive of sporadic and recurrent miscarriage (Haddock et al., 2021). Neutral comet threshold of 77.5% has been shown to be associated with recurrent pregnancy loss (Ribas-Maynou et al., 2012), however, larger clinical studies are required to elucidate a clinically relevant threshold for the neutral comet assay.

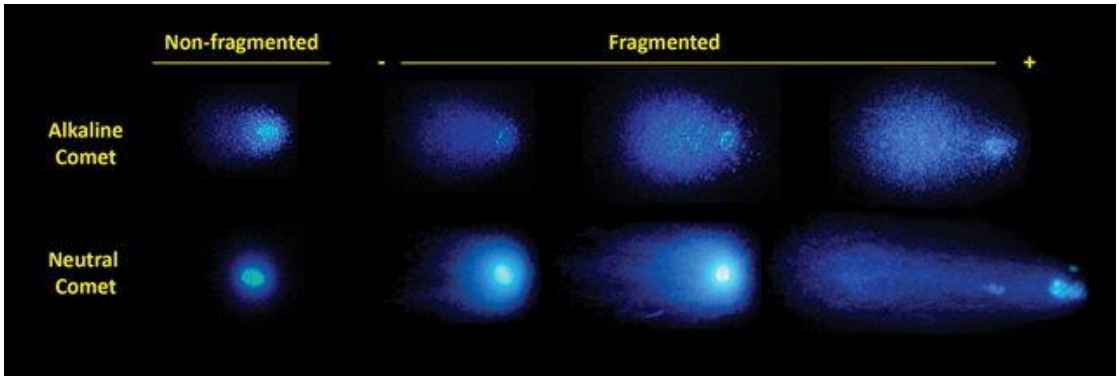


Figure 15. Photomicrograph of non-fragmented and fragmented spermatozoa in alkaline and neutral comet (Ribas-Maynou et al., 2012). Varying levels of sperm DNA fragmentation (SDF) are shown for the fragmented spermatozoa (stained using DAPI).

1.7.6. Limitations of sperm DNA damage testing

Sperm DNA damage testing allows for the assessment of DNA integrity and provides valuable information about male fertility and potential reproductive outcomes. However, there are several limitations when it comes to sperm DNA damage testing, including a lack of standardised testing protocols (Durairajanayagam, 2017). There is currently no consensus on methodology to conduct sperm DNA damage testing, making it difficult to compare results between different studies. The main advantages and disadvantages of the different sperm DNA damage tests are reported in **Table 3**. Additionally, there is variability in results based on the different external factors including method followed and laboratory used. Sperm DNA damage testing methods are also often criticised for their inability to differentiate the exact type of DNA break as sperm DNA breaks occur naturally during spermatogenesis (Agarwal et al., 2017). Therefore, the lack of identification of the particular DNA breaks can impair the clinical significance of such test. However, multiple studies have assessed the correlation

between sperm DNA damage and reproductive outcomes using a wide variety of sperm DNA damage tests. Therefore, despite there being a lack of standardisation for sperm DNA damage testing, this should not underestimate the clinical benefit sperm DNA damage testing has as a complimentary test to a standard semen analysis.

Table 3. Summary of principle, advantages and disadvantages of common sperm DNA fragmentation tests.

Assay	Types of DNA breaks detected	Basis	Advantages	Disadvantages
TUNEL	SSB DSB	dUTP staining of DNA strand breaks	• Standardised protocol • Low inter-observer variability • Specific detection of DNA fragmentation.	• Specialised equipment training • Time consuming • TdT is a bulky enzyme resulting in limited access to sperm chromatin
SCSA	SSB DSB	Acridine orange staining	• Standardised protocol • Low inter-observer variability • Rapid evaluation of sperm DNA damage levels • Acridine orange is a small molecule and access to sperm chromatin should not be limiting	• Specialised equipment training • Indirect evaluation of DNA damage
SCD	SSB DSB	Staining of DNA loops embedded in agarose	• Standardised protocol • Technically simple • Inexpensive	• High inter-observer variability • Labour intensive • Time consuming • May result in artifactual DNA breaks
Neutral Comet	DSB	Electrophoresis measurement of DNA fragments	• Can be done using low sperm counts • Quantifies actual DNA damage of	• High inter-observer variability • Time consuming

			each spermatozoon	• May result in artifactual DNA breaks
Alkaline Comet	SSB DSB	Electrophoresis measurement of DNA fragments	• Can be done using low sperm counts • Quantifies actual DNA damage of each spermatozoon	• High inter-observer variability • Time consuming • May result in artifactual DNA breaks

1.8. Strategies to minimise sperm DNA damage

Female partners of males presenting with high sperm DNA damage levels, have a higher likelihood of lower pregnancy success rates and higher miscarriage rates, even following ART procedures (Chua et al., 2023; Malić Vončina et al., 2021; Robinson et al., 2012). Therefore, utilising methods that can help prepare and select sperm with better chromatin can help increase the chances of reproductive success. Where possible, men are advised to adopt certain lifestyle changes to improve sperm parameters.

1.8.1. Sperm Preparation Techniques

Sperm preparation is an important step for ART procedures. The goal of sperm preparation is to select sperm having high motility, normal morphology, and low sperm DNA damage, to increase likelihood of achieving good reproductive outcomes (Pinto et al., 2021). A variety of methods have been used to select sperm of high quality, with the most common techniques being density gradient centrifugation and swim up. Density gradient centrifugation (DGC) is considered the gold stand for sperm preparation. It is

based on centrifugation of semen on different levels of solutions that have varying density. Following centrifugation, the sperm separate at the gradient layer of the most appropriate density. Mature morphologically normal sperm have a density of around 1.10 g/ml while immature morphologically abnormal sperm have a lower density of around 1.06 – 1.09 g/ml (Sharma & Agarwal, 2020). The swim up method relies on the ability to separate a semen sample into motile and less motile fractions. In swim up, a sperm pellet obtained from centrifugation is placed on an overlaying culture medium and incubated at a 45-degree angle. Motile spermatozoa swim up through the medium while non-motile spermatozoa remain in the bottom layer.

A major limitation of both DGC and swim-up techniques is that they rely on centrifugation steps. Centrifugation has been suggested to increase the level of ROS resulting in increased sperm DNA damage levels (Aitken et al., 2010; Z. Li et al., 2012). As a result, microfluidic based sperm preparation has emerged as an alternative sperm preparation technique (**Figure 16**), that allows for sperm to be naturally selected based on motility, morphology and DNA integrity (Nosrati et al., 2017). Microfluidic devices consist of an array of microchannels through which motile sperm move and are collected at the outlet port. Since it does not rely on centrifugation for sperm separation, studies have reported lower DNA fragmentation levels compared to swim up and density gradient centrifugation (Gode et al., 2020; Nosrati et al., 2014; Quinn et al., 2018).

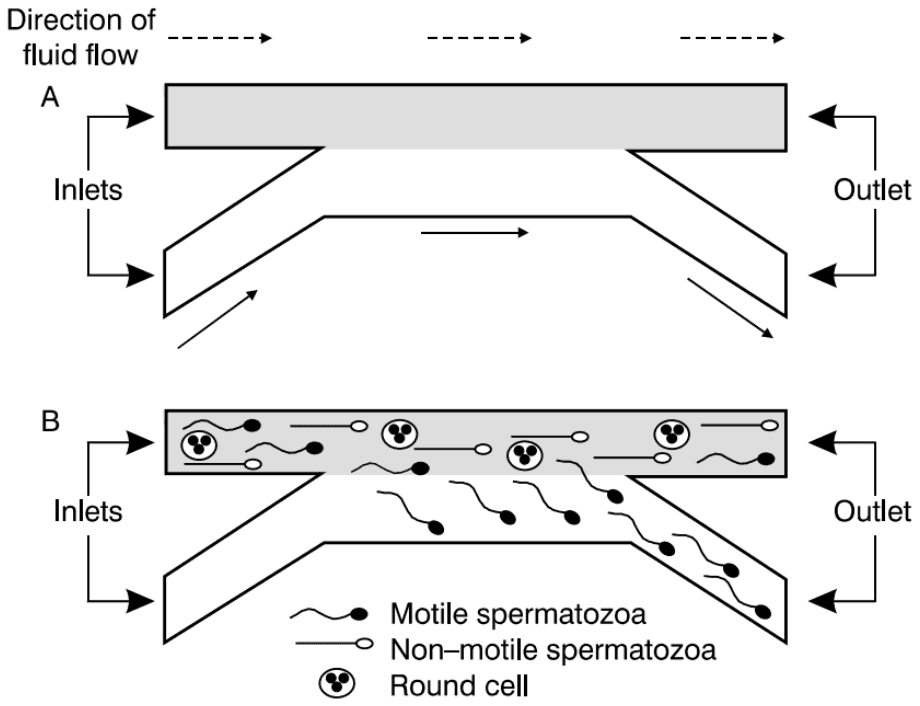


Figure 16. **Microfluidic sperm separation mechanism** (Schuster et al., 2003)**.** A passively driven microfluidic device for sperm sorting utilizing microfluidic channels to separate sperm cells based on their motility. It relies on the natural flow of fluids to drive the separation process. The flow direction shown in (A) and (B) shows the sorting process of sperm. Highly motile sperm are able to swim across the stream more readily than less motile sperm.

1.8.2. Lifestyle modifications

Several lifestyle factors including obesity, caffeine intake and smoking can have been correlated to reduced sperm quality.

1.8.2.1. Overweight and Obesity

Obese men have been shown to have increased levels of seminal oxidative stress, resulting in increased levels of DNA damage when compared to normal weight men (Palmer et al., 2012). This could be a direct result of increased scrotal temperatures and

endocrine imbalance, reducing overall sperm quality. The increased levels of sperm DNA damage in obese men have been associated with lower pregnancy rates, increased miscarriage rates, and reduced live birth rates (Bakos et al., 2011; Keltz et al., 2010). Weight loss was found to decrease levels of sperm DNA damage, potentially due to restoring the endocrine balance of the hypothalamic-pituitary-gonadal axis (Samavat et al., 2018; Sultan et al., 2020). Therefore, for obese patients, weight loss is recommended prior to ART treatment and to increase chances of natural conception.

1.8.2.2. Smoking

Cigarette smoking has been linked to increased levels of sperm DNA damage (CUI et al., 2016). Tobacco smoke contains over 4700 chemical compounds and several compounds found in the smoke are considered hazardous including nicotine and its metabolites (Talhout et al., 2011). Enzymatic oxidation of nicotine can result in the formation of cotinine that can be further metabolized into trans-3'-hydroxycotinine (Harlev et al., 2015). The metabolite trans-3'-hydroxycotinine, can undergo redox cycling which can enhance ROS levels (Kanamori et al., 2022) and result in DNA damage (Morgan, 1995). Additionally, tobacco smoking has been directly associated with increased seminal oxidative stress as a result of increased seminal leukocyte concentrations and decreased antioxidant capacity (Rehman et al., 2019; Saleh et al., 2002). Studies have shown that smoking cessation improves sperm parameters (Kulaksiz et al., 2022) and reduces the risk of failure during an ART cycles, by up to 4%, each passing year since a man quit smoking (Vanegas et al., 2017).

1.8.2.3. Air pollution

High levels of air pollution have also been indicated to alter sperm quality, with reduced sperm motility (Najafi et al., 2015) and increased sperm DNA damage levels reported (Rubes et al., 2005; Selevan et al., 2000). However, limited information is available on the impact of air pollutants on sperm quality. More environmental epidemiology studies are needed to anlayse the association between ambient air pollution and sperm quality.

1.8.2.4. Antioxidant intakes

Excessive ROS levels can overcome the physiological antioxidant defenses, resulting in oxidative stress. Antioxidant oral supplementation could help counterbalance excess seminal ROS (Agarwal & Majzoub, 2017; Martínez-Holguín et al., 2020). The physiological antioxidant system consists mainly of micronutrients (including zinc and selenium), enzymatic factors (including superoxide dismutase, catalase and glutathione-peroxidase) and non-enzymatic factors (vitamin E and C, and L-carnitine) (Martínez-Holguín et al., 2020). The combinatory actions of these antioxidant systems help maintain seminal redox balance.

Several studies have analysed the relationship between antioxidant intake and sperm DNA damage levels and have reported conflicting results. Numerous studies have found that intake of supplementary antioxidants improves sperm parameters (Ahmadi et al., 2016; Ilić et al., 2018; Li et al., 2022; Ligny et al., 2022; Xu et al., 2003), however, some studies found no such association (Appasamy et al., 2007; Steiner et al., 2020). A reduction in sperm DNA damage levels has also been reported following antioxidant treatment. A study by Fraga et al., found that vitamin C supplementation helped lower 8-hydroxy-2'-deoxyguanosine levels (Fraga et al., 1991). Vitamin C is considered to be

the main natural semen antioxidant and is found in relatively high concentrations in semen. Poor sperm parameters have been associated with low vitamin C levels and improved sperm motility has been associated with vitamin C supplementation (Dawson et al., 1992). Overall, the effects of several individual antioxidants have been assessed to analyse their effect on sperm parameters, however, it is likely that the antioxidants work together in synergy (Caroppo & Dattilo, 2022; Kowalczyk, 2021), therefore multi-antioxidant complex therapies might prove more beneficial in helping maintain the balance between ROS production and clearance.

Although high ROS concentration can result in sperm pathologies, ROS concentration at physiological levels are necessary for normal sperm function including capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion (de Lamirande et al., 1997). Therefore, although antioxidant supplementation can help reduce seminal oxidative stress levels, it is important to ensure that ROS levels are not below physiological levels, as reductive stress can have deleterious effects on sperm function (Selvam et al., 2020).

1.8.2.5. Exercise

Although few study are available that analyse the benefits of exercise on male reproductive health, it is generally considered beneficial (Vaamonde et al., 2017), and positive correlations have been demonstrated between exercise and sperm count (Lalinde-Acevedo et al., 2017; Vaamonde et al., 2012). However, cycling has been found to have the opposite effect, likely due to increased testicular friction resulting in increased sperm DNA damage levels (Hajizadeh & Tartibian, 2015). Other activities that

result in increased scrotal temperatures are also to be avoided, including long hot baths and saunas, since these can also result in reduced overall sperm quality (Garolla et al., 2013).

1.8.2.6. Caffeine Intake

There is limited studies analyzing the effect of caffeine on sperm quality, and there is no established agreement between studies analysing the effect of caffeine and number of coffee cups in one day on sperm quality (Ricci et al., 2017). However, it is still recommended to avoid caffeine or consuming too much caffeine (>2 cups of coffee a day) when trying to conceive.

1.8.2.7. Varicocele repair

The presence of varicocele is often associated with reduced fertility. In a study by Nieschlag, 2001, varicocele was found to have a clinical prevalence of 16% of 7,802 men referred for infertility. The exact mechanism behind the influence of varicocele on fertility, is largely unknown, however it has been associated with raised scrotal temperatures (Goldstein & Eid, 1989), hormonal profile abnormalities (Bellastella et al., 2022) and tissue hypoxia (Gat et al., 2005). The increased testicular temperature can increase seminal oxidative stress levels, resulting in elevated sperm DNA damage levels (Smith et al., 2006; Wang et al., 2022). Surgical repair of varicocele has been shown to restore testicular temperature and improve sperm parameters (Schlesinger et al., 1994), however further research is required to fully elucidate the effect of varicocele surgical correction on sperm quality.

1.9. Thesis prospectives

Taken together, it is clear that there are substantial challenges to overcome in both the diagnosis and treatment of male factor fertility problems. These can be broadly grouped under two headings: firstly, to ensure that the best use is made of *currently available* diagnostics and remedies, and secondly the development of *new* methods to address issues for which limited/no current solution exists.

1.9.1. Key questions addressed in this thesis

This thesis is comprised of five related projects. The first project (**Chapter 3**) is aimed at understanding the barriers to uptake of current advanced semen testing methods. In this, I analyse urologists' perceptions of these tests and the reasons for their use, or lack of use. The second project (**Chapter 4**) focuses on understanding the combinatory results of two seminal oxidative stress tests (ROS and ORP tests) and seeks to understand how these relate to sperm DNA damage and other sperm parameters. The third project (**Chapter 5**) focuses on improving access to diagnostic testing, through the validation of a rapid sperm DNA damage test to be provided as a commercially available service. The fourth project (**Chapter 6**) turns to the development of novel treatments for male fertility problems, and studies the use of microfluidic sperm preparation techniques to reduce sperm DNA damage levels in an animal model system (boar semen). The fifth and final project (**Chapter 7**) assesses the safety of broadband infrared sources on boar and human spermatozoa – while not directly used for male fertility treatment, these are proposed for use in optical coherence tomography to monitor

fertilization and embryo development and so, understanding the effect on sperm is crucial to their deployment.

2. Chapter 2: Methods

2.1. Survey Methodology (for Chapter 3)

A web-based survey was conducted between 13th October 2020 and 1st December 2021, to investigate how urologists use standard and advanced semen analysis data to diagnose and manage subfertility in male patients. Urology registrars, fellows and consultant urologists that specialise in male reproductive health were invited to participate in this study.

A total of three sections which comprised 13 multiple choice and open-ended questions were included in this survey. Section A analysed what can be diagnosed from routine semen analysis (to choose whether they consider a semen analysis an important indicator of (i) obstruction of male reproductive tract, (ii) male accessory gland dysfunction, (iii) testicular dysfunction, (iv) infection/inflammation, (v) immunological infertility, (vi) toxin exposure and (vii) genetic infertility) and which semen parameters (sperm motility, abnormal % morphology, semen volume, seminal pH, % vitality, sperm concentration, % round cells and leukocyte number) the respondents considered relevant. Section B investigated what follow-up tests (sperm aneuploid testing, sperm DNA fragmentation testing and sperm oxidative damage testing) the respondents use in their current practice and the referral frequency of these tests. Section C aimed to understand what the urologists want to test for, particularly in relation to sperm DNA damage measurement.

2.1.1. Survey Data Collection

The survey was primarily distributed through the LinkedIn Media platform and data was collected on Jisc Online surveys (www.onlinesurveys.ac.uk). We specified that the survey was intended only for urologists dealing with male subfertility patients and we included a question to confirm that the participants are urologists that work in urology and/or andrology clinics. Participants were implied to have consented to participate upon registration and providing responses to the survey. All survey responses were accessible only to the investigators.

2.2. Retrospective study methodology (for Chapter 4)

The study analyzing the impact of reactive oxygen species (ROS) and oxidative reduction potential (ORP) on seminal parameters was a retrospective cross-sectional study involving a total of 1278 patients attending TDL Andrology, London, UK, for seminal oxidative stress testing between January 2019 and March 2022. Out of the 1278 patients, 183 patients also had sperm DNA damage testing performing in conjunction to seminal oxidative stress testing. Exclusion criteria included incorrect abstinence period (advised to have 2 to 3 days of sexual abstinence), less than 10^6 sperm per mL due to unreliable oxidative-reductive potential (ORP) and reactive oxygen species (ROS) measurements. Patients having had fever during the previous 12 week were also excluded. Semen analysis at TDL Andrology, London was done according to WHO 2010 guidelines. Polymorphonuclear leukocytes were identified using a peroxidase screen (LeucoScreen, FertiPro N.V., Beernem, Belgium).

2.3. Ethical Clearances (required for all except Chapter 6)

2.3.1. Chapter 3 patients and ethical clearance

Ethical approval for this study was not required as it does not fall within the requirement for a full research ethics review under University of Kent policy.

2.3.2. Chapter 4 patients and ethical clearance

This study was approved by the Faculty of Sciences Research Ethics Advisory Group for Human Participants at the University of Kent (CREAG113-10-22) and adhered to the current legislation on research involving human subjects in the UK.

2.3.3. Chapter 5 patients and ethical clearance

This study was approved by the Faculty of Sciences Research Ethics Advisory Group for Human Participants at the University of Kent (CREAG074-05-22) and adhered to the current legislation on research involving human subjects in the UK.

2.3.4. Chapter 7 patients and ethical clearance

This study was approved by the Faculty of Sciences Research Ethics Advisory Group for Human Participants at the University of Kent (CREAG078-06-22) and adhered to the current legislation on research involving human subjects in the UK.

2.3.5. Semen samples analysed in this thesis (Chapters 4-7)

Sperm DNA fragmentation data and ROS/ORP measurements in **Chapter 4** are a retrospective reanalysis of data captured during routine diagnostic testing at The Doctors Laboratory (TDL) Andrology clinic (Wimpole Street, London, UK). Anonymised data was provided by the clinic following ethical approval via the appropriate University of Kent Research Ethics Advisory Group (REAG) – see above. DNA fragmentation data in **Chapter 5** is a comparison of data provided by TDL to our own in-house tests, using human semen samples provided by TDL. DNA fragmentation and viability/acrosome integrity data in **Chapter 6** and **Chapter 7** are based on our own in-house assays, using a boar model system and/or volunteer samples collected at Kent.

2.3.6. Human patient semen sample collection at The Doctors Laboratory Andrology clinic for measurement of sperm DNA fragmentation (Chapters 4-5)

Semen samples were obtained from patients attending The Doctors Laboratory Andrology clinic (Wimpole Street, London, UK) for sperm DNA damage testing by the sperm chromatin structure assay (SCSA). Patients were advised to abstain from ejaculation for 2 to 3 days (abstinence period of longer than 5 days were not accepted) before the test. Semen samples were obtained by masturbation at the clinic and frozen within 60 minutes of ejaculation. Two 250 µl – 500 µl aliquots of semen were placed in 1.8 mL cryovials. Samples were not accepted if the sperm count was below 0.1 x 10^6 sperm cells/mL. The vials were labelled and snap frozen in dry ice and kept in a dry ice container until shipment. One vial was then sent for SCSA testing to an external provider (SCSA Diagnostics, Brookings, South Dakota, USA, data used in **Chapter 4** and **Chapter 5**

and the other vial (for **Chapter 5** work only) was sent to the School of Biosciences in Kent for sperm DNA damage testing. Samples that arrived at the University of Kent were kept in a -86 ^{o}C Ultra Low Temperature (ULT) freezer.

2.3.7. Human volunteer sperm collection at Kent (Chapter 7)

Human semen samples were collected by masturbation on the day of testing and placed in an incubator at 37 ^{o}C to liquify. The samples were prepared for testing within 30 minutes after collection.

2.3.8. Commercial boar sperm collection (Chapters 6-7)

Boar semen samples used for artificial insemination were supplied by JSR genetics (East Yorkshire, United Kingdom). Samples were shipped in semen extender at room temperature. Once received, the samples were placed in a cool box set at 17 ^{o}C for up to three days before use.

2.4. Measurement of Seminal Oxidative Stress (Chapter 4)

2.4.1. Measurement of Reactive Oxygen Species

ROS levels were measured by staff at The Doctors Laboratory Andrology (London) using a single tube luminometer (Turner Biosystems Instrument Modulus Model no. 9200-001, Sunnyvale, California, USA). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; SigmaAldrich) was used as the probe (Vessey et al., 2014). A stock 100 mmol/L luminol solution in dimethylsulfoxide (DMSO) was prepared in a 15 mL polystyrene falcon tube

in the dark and wrapped in aluminium foil. A luminol working solution was prepared daily (5 mmol/L luminol in DMSO) and kept in the dark. Positive controls (395 µl PBS, 5 µl 30% H_2O_2 (VWR) and 10 µl 5mmol/L luminol working solution) and negative controls (400 µl PBS and 10 µl 5 mmol/L luminol working solution) were run daily before assaying. Reagents were brought to room temperature prior to assaying. For measuring ROS in semen, 400 µl of liquified semen and 10 µl of luminol working solution were added together and gently mixed before reading in the luminometer. Chemiluminescence results were reported as relative light units (RLU) per second normalised to the sperm concentration (RLU/s/10^6 sperm). RLU readings for control and test results were noted at 1-minute intervals for a total of 10 minutes. The negative control mean value was subtracted from the test mean value to remove background variation.

2.4.2. Measurement of oxidation reduction potential (ORP)

The MiOXSYS System (MiOXSYS, Aytu BioScience Inc., Englewood, CO, USA) was used for ORP measurements (Agarwal et al., 2016; Rael et al., 2015) and was measured by staff at The Doctors Laboratory Andrology (London). 30µl of liquified semen at room temperature was pipetted on the sample aperture (**Figure 17**) and inserted in the MiOXSYS analyser. Once the MiOXSYS sensor was inserted, a 2-minute sample detection countdown started and a reading in milli Volts (mV) was displayed on the MiOXSYS analyser screen. The mV readings were normalised to the sperm concentration (mV/10^6 sperm/mL). Duplicate measurements were taken, and an average calculated.

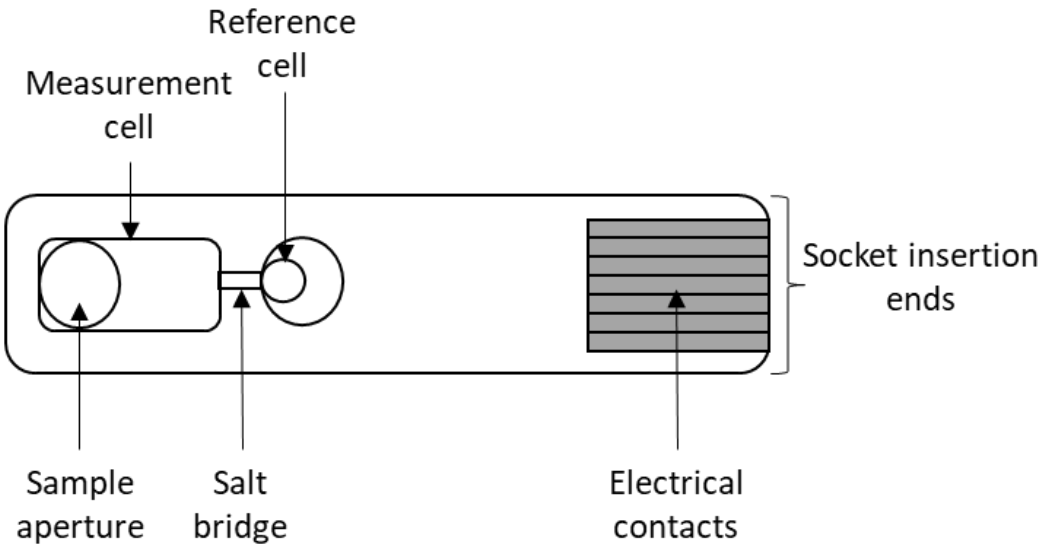


Figure 17. Illustration of the MiOXSYS sensor used for insertion in the MiOXSYS system for measurement of oxidative reductive potential.

2.5. Sperm DNA fragmentation testing (for Chapters 4-7)

2.5.1. Sperm DNA fragmentation measurement by external provider (Chapters 4-5)

Samples were shipped to SCSA diagnostics (South Brookings, South Dakota, USA) for SCSA testing. The sperm chromatin structure assay (SCSA) was used, and the reported parameters were the level of sperm DNA fragmentation (reported as %DNA Fragmentation Index, DFI) and the number of immature spermatozoa (reported as HDS – high DNA stainability) (Evenson, 2013).

2.5.2. AOFT testing solutions and buffers (Chapters 5-7)

Double distilled (ddH_2O) water was used for preparation of all solutions. All solutions and buffers were stored at 4^oC (unless specified otherwise).

Acridine orange (AO) stock solution (1.0 mg/ml) was prepared by dissolving purified acridine orange (ThermoFisher, A1301) in ddH_2O. Acid detergent solution was prepared by adding 20.0 mL of 2.0N hydrochloric acid (HCl) (SigmaAldrich, 258148), 4.39 g of 0.15M sodium chloride (SigmaAldrich, 59222C), 0.5 mL of 0.1% Triton X-100 (ThermoFisher, HFH10) and ddH_2O up to a final volume of 500 mL. pH of acid detergent was adjusted to 1.2 using 5.0 N HCl (ThermoFisher, AA35638K2). 0.1 M citric acid buffer was prepared by adding 21.01 g/L citric acid monohydrate (SigmaAldrich, C19009) to 1.0L H_2O. 0.2 M Sodium phosphate buffer was prepared by adding 28.4 g sodium phosphate (Na_2PO_4) (SigmaAldrich, 342483) debased to 1.0L H_2O. Staining buffer was prepared by adding 370 mL of 0.1 M citric acid buffer, 630 mL of Na_2PO_4 buffer, 372 mg 1 mM Ethylenediaminetetraacetic acid (EDTA) disodium (SigmaAldrich, E7889) and 8.77 g 0.15 M NaCl (SigmaAldrich, 59222C). This was stirred for 6 hours using a stirrer plate to ensure complete dissolution of EDTA. pH was adjusted to 6.0 using sodium hydroxide (NaOH) (ThermoFisher, 10306323) pellets.

Acridine orange (AO) staining solution was prepared by adding 600 µl of AO stock solution to 100 mL of staining buffer. The AO staining solution was kept in amber glass vials for a maximum of two weeks. The AO equilibration buffer was prepared by combining 400 µl acid-detergent solution with 1.2 mL AO staining solution. TNE buffer (10x) was prepared by adding 9.48 g Tris-HCl (Roche, 10812846001), 52.6 g NaCl (SigmaAldrich, 59222C) and 2.23 g disodium EDTA (SigmaAldrich, E7889) to 600 mL ddH_2O. pH of 10x TNE buffer was adjusted to 7.4 using 2N NaOH (ThermoFisher, 11963233). 1x TNE buffer (working solution) was prepared by adding 60 mL of 10x TNE and 540 mL of ddH_2O. pH of 1x TNE was adjusted to 7.4.

2.5.3. AOFT Staining Protocol (Chapters 5-7)

Prior to testing, semen samples were thawed at room temperature for 15 to 30 minutes. Sperm concentration was assessed using the haemocytometer with improved Neubauer ruling.

The semen sample were diluted to a concentration of 1-2 x 10^6 cells/mL with TNE buffer for a total volume of 200 µl in a 12x75 mm conical test tube. 400 µl of the acid-detergent was added and the stopwatch started. Exactly 30 seconds later, 1.2 mL of AO staining solution was added to the semen sample. The sample tube was placed into the flow cytometer sample chamber and the sample flow was measured after placing it in the sample holder. The sperm flow rate was checked to be <250 cells/sec. If the flow rate was higher, a new sample was prepared. Samples were measured in triplicates and an average taken. The flow cytometer was set to perform a sip rinse with ddH_2O between samples and a sip clean involving FACSclean solution (BD Biosciences, 660322) was done at the end of testing.

2.5.4. AOFT Flow cytometry Quality Controls (Chapters 5-7)

Three quality control checks were performed during each testing cycle. The first quality control check was to track the performance of the flow cytometer lasers using BD™ CS&T RUO calibration beads (BDBiosciences, 661414). The second quality control check was made using reference samples of known sperm DNA fragmentation levels (refer to section 2.5.1.). AOFT was performed on these samples of known reference ranges, for analysis of reagents. Up to 5% coefficient of variation difference between samples was considered acceptable. The third QC involved the addition of 5 µl 30% H_2O_2 (SigmaAldrich, 8222871000) to a semen sample that had sperm DNA damage levels

assessed during that day (**Figure 18**). The sample was incubated for 1 hour at 50°C and an increase in sperm DNA fragmentation levels should have been observed (**Figure 18**).

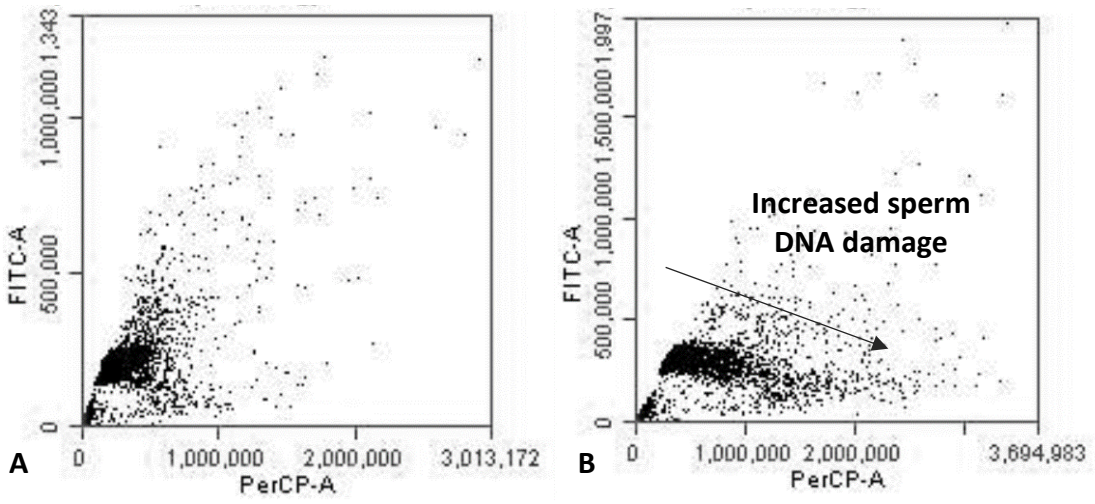


Figure 18: Assessment of sperm DNA damage changes following hydrogen peroxide addition, cytogram obtained after acridine orange staining. Green (Fitc) represents double-stranded DNA versus red (PerCP) represents single-stranded DNA. A shows a dot plot for a control a semen sample (% DFI: 6.4 ± 0.21) obtained prior to addition of 5 µl 30% H_2O_2 and B is the dot plot for the semen sample following addition of the H_2O_2 (% DFI: 39.04 ± 5.76).

2.5.5. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL) assay (Chapter 5)

The APO - DIRECT Kit (BD Pharmingen, San Diego, California, 556381) was used for the TUNEL assay. Prior to testing, samples were thawed at room temperature for 15 to 30 minutes. Sperm concentration was assessed using the haemocytometer with improved Neubauer ruling. Sperm concentration was adjusted based on the following calculation:

$$\frac{2.5}{(\text{Sperm Concentration } (10^6/\text{mL})} \text{x}1000 = \text{x µl}$$

The required volume of semen sample was added into a 1.5 mL Eppendorf tube and centrifuged at 600 g for 7 minutes. The supernatant was removed and 1 mL of 1X PBS (ThermoFisher, 18912014) was added. The sample was centrifuged again at 600 g for 7 minutes and supernatant was discarded, and 1 mL of PBS was added.

2.5.6. TUNEL - Spermatozoa Positive Control (Chapter 5)

A 9.8 M H_2O_2 stock (SigmaAldrich, 8222871000) was diluted 1:15 using 1X PBS. To a sample, 1 mL of the diluted H_2O_2 was added to the pellet. The tube was placed in a water bath set at 50^{o}C for 1 hour. Following incubation, the sample was centrifuged at 600 g for 7 minutes. The supernatant was removed and replaced with 1 mL of PBS. The PBS washing step was repeated twice more. The sample was centrifuged for 7 minutes at 600 g and supernatant discarded.

2.5.7. TUNEL - Internal Control and Kit Control Samples (Chapter 5)

The negative (BD Pharmingen, 6553LZ) and positive kit (BD Pharmingen, 6552LZ) controls (**Figure 19**) were vortexed. 2 mL aliquots of the negative and positive control cell suspensions were placed in 1.5 mL Eppendorf tubes and returned to -20^{o}C. Two samples of known DNA damage semen samples were centrifuged at 600 g for 7 minutes and supernatant discarded. The positive and negative kit control cell suspensions were centrifuged for 5 minutes at 600 g and supernatant removed. To the control and test samples, 1.0 mL of the wash buffer provided with the kit was added and vortexed. The control and test samples were centrifuged at 600 g for 7 minutes. This wash buffer step

was repeated, and following centrifugation, the supernatant was again discarded. The tubes were labelled accordingly.

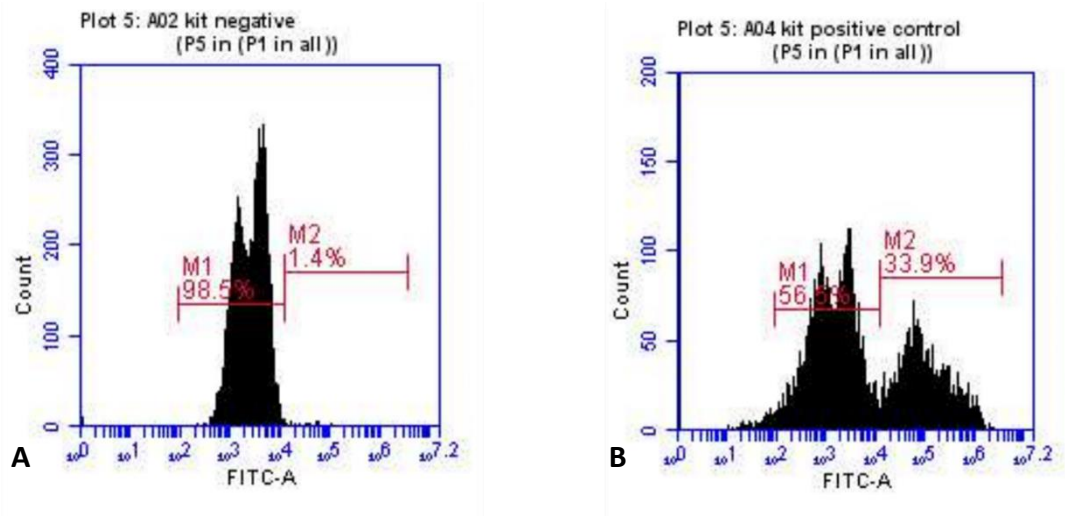


Figure 19. **Negative and Positive Controls**. Results for negative (A) and positive (B) control cells. The presence of DNA damage is indicated by the increased fluorescence in the M2 gate.

2.5.8. TUNEL Staining protocol (Chapter 5)

The appropriate volume of stain was prepared using the volumes indicated in **Table 4**. The preparation of stain and all subsequent steps were carried out in the dark. The stain was kept at 4^{o}C until used.

Table 4. **Preparation of staining solution**.

Staining solution	1 assay
Reaction buffer	10 µl
TdT enzyme	0.75 µl
FITC-dUTP	8.00 µl
ddH_2O	32.25 µl
Total Volume	51.00 µl

The pellets from the test samples and control samples were resuspended in 50 µl of staining solution. The samples were covered with aluminium foil and incubated in staining solution for 60 minutes at 37^{o}C in a water bath. At the end of incubation time, 1 mL of Rinse buffer was added to each tube and centrifuged at 600 g for 7 minutes. The supernatant was discarded. The cell rinsing was repeated, and supernatant was discarded again. The pellet was resuspended in 0.5 mL of PI/RNase staining buffer. The cells were incubated in the dark for 30 minutes and then analysed with flow cytometry.

2.6. Flow cytometry (Chapters 5-7)

Flow cytometry analysis was done using BD Accuri C6 Plus flow cytometer and BD Accuri C6 Plus flow cytometer with CSampler Plus attachment flow cytometer (BD Biosciences, Wokingham, Berkshire, United Kingdom). The BD Accuri software was used for plot generation (BD Accuri C6 Software v.1.34.1). The settings were set at 'slow' flow rate of <100 cells per seconds.

2.6.1. AOFT flow cytometer gating (Chapters 5-7)

The AOFT measures the %DNA fragmentation index (DFI) and the %High DNA stainability (sperm exhibiting high DNA stainability have higher histone number). For measurement of % DFI, an AO-stained sample was placed in the flow cytometer. The first cytogram consisted of an elliptical gate on the forward scatter vs side scatter plot, set to exclude debris. The second cytogram had a hinge gate set at a 45-degree angle with the bottom left hinge set to preclude the tailing debris beneath the main sperm population. The % DFI was measured by calculating the number of sperm in the H3-3 hinge region divided by the sum of sperm in H3-1 and H3-3 hinge region of **Figure 20.B**. The %High DNA stainability (HDS) represents the fraction of sperm that have increased levels of green fluorescence, reflecting a higher ratio of histones to protamines. The %HDS was measured by dividing the number of sperm that have higher green fluorescence (R1) by the number of sperm in P2 region of **Figure 20.C**.

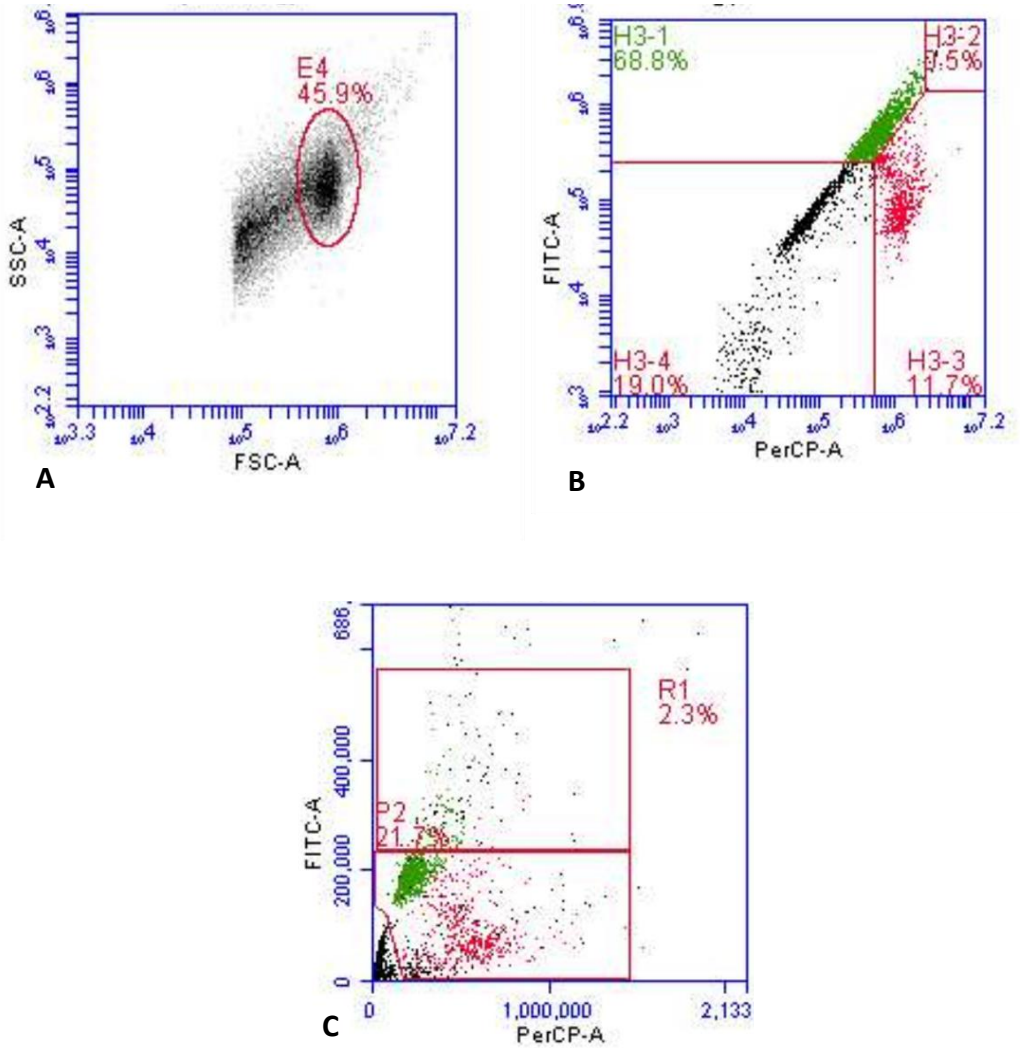


Figure 20. AOFT cytograms for measuring sperm DNA damage levels. (A) Forward scatter (FSC) vs side scatter (SSC) dot plot. The region E4 is established to exclude debris and larger cells. (B) PerCP (red) vs FITC (green) dot plot. Acridine orange (AO) emits a red fluorescence when bound to single stranded DNA and green when bound to double stranded DNA. The percentage of spermatozoa in region H3-3 relative to the total sperm population (regions H3-1 and H3-3) correspond to the % DNA fragmentation index. (C) PerCP (red) vs FITC (green) dot plot. The percentage of sperm in R1 relative to the total number of sperm in P2 correspond to % high DNA stainability.

2.6.2. TUNEL Flow Cytometer gating (Chapter 5)

The TUNEL assay measures the sperm DNA damage levels using the APO-Direct Kit (Pharmingen). For measurement of % DFI, a forward scatter versus side scatter plot was generated and a gate was set around the sperm cells to exclude any debris. A second dot plot of PI vs FITC was then generated, and a gate was set around the PI positive cells, allowing further exclusion of any debris material or apoptotic bodies. A final dot plot of FITC vs PE was generated, and quadrant gates were set, with the upper quadrant as shown in **Figure 21** representing the % of sperm that are PI and TUNEL positive cells (Sharma et al., 2021).

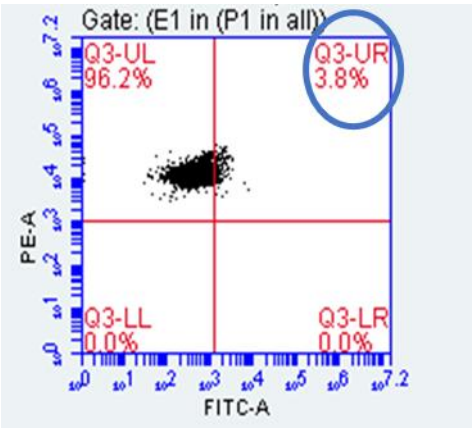


Figure 21. FITC (green) vs PE (red) dot plot, where TUNEL - positive cells are calculated. The percentage of sperm cells which are PI and TUNEL positive are revealed in the upper right quadrant (Q3-UR).

2.7. Sperm Preparation

2.7.1. Microfluidic Sperm Separation (Chapter 6)

The ZyMot™ Multi (850µl) sperm separation device (DxNow, Inc. ZMH0850) was used for microfluidics chip-based sperm separation (MCSS). A 1 mL Luer-tip syringe was used to gently draw 850µl aliquot of the semen sample. The syringe was then gently inserted in the inlet port (**Figure 22**), the sample was then slowly injected in the device. Using a fresh 1 mL syringe, 750 µl of porcine gamete medium (Yoshioka et al., 2008) was ejected into the surface of the upper membrane. Using the tip of the syringe, the media was manipulated to cover all the edges of the upper chamber. The device was covered with a Petri dish lid and left at room temperature. After 30 minutes, a 1 mL syringe was inserted into the outlet port and 500 µl was gently aspirated and placed in a 1.5 mL Eppendorf tube.

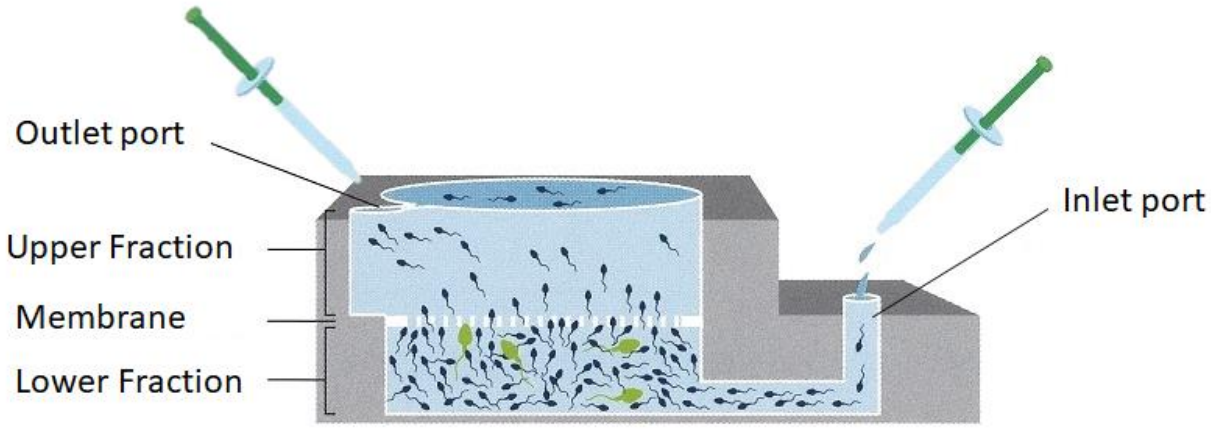


Figure 22. Schematic diagram of the ZyMot™ Multi sperm separation device (Image modified from ZyMot Fertility, 2019). A sperm sample is injected in the inlet port, and after an incubation time of 30 minutes, sperm is extracted from the outlet port (the green sperm represent morphologically abnormal spermatozoa).

2.7.2. Density Gradient Centrifugation (Chapter 6)

The BoviPure™ System (Nidacon, BP-100) was used for separating sperm by density gradient centrifugation. 2 mL of 80% diluted BoviPure™ (prepared by adding 8 mL of BoviPure™ and 2 mL of BoviDilute™) was transferred to a 15 mL conical centrifuge tube. 2 mL of 40% BoviPure™ (prepared by adding 4 mL of BoviPure™ and 6 mL of BoviDilute™) was carefully layered on top of the 80% diluted BoviPure™. 2 mL of semen was carefully layered on top, ensuring that the layers were not disrupted. The tube was centrifuged at 300 g for 30 minutes at room temperature. The supernatant was then removed, and the pellet was resuspended in 1 mL BoviWash™ in a 15 mL conical tube.

2.8. Semen Analysis

2.8.1. Boar sperm - computer assisted semen analysis (for Chapters 6 -7)

The iSperm® computer assisted semen analysis (Aidmics Biotechnology Co. Ltd, Taipei City 10647, Taiwan) was set up according to the manufacturer's guidelines for boar semen. 7.5 µl of semen was pipetted onto the surface of an iSperm® base chip. A cover chip was used to lock the base chip and then the locked chip was inserted in the heated chamber attached to the iPad camera and analysed. For each sample, two replicates were taken. The concentration and kinetic parameters were recorded: progressive motility, total motility, average path velocity, straight line velocity, curvilinear velocity, straightness, and linearity (**Figure 23**).

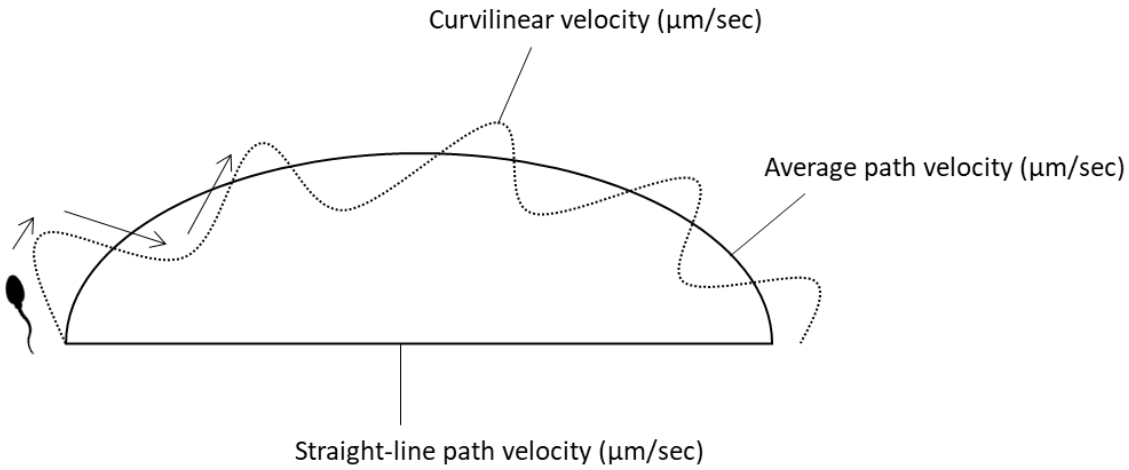


Figure 23. Sperm kinetic parameters measured by the iSperm® computer assisted semen analysis. The straight-line velocity (VSL) is the time-average velocity of a sperm head along the straight line between its first and last detected positions. Curvilinear velocity (VCL) is the average velocity measured over the actual point-to-point track followed by the cell. The average path velocity (VAP) measures the sperm head along its spatial average trajectory.

2.8.2. Human sperm - computer assisted semen analysis (Chapter 7)

Automated semen analysis (LensHooke® X1 PRO [X1 PRO], Bonraybio, Taichung, Taiwan) was used for further evaluation of human sperm kinetics (Agarwal et al., 2021). 40 µl of semen samples was loaded into the semen test cassettes and inserted into the Lenshooke® test unit. Duplicate measurements were taken. Results were automatically generated.

2.8.3. Human sperm – basic semen analysis (Chapter 7)

The samples were prepared for testing within 30 minutes after collection. Sperm concentration and vitality were performed according to WHO 6th edition criteria via the

manual method (World Health Organization, 2021). Sperm motility was performed according to WHO 5th edition criteria via the manual method using a heated stage set at 37 ^{o}C (World Health Organization, 2010).

Sperm motility was assessed by placing 2 x 10 µl drops of semen on opposite sides of a microscope slide and covered using 22 mm by 22mm coverslips. A positive displacement pipette was used to handle human semen. Sperm motility was assessed using a x 40 phase objective (x 400 total magnification) and % progressively motility, % non-progressive motility and % immotile was recorded using an appropriately labelled blood cell counter. 200 sperm cells were counted in each semen drop, and the average of the replicate results were compared to the acceptable differences criteria (if not, another 200 sperm cells were counted using a freshly prepared slide and a third count performed), as shown in **Table 5**.

Vitality was assessed using 0.5% Eosin Y in 0.9% Sodium chloride (Gurr, 34197). 5 µl of semen was mixed with 5 µl of the dye on a slide. A 22 mm by 22 mm coverslip was appropriately placed and examined under a bright field microscope. Colourless cells were counted as viable while pink stained cells were counted as non-vital. 200 sperm cells were counted. Another slide for vitality was done to count another 200 sperm cells and the average was taken to be compared to the acceptability criteria indicated in WHO laboratory manual for the examination and processing of human semen, 2021 (if not, another 200 sperm cells were counted using a freshly prepared slide and a third count performed).

Table 5. Acceptable differences between two replicates counts of 200 sperm and their average (based on 95% confidence interval). Table taken from the 5th Edition WHO Manual (World Health Organization, 2010).

Average (%)	Acceptable difference
1	2
2-3	3
4-6	4
7-9	5
10-13	6
14-19	7
20-27	8
28-44	9
45-55	10
56-72	9
73-80	8
81-86	7
87-90	6
91-93	5
94-96	4
97-98	3
99	2

An initial subjective observation during motility assessment was done for initial examination of count to aid in determining the correct dilution factor for sperm concentration. Sperm concentration was calculated using the improved Neubauer

chamber. Appropriate dilution was performed based on the number of sperm cells in the field of view. Dilution was done using a fixative containing 0.6 M sodium bicarbonate and 0.14 M formalin. Following dilution, 10 µl of the mixed diluted samples was loaded into each of the counting chambers and placed in a Petri dish containing moist paper for 5 minutes. After this, sperm was counted using a x 40 phase objective (x 400 total magnification). Pinheads or sperm heads only were not counted (but presence was reported). A minimum of 200 sperm cells were counted in a known number of large squares in each of the two hemocytometer chambers. The sum and difference of the chamber counts were calculated. The results were checked to ensure they are within the83abptable range of difference between the replicate counts shown in **Table 6** (if not, another 200 sperm cells were counted, and a third count performed).

Table 6. Acceptable difference between two replicate counts for a given sum (World Health Organization, 2021).

Sum	Acceptable Difference*	Sum	Acceptable Difference*
144–156	24	329–346	36
157–169	25	347–366	37
170–182	26	367–385	38
183–196	27	386–406	39
197–211	28	407–426	40
212–226	29	427–448	41
227–242	30	449–470	42
243–258	31	471–492	43
259–274	32	493–515	44
275–292	33	516–538	45
293–309	34	539–562	46
310–328	35	563–587	47

2.8.4. Boar Sperm Vitality and Acrosome Reactivity (Chapters 6-7)

Sperm vitality and acrosome reactivity was assessed using staining solution consisting of 1.5 µM Propidium Iodide and 1 µg/mL fluorescein isothiocyanate-labelled peanut agglutinin in phosphate-buffered saline (Robles & Martínez-Pastor, 2013). 500 µl of staining solution was pipetted into a flow cytometer tube. The sperm was diluted in the staining solution to achieve a concentration of 1-2 x 10^6 sperm cells/mL. The sample was incubated in the dark for 15 minutes at room temperature. The sample was then analysed using the flow cytometer. Positive control samples were placed in dry ice for 1 hour before staining (**Figure 24**). Following staining, the positive control should have shown that >90% of sperm is dead and acrosome reacted (if not, another staining solution would be prepared).

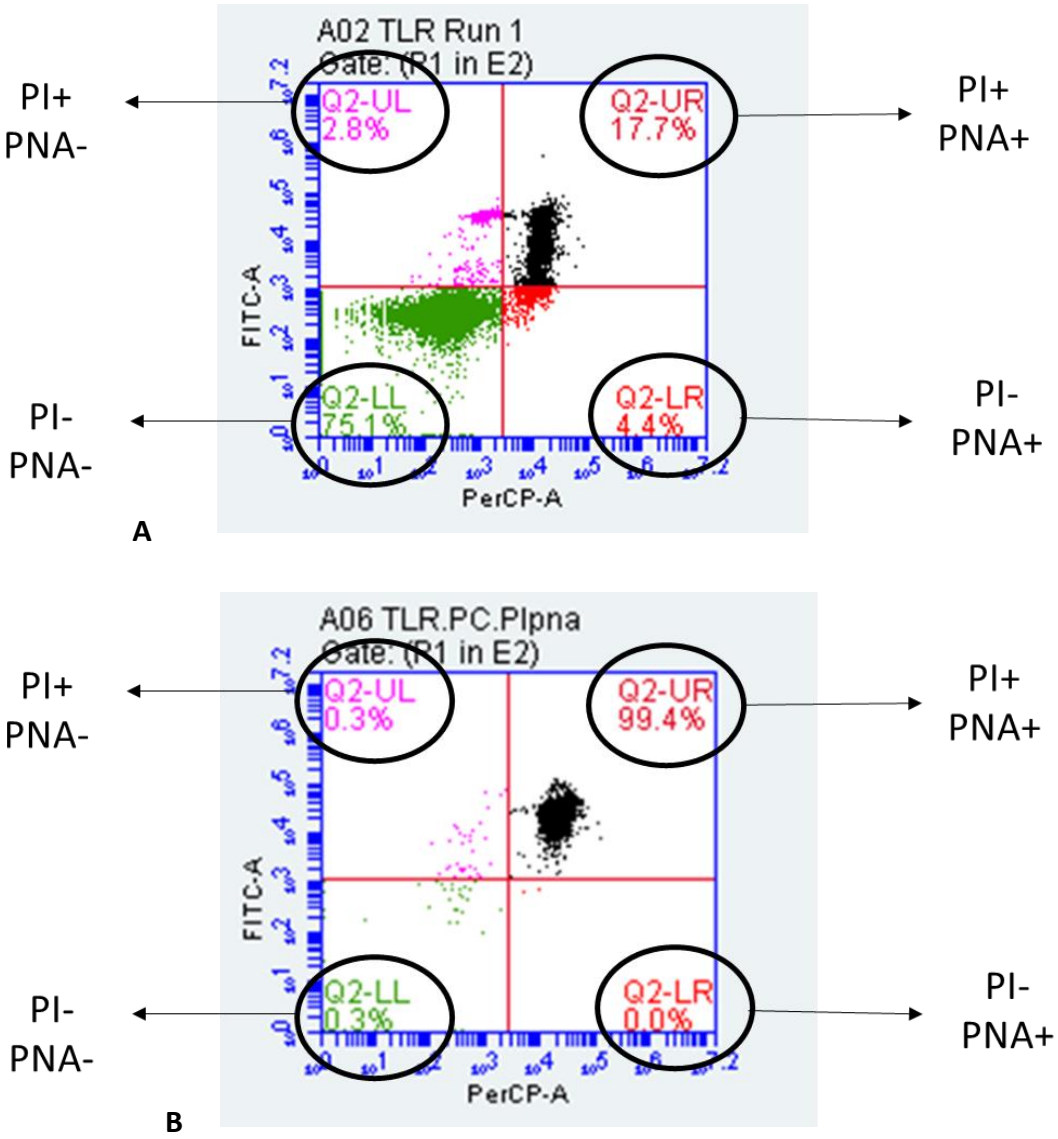


Figure 24. **Flow cytometric analysis of boar sperm labelled with FITC-PI/PNA.** In (A) the upper right quadrant represents dead acrosome-reacted sperm, the upper left quadrant represents viable acrosome-reacted sperm, the lower right quadrant represents dead acrosome-intact sperm and the lower left quadrant represents viable acrosome-intact sperm. (B) is a cytogram of a positive control for the FITC-PI/PNA labelling. >90% of sperm are dead acrosome-reacted after being placed in dry ice for one hour.

2.9. Imaging and irradiation protocol (Chapter 7)

Imaging and irradiation of sperm and oocytes was performed by the Optics Group at University of Kent. The optical system used for this study was a Swept Source Optical

Coherence Tomography (SSOCT) setup designed for embryo imaging, in which the sample arm was contained in a sealed imaging probe (IP) for integration into an incubator.

The goal of the irradiation setup was to mimic OCT scanning patterns, but without performing any imaging. In the irradiation setup, a superluminescent diode (Superlum MOPA) with an emission centered at 1077.9 nm and a full width at half maximum (FWHM) bandwidth of ~20 nm was used. Light from the source was directly injected into the imaging box via fibre and directed towards the sample.

The irradiation protocol for boar semen samples was designed to mimic the operation of a point scanning OCT system, with 3 different scanning protocols, referred to as protocol A, B and C. Protocols A and B simulated the scanning pattern of a raster (zig-zag pattern) scanning OCT system. In protocol A, the time for scan the entire field of view was kept unchanged by the overall time of acquisition was varied from 20 to 60 seconds. In protocol B, the total time was kept at 60 seconds, but sampling density was changed. The total irradiation dose received by the sample was kept unchanged, however, the instantaneous energy absorption increased with higher sampling density as the beam moved over a given area at a slower pace. Protocol C simulated different OCT scanning protocols that are commonly used for functional characterization of dynamic samples and is termed OCT angiography (OCT-A). In OCT-A, the rapid galvanometric scanners scan the same line multiple times before the slow scanner steps discreetly to the next position.

The irradiation protocol for human semen samples was performed with a constant 300x300 sampling density and 60 seconds exposure time. The semen samples were

separated into three groups: 0 mW, 3 mW power and 20 mW power at 1055 nm. Power values were measured with a power meter (Thorlabs).

2.10. Statistical Analysis

Statistical analysis was performed using the SPSS 28.0 software (SPSS Inc., Chicago, IL, USA), unless stated otherwise. For all statistical comparisons, significance was considered as p-value < 0.05.

2.10.1. Chapter 3 Statistical analysis

The general outcome for the survey was to understand the urologist perception and understanding of advanced sperm analysis specifically SDF testing. Further analysis was done to understand the difference (if any) among the perception of urologists practicing in different geographical regions on the use of conventional and advanced semen analysis testing.

The responses for Likert scale questions were converted into numerical variables with a total score to calculate the mean and the standard deviations. The quantitative data were tested using Anova and Chi-Square tests. The Kolmogorov-Smirnov test was used to assess normality. Considering that the data was not normally distributed, Spearman Rank correlations were calculated. Groups were compared using the Kruskal-Wallis test. The Chi-square test was used for analyzing the distribution of categorical variables. Group pairings test was conducted using the Bonferroni correction adjusted significance.

2.10.2. Chapter 4 Statistical analysis

The Kolmogorov-Smirnov test was used to assess normality. Correlations were assessed using the Spearman correlation tests reported as r and p values. R^2 denotes co-efficient of determination determined by linear regression models. Since the data was not normally distributed (using the Kolmogorov-Smirnov test), groups were compared using the Kruskal-Wallis test. The Chi-square test was used for analyzing the distribution of categorical variables. The group pairings test was conducted using the Bonferroni correction adjusted significance.

Samples were allocated into different groups according to whether they fell within or outside acceptable threshold limits (ORP: ≤1.34 mV/10^6 sperm/mL; ROS: ≤13.8 RLU/sec/10^6 sperm/mL). Samples were grouped as follows: group 1 (low ORP and low ROS); group 2 (low ORP and high ROS); group 3 (high ORP and low ROS) and group 4 (high ORP and high ROS).

2.10.3. Chapter 5 Statistical analysis

Validation of sperm DNA damage testing involved 3 categories (1) the analysis of sperm DNA damage variation between SCSA diagnostics and University of Kent, (2) the intra and inter-operator variability of sperm DNA damage testing and (3) the comparison between two different sperm DNA damage techniques (acridine-orange based flow cytometric testing vs terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling – TUNEL). The variability for the 3 categories was assessed by calculating the coefficient of variation. Bland-Altman plots were created using DATAtab (Graz, Austria) to compare the differences from the mean and from

upper and lower limits of acceptability. Test of normality were measured using the Shapiro-Wilk test.

2.10.4. Chapter 6 Statistical analysis

Statistical analysis was performed to evaluate the difference between two different sperm separation techniques: microfluidics sperm separation and density gradient centrifugation. The Kolmogorov-Smirnov test was used to assess normality and groups were compared using the Kruskal-Wallis test.

2.10.5. Chapter 7 Statistical analysis

Statistical analysis was performed to evaluate whether irradiated samples (at different imaging protocols) showed significant differences between their matched manipulation control. The Kolmogorov-Smirnov test was used to assess normality and groups were compared using the Kruskal-Wallis test.

3. Chapter 3. Urologist perception of advanced semen analysis testing to diagnose and manage subfertility

3.1. Introduction

Male factor subfertility has been widely recognised to influence assisted reproduction technology (ART) outcome (Sharma et al., 2015; Tandara et al., 2014; Zheng et al., 2018) and is solely responsible for about 20% of infertility cases and an additional 30% to 40% as a contributing cause (Hull et al., 1985). Males typically undergo a routine semen analysis consisting of assessing sperm morphology, motility and concentration, however, this is a minimum contributor for assessing male related subfertility relative to female pathology assessment (Krausz et al., 2015).

One parameter that is not examined in a standard semen analysis is the degree of sperm DNA fragmentation (SDF). However, sperm DNA quality is increasingly understood to be a key factor in fertilisation success (Ribas-Maynou & Benet, 2019; Zheng et al., 2018; Zhu et al., 2022). In particular sperm exhibiting above threshold levels of sperm DNA fragmentation (SDF) have a reduced ability to fertilise the oocyte, negatively influencing ART outcomes (González-Marín et al., 2012; Le et al., 2019). SDF in the form of double-strand breaks have been linked to higher implantation failures during ICSI cycles and may represent lethal alterations during early-stage embryogenesis. Both the paternal and maternal pronucleus remain separated at this stage and DNA repair is limited (Ribas-Maynou & Benet, 2019).

Various techniques that use different technologies to identify and quantify sperm DNA damage are available (**Figure 25**), the most common being the Terminal deoxynucleotidyl transferase dUTP nick end 91abelling (TUNEL) assay and sperm chromatin structure assay (SCSA) (Baskaran et al., 2019). Other tests include sperm Comet assay, sperm chromatin dispersion test (SCD) and acridine orange test.

The TUNEL assay measures the extent of single and double strand breaks by using terminal deoxynucleotidyl transferase which catalyses the addition of fluoresceinated-dUTP to 3'-OH ends of DNA fragments (Mitchell et al., 2011; Sharma et al., 2021). The SCSA and Acridine Orange test measure the susceptibility of DNA to damage, as induced by acid denaturation, which is then quantified using the metachromatic dye acridine orange. Acridine orange emits red fluorescence when bound to single stranded DNA and emits green fluorescence when bound to double stranded (non-denatured) DNA regions (Evenson, 2022). The SCD assay assesses the dispersion of the central core and peripheral halo following denaturation (Fernández et al., 2003). The DNA breaks causes expansion of the halo which is observed using fluorescence/bright field microscopy. The Comet assay assess the DNA migration using electrophoresis following lysis of the sperm membrane and can be performed under neutral or alkaline conditions (Simon & Carrell, 2013).

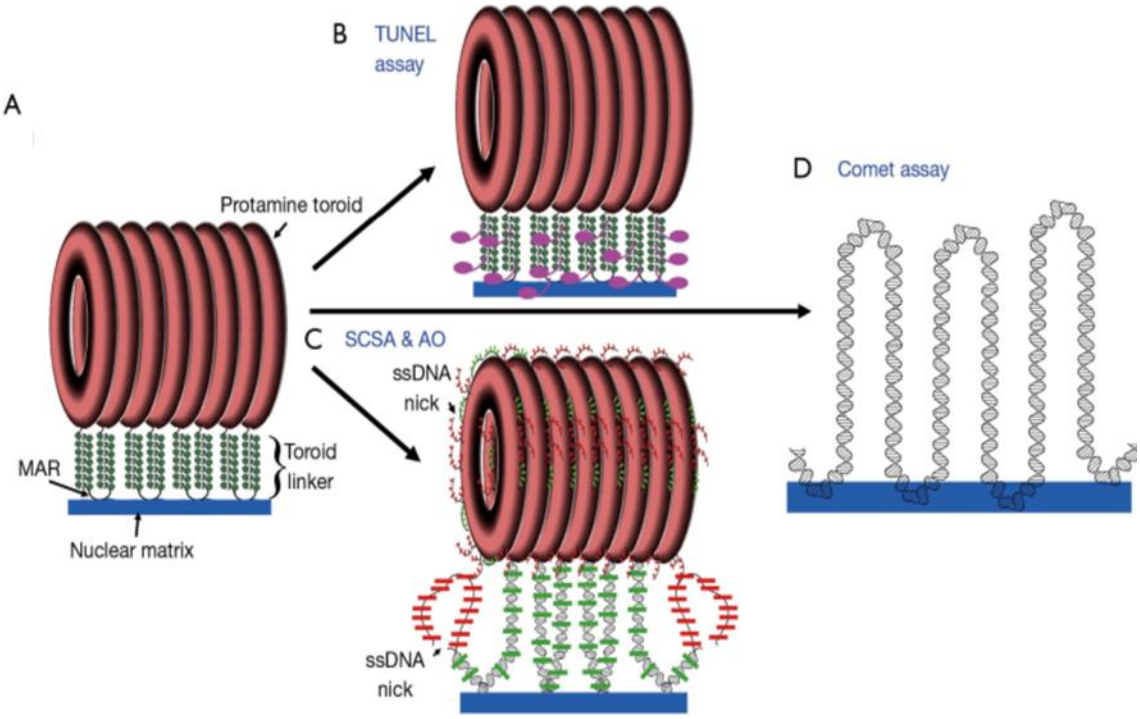


Figure 25. **Schematic representation of the abilities of different sperm DNA damage tests in detecting sperm DNA damage** (Ward, 2017). A) sperm DNA is condensed into protamine-bound toroids that are anchored by nuclease sensitive linkers to the nuclear matrix. B) TUNEL assay accesses the linker regions but not the protomine toroid structures. C) SCSA and AO accesses most part of the sperm chromatin to stain double strand DNA green and single stranded DNA red. D) Comet assay assesses the toroid free DNA.

Despite the benefits of offering SDF tests, it is not routinely presented to the patients due to the cost of tests and limited centres offering SDF services. However, despite the perceived cost of screening for sperm DNA damage, it can ultimately reduce the overall expense and time to pregnancy for ART patients as initial screening for sperm DNA damage can help in patient management (**Figure 26**) and may help root early on the cause of subfertility (O'Neill et al., 2018).

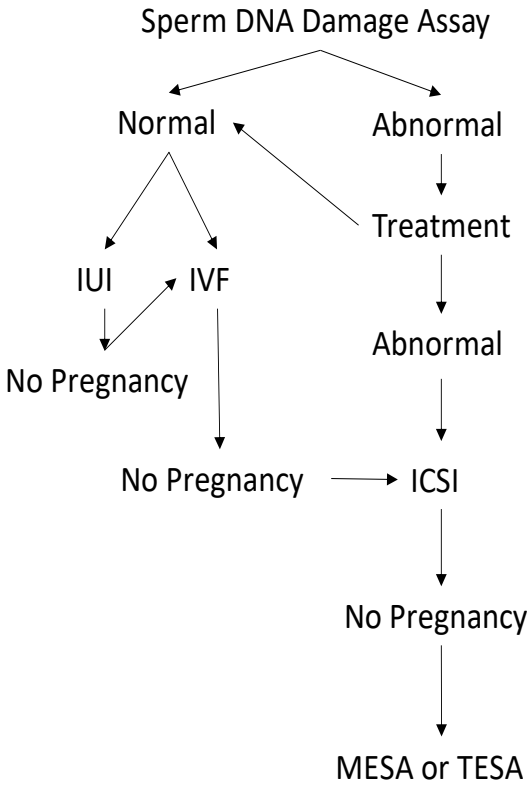


Figure 26. Flow chart showing a treatment algorithm based on sperm DNA damage results. Adapted and modified from (O'Neill et al., 2018).

3.2. Aims

A survey was conducted to analyse the perception of urologists practicing in different geographical regions towards SDF testing, to further understand their knowledge and understanding of sperm DNA damage and other available test. Urologists were chosen as the survey population as they specialize in diagnosing and treating disorders of the male reproductive system. While obstetrician-gynecologists are typically the first healthcare providers that evaluate couples for infertility, urologists play a key role in thoroughly assessing the male partner and identifying contributors to subfertility or

infertility specifically in men. A special emphasis on the United Kingdom (UK) was present in the analysis of the survey to gauge the understanding of and demand for sperm DNA damage screening in the UK.

3.3. Results

Between 13th October 2020 and 1st December 2021, a total of 117 urologists completed the survey. Demographic details are summarised in **Table 7**. One of the respondents was removed from the geographical location sub-analysis due to practicing in two different geographical regions. There is a subdivision of Europe to include the United Kingdom. The majority of participants were from Europe, followed by Asia and North America.

Table 7. Classification of survey participants according to geographical origin and practice settings.

Continent	n (N=117)	%
Asia	40	34
Europe	56	48
United Kingdom	24	21
North America	14	12
South America & Africa	6	5
Mixed continents	1	1
Type of hospital/institution	n (N=117)	%
Government-run/public health urology clinic	19	16
Private urology clinic	24	21
Government-run/public health andrology clinic	12	10
Private andrology clinic	14	12
Mixture of public and private practices	48	41

3.3.1. Section A: Use of semen analysis in your practice

The first questions asked why urologists consider a semen analysis to be important (see **Appendix Section**). The survey was limited to urologists as they specialise in the reproductive system and could provide richer insights on sperm tests, specifically, advanced sperm tests. Respondents were given a choice of 7 options (of which multiple could be selected): indicator of obstruction of the male reproductive tract, indicator of male accessory gland dysfunction, indicator of testicular dysfunction, indicator of infection/inflammation, indicator of immunological infertility, indicator of toxin exposure and indicator of genetic infertility. A free-text "other" option was also available. Indication of obstruction of the male reproductive tract and indicator of testicular dysfunction were most frequently selected by the survey respondents, followed by indicator of male accessory gland dysfunction and indicator of infection/inflammation (**Table 8**). In general, there was no major difference between geographic regions in the main reasons urologists refer patients for semen analysis, apart from (i) indicator of infection/inflammation and (ii) indicator of genetic infertility. Post hoc analysis (Tukey honestly significant difference test) revealed that urologists practicing in Asia and North America selected semen analysis as an indicator of infection/inflammation significantly less than the urologists practicing in South America & Africa. Urologists practicing in Asia and South America & Africa selected semen analysis as an indicator of genetic infertility significantly less than the urologists practicing in North America. For the free text options, two survey participants said that a semen analysis is also a marker for prostatitis and one survey participant wrote that a semen analysis is important for investigation of pretesticular and testicular causes of infertility.

Table 8. Significance of a semen analysis according to the respondents. Anova testing was used for statistical comparison between geographical regions.

Significance of semen analysis	Total N = 116	Asia N = 40	Europe N = 56		North America N = 14	South America & Africa N = 6	*p*
			All	United Kingdom N = 24			
Indicator of obstruction of the male reproductive tract	93% (108)	90% (36)	97% (54)	96% (23)	93% (13)	83% (5)	.49
Indicator of male accessory gland dysfunction	74% (86)	73% (29)	79% (44)	75% (18)	57% (8)	83% (5)	.40
Indicator of testicular dysfunction	92% (107)	93% (37)	95% (53)	95.8% (23)	86% (12)	83% (5)	.59
Indicator of infection/inflammation	71% (82)	58% (23)	80% (45)	75% (18)	57% (8)	100% (6)	.02
Indicator of immunological infertility	52% (60)	43% (17)	57% (32)	50% (12)	50% (7)	67% (4)	.47
Indicator of toxin exposure	28% (32)	30% (12)	23% (13)	21% (5)	29% (4)	50% (3)	.55
Indicator of genetic infertility	48% (56)	30% (12)	55% (31)	71% (17)	79% (11)	33% (2)	.01

The second question asked what the respondents consider relevant parameters in a semen analysis. For this question a four-point Likert scale was used ranging from “very relevant” to “not at all relevant”. Of the presented semen analysis parameters (motility, morphology, appearance, pH, volume, antibodies, leukocytes, round cells and vitality), motility followed by morphology, volume and vitality were selected as overall the most relevant parameters (**Table 9**). No parameters were selected as being not at all relevant. Respondents practicing in Europe and North America selected round cells (leukocytes or immature germ cells) to be slightly relevant parameters when conducting a semen analysis.

Table 9. Mean and standard error of Likert scale scores on different semen parameters across the different geographical areas. Chi-square tests were used for comparison between the different regions. Different colours represent different Likert scale parameters.

<1.4	Very Relevant	1.5 - 2.4	Moderately Relevant	2.5 - 3.4	Slightly Relevant	>3.5	Not at all Relevant

	Asia	Europe		North America	South America & Africa	All	*p*
		All	United Kingdom				
Motility	1.0 ± 0.4	1.3 ± 0.3	1.3 ± 0.4	1.0 ± 0.4	1.0 ± 0.4	1.1 ± 0.4	.6
Morphology*	1.3 ± 0.3	1.7 ± 0.3	1.7 ± 0.2	2.1 ± 0.1	1.0 ± 0.4	1.6 ± 0.2	**.02**
Appearance	2.1 ± 0.3	2.3 ± 0.4	2.4 ± 0.3	2.9 ± 0.6	2.0 ± 0.5	2.3 ± 0.3	.051
pH	1.7 ± 0.2	2.1 ± 0.4	2.0 ± 0.5	2.2 ± 0.6	1.7 ± 0.5	2.0 ± 0.3	.09
Volume**	1.5 ± 0.2	1.8 ± 0.2	1.6 ± 0.3	1.6 ± 0.5	1.0 ± 0.4	1.6 ± 0.2	**.04**
Antibodies***	1.9 ± 0.3	2.3 ± 0.3	2.3 ± 0.3	2.9 ± 0.5	2.0 ± 0.3	2.2 ± 0.3	**.008**
Leukocytes	1.8 ± 0.2	2.0 ± 0.3	2.1 ± 0.2	2.4 ± 0.4	1.3 ± 0.3	1.9 ± 0.2	.07
Round Cells	2.3 ± 0.4	2.5 ± 0.4	2.7 ± 0.3	2.7 ± 0.5	2.0 ± 0.5	2.4 ± 0.4	.27
Vitality	1.4 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	2.4 ± 0.3	1.0 ± 0.4	1.6 ± 0.1	**.037**

* South America & Africa was significantly different to Europe (All), United Kingdom and North America.

** South America & Africa was significantly different to Europe (All).

*** North America was significantly different to Asia and South America & Africa.

3.3.2. Section B: Types of follow up tests in your practice

When asked if the respondents refer any patients for additional follow-up tests, sperm DNA fragmentation testing was the most popular follow-up test, with approximately 35% of respondents making 26+ referrals per year for this test (**Table 10**). Sperm oxidative damage testing and sperm aneuploidy testing were equally offered by 21% of the urologists that commonly make 1-5 referrals per year of these tests. Sperm Aneuploidy testing is most popular in South America and Africa, followed by Europe. Semen oxidative stress testing is most popular in Asia while sperm DNA fragmentation is most popular in South America and Africa followed by North America.

Table 10. Referral frequency of advanced semen testing conducted by the urologists. Anova testing was used for statistical comparison between geographical regions.

		Asia (%)	Europe (%)		North America (%)	South America & Africa (%)	All	*p*
			All	United Kingdom				
Sperm Aneuploidy Testing	**Offer test**	**20.5**	**25.5**	**17.4**	**7.1**	**25**	**21**	.8
	1-5	25	53.8	75	0	0	38	
	6-10	12.5	15.4	25	0	50	17	
	11-25	37.5	7.7	0	0	50	21	
	26+	25	23.1	0	0	0	21	
Sperm DNA fragmentation testing	**Offer test**	**68.4**	**67.3**	**58.3**	**71.4**	**75**	**67**	.98
	1-5	23.1	31.4	53.6	11.1	16.7	23	
	6-10	26.9	11.4	14.3	11.1	0	16	
	11-25	23.1	25.7	21.4	33.3	16.7	25	
	26+	26.9	31.4	28.6	44.4	66.7	35	

Sperm oxidative damage testing	**Offer test**	**25.6**	**21.6**	**21.7**	**21.4**	**12.5**	**21**	.95
	1-5	40	63.6	60	0	100	46	
	6-10	30	0	0	0	0	13	
	11-25	0	9.1	0	66.7	0	13	
	26+	30	27.3	40	33.3	0	29	

When asked for the reasons as to why the urologists do not offer sperm aneuploid testing, 37% said that it is due to unfamiliarity to the test, 24% said that they would use the test if it is more readily available, 12% said they would use the test but it is not cost effective and 11% said that they are unaware that this test is available. When asked for the reasons as to why the urologists do not offer sperm DNA fragmentation testing, 26% said that it is due to unfamiliarity to the test, 26% said they would use the test if it were more readily available, 18% said the test is never useful/not medically informative and 16% said that they would use the test but it is not cost effective. When asked for the reasons as to why the urologists do not offer sperm oxidative damage testing, 31% said they are unfamiliar with the test, 31% said they would offer the test if it were more readily available, 14% said that the test is never useful/is not medically informative and 12% said they would use this test but it is not cost effective.

In the United Kingdom, when asked for the reasons as to why the urologists do not offer sperm aneuploid testing, 26% said due to unfamiliarity, 16% said they were unaware of the test being available and 16% said it is not appropriate for their patients. When asked

for the reasons as to why the urologists in the UK do not offer sperm DNA fragmentation testing, 30% said they would use it if the test was more readily available, and 20% said they would refer patients for sperm DNA fragmentation testing if it were more cost effective. When asked the reasons as to why the urologists did not offer sperm oxidative damage testing, 28% said they would if the test was more readily available, 22% claimed they were unfamiliar with the test and 17% said that that the test is never useful/is not medically informative.

3.3.3. **Section C: Which sperm test are most useful to you and why?**

When asked which types of DNA damage are most important to measure, a high proportion of respondents answered 'don`t know' for the different types of DNA damage presented in the survey: 35% for double strand breaks, 40% for single strand breaks, 44% for oxidised bases, 59% for adducts and 57% for abasic sites. The rest of the respondents stated that double stand breaks and single strand breaks are moderately relevant parameters to measure, while oxidised bases, adducts and abasic sites are only slightly relevant to measure (when looking at the total data) (**Table 11**). Double strand breaks were voted as the most significant DNA damage type to measure across all regions.

Table 11. Mean and standard error of Likert scale scores on the respondents perception of different types of sperm DNA damage across the different geographical areas.

	Asia	Europe		North America	South America & Africa	All	p
		All	United Kingdom				
Double Strand Breaks	1.8 ± 0.3	1.7 ± 0.3	1.9 ± 0.3	1.9 ± 0.4	1.5 ± 0.3	1.8 ± 0.2	.65
Single Strand Breaks	2.6 ± 0.4	2.0 ± 0.3	2.3 ± 0.2	2.4 ± 0.4	2.5 ± 0.3	2.3 ± 0.2	.44
Oxidised Bases	2.6 ± 0.4	2.3 ± 0.2	2.5 ± 0.3	2.8 ± 0.9	1.7 ± 0.5	2.5 ± 0.3	.08
Adducts	2.9 ± 0.7	2.8 ± 0.8	2.8 ± 0.9	3.3 ± 1.5	1.9 ± 0.7	2.9 ± 0.8	.57
Abasic sites	3.1 ± 0.8	3.0 ± 0.8	3.1 ± 1.0	3.0 ± 1.1	2.9 ± 1.0	3.0 ± 0.8	.95

<1.4	Very Relevant	1.5 - 2.4	Moderately Relevant	2.5 - 3.4	Slightly Relevant	>3.5	Not at all Relevant

When asked which sperm DNA damage assays the respondents use, the sperm chromatin structure assay was most popular, followed by TUNEL and sperm chromatin dispersion assay (**Figure 27**).

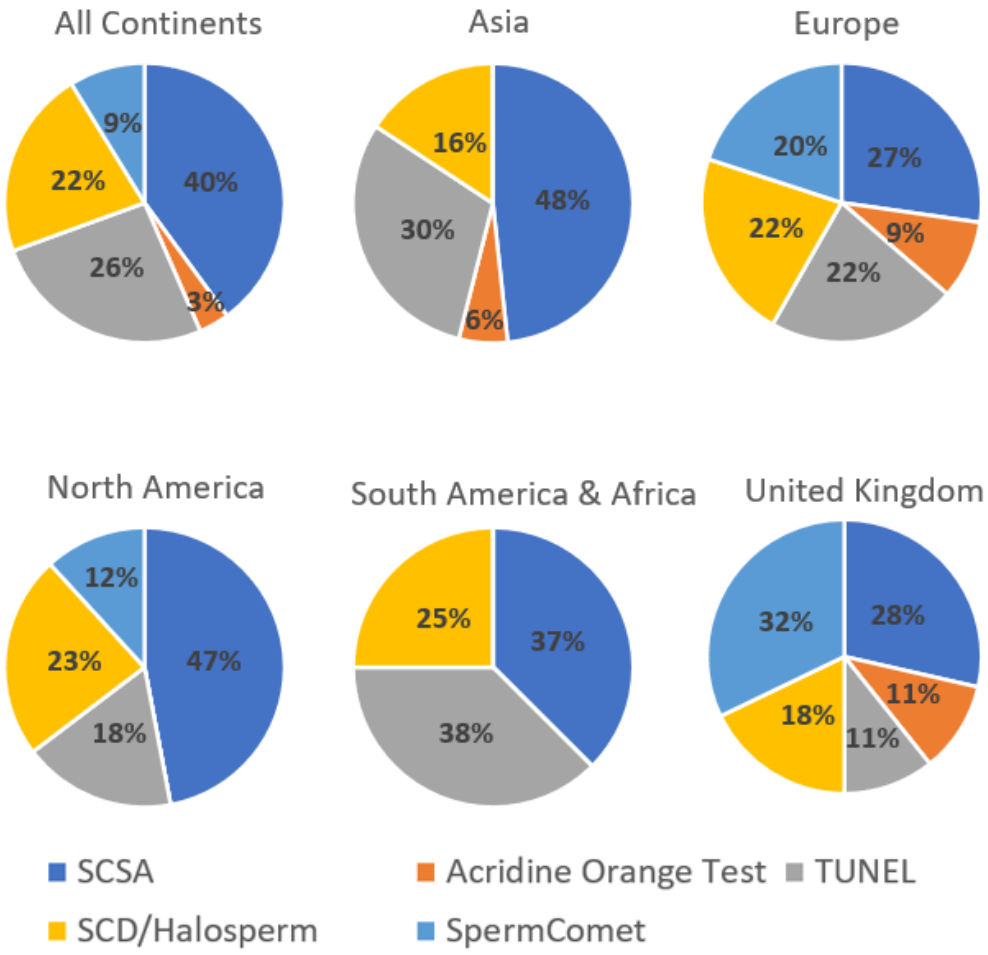


Figure 27. Pie charts depicting the percentage of the different sperm DNA damage tests conducted at the different geographical regions.

When asked if it will be useful to provide a "suite" of different DNA fragmentation tests, interestingly 59% said it will be useful. In the UK, 47.8% of respondents said it would be useful to provide a range of DNA fragmentation tests.

3.4. Discussion

To the best of our knowledge, this study is the first survey that focuses solely on the urologists perception and understanding of advanced sperm testing. Data from this survey provides a representative picture of the opinions and current practices of urologists across diverse global regions on their view of advanced semen analysis testing to diagnose and manage subfertility. Regarding the demographics of our survey respondents, 48% were from Europe, 34% from Asia, 12% from North America and 5% from South America and Africa. The survey respondents have reasonable representativeness globally in terms of types of hospital and institution. The survey was composed of a three-category classification incorporating questions on the use of semen analysis in the respondents practice, types of follow-up tests used and what the respondents recommend and use for analyzing sperm damage testing. The results demonstrate that majority of respondents had similar responses on what they consider relevant indicators and parameters of a semen analysis, independent of which geographical region they practice. The most popular advanced sperm testing technique offered by the participating urologists was SDF testing with the most popular techniques being SCSA and TUNEL. The main reasons given by the urologists for not offering advanced sperm testing were unfamiliarity to the tests, not readily available and not being cost effective. The results demonstrated that despite 67% of urologists offering sperm DNA damage testing, when asked what sperm DNA damage type is most important to measure, a high proportion of respondents were uncertain.

In general, when looking at what urologists practicing in different regions consider important indicators of a semen analysis, there was not a significant difference in the

answers given. A main discrepancy was that 79% of urologists practicing in North America consider that semen analysis may be an important indicator of genetic infertility, while this response was much less popular among the participants practicing in the other regions (ranging from 30% - 55%). A semen analysis of Klinefelter syndrome, balanced reciprocal translocation, Robertsonian translocation and structural abnormality of Y chromosome typically show moderate to severe abnormalities in semen analysis (Kuroda et al., 2020). With around 60% of carriers of autosomal translocations having at least one abnormal parameters in their semen analysis (Mayeur et al., 2019). However with reports of chromosomal abnormalities accounting for only around 5% of subfertility in males, this could be the reason that semen analysis is not being widely associated with genetic subfertility (O'Brien et al., 2010). The most relevant parameter in a semen analysis according to the respondents is sperm motility. Seminal volume was considered to be moderately relevant, which is interesting since over 90% of respondents stated that a semen analysis is an important indicator of obstruction of male reproductive tract and this commonly correlated with a low seminal volume (Meacham et al., 1993; Roberts & Jarvi, 2009).

When asked which types of follow-ups tests the respondents use, SDF testing was the most popular, being offered by 68% of the respondents. The test is most popular by the urologists practicing in North America, South America & Africa with 44% of the urologists offering SDF testing in North America making 26+ referrals per year. SDF testing was least popular in the UK, being offered by 58.3% of respondents. 30% of UK urologists that are not currently offering SDF testing stated that they would offer it if the test became more readily available while 20% stated that that the test is not cost effective. Further reasons UK urologists provided for not offering SDF testing include test

unfamiliarity (10%), test not being medically informative (20%), no consensus on SDF threshold (10%).

The most popular techniques for measuring sperm DNA damage were SCSA followed by TUNEL and SCD/Halosperm. Sperm aneuploid testing was most popular in Europe, however, the majority of respondents that offer it only refer 1-5 cases per year. Sperm oxidative damage testing is most popular in Asia with the majority of urologists that offer it making 1-5 referrals per year for this test. Unfamiliarity, cost, lack of testing availability and lack of awareness were the main reasons for the mentioned tests not being offered to the patients.

When asked what types of sperm DNA damage are most important to analyse, a high proportion of respondents answered 'don`t know'. Of those that answered, single and double strand breaks were commented as being moderately relevant to measure, while oxidised bases, adducts and abasic sites were voted as only being slightly relevant parameters to measure (when looking at the total replies).

A separate study revealed that SDF was the most commonly considered test for couples opting for ART treatment, with SDF being offered by 79.6% (39/49 respondents) by the fertility specialists (Majzoub et al., 2017). A high majority of the respondents in the study conducted by Majzoub et al. considered SDF as an important tool in understanding the cause of IUI, IVF and ICSI failure and in understanding the cause of recurrent pregnancy loss. The majority of the participants in the survey acknowledged the value of SDF testing for couples undergoing ART and this could well be due to the compelling evidence extracted from systemic reviews and meta-analysis suggesting that SDF has a significant impact on the ART success rate and its significant correlation with pregnancy

loss (Ribas-Maynou et al., 2021; Robinson et al., 2012; Zhao et al., 2014; Zini & Sigman, 2009). With the emerging evidence on the links between sperm DNA damage and ART outcomes, the sixth edition of the WHO Manual for the Laboratory Examination and Processing of Human Semen has included SDF testing as an extended examination in the guide to the clinical characterisation of fertile or infertile men (World Health Organization, 2021).

However, despite many benefits of testing for sperm DNA damage, there are still limitations when it comes to applying it routinely in a clinical setting (Mehta, 2017). As sperm DNA damage testing are considered add-ons to the routine semen analysis offered by fertility specialists, patients would incur extra costs for such tests. One way of reducing the costs in clinics is to do in house sperm DNA damage testing, this could allow for consistent and cost-effective methods to screen couples. Another limitation is that the absence of a gold standard for measuring sperm DNA damage levels and the optimal thresholds for the available SDF tests have yet to be determined. However, there is reliable evidence to suggest that the common tests for measuring SDF – SCSA, Comet, SCD and TUNEL provide reliable information on sperm DNA integrity that can guide patient treatment pathway.

Furthermore, 59% of the respondents said that it will be useful to provide a “suite” of different DNA fragmentation tests. The different SDF tests available have different mechanisms for measuring sperm DNA damage levels and therefore the type of DNA damage as measured by each assay may be complementary to each other.

Although the exact causes of the overall high sperm DNA damage levels in infertile men relative to fertile men are not fully understood, existing evidence suggests that oxidative

stress plays a crucial role in SDF-related infertility. There are several laboratory tests available for measuring seminal oxidative stress levels, with prominent methods being: (1) a luminol-based chemiluminescence assay for measurement of reactive oxygen species and (2) oxidation-reduction potential measurement using the MiOXSYS System (**Chapter 4**). Antioxidant therapy, varicocele repair and lifestyle recommendations could help reduce the levels of oxidative stress induced sperm DNA damage and improve the chances of reproductive success.

While this study focused on surveying urologists, future work could benefit from also including obstetricians and gynecologists who specialize in fertility. As first-line users of diagnostic testing, obstetricians and gynecologists could provide valuable clinical insights into the utility and interpretation of advanced sperm tests for understanding male factor infertility contributions. Widening the survey pool to incorporate obstetricians and gynecologists perspectives along with urologists may enable a more comprehensive analysis of how advanced male fertility testing is viewed across the clinical fertility field. Additionally, comparing responses between specialties could reveal interesting similarities or differences in knowledge, beliefs, and practices related to advanced sperm tests. Furthermore, given the significant influence of private testing companies and the commercial aspects of private medicine, it is essential to critically evaluate the role of advanced sperm testing in couple management. Further research could explore the division of subjects into private versus publicly owned institutions, shedding light on disparities in access, cost, and the overall impact of advanced male fertility testing on patient care and fertility outcomes. This consideration raises important questions about the equity and effectiveness of these tests within different healthcare systems and settings.

3.5. Conclusion

In summary, this study sheds light on urologists' perspectives on advanced sperm testing, highlighting areas of consensus and variation in different regions. The results emphasize the importance of further research, standardization, and cost-effective approaches to incorporate advanced sperm testing into routine clinical practice. Offering a suite of complementary DNA fragmentation tests can provide valuable insights into sperm quality and guide treatment decisions for individuals experiencing subfertility.

4. Chapter 4: Analysis of two assays for measuring seminal oxidative stress and their combinatory relationship with semen parameters and sperm DNA damage

4.1. Introduction

Infertility is defined as the failure to achieve pregnancy following 12 months of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2017). It can be caused by female factors, male factors or a combination of both; therefore, a thorough investigation and management of both partners is necessary. Infertility impacts at least 48 million couples worldwide (Health, 2022) and male related factors account for approximately 40% - 50% of these cases (Kumar & Singh, 2015). The main causes of male infertility include endocrine imbalance, genetic abnormalities, urogenital tract infection, varicocele and immunological conditions (Punab et al., 2017). Male factor subfertility has been widely recognised to influence assisted reproduction technology (ART) outcome; however, the male is still often overlooked during evaluation and treatment of subfertility. Typical diagnosis of male factor infertility relies on a standard semen analysis test (Nallella et al., 2006) with no further investigation, and this is a minimum contributor for assessing male related subfertility relative to female pathology assessment (Krausz et al., 2015). This results in a significant number of men being labelled as having unexplained or idiopathic infertility. Males presenting with idiopathic infertility are challenging to diagnose and manage since the root cause of abnormal semen parameters is often not identified. Unexplained infertility is even more challenging as semen parameters in this group of individuals are normal. Many studies

indicate that seminal oxidative stress could be a factor in idiopathic and unexplained male infertility (Aktan et al., 2013; Mannucci et al., 2022; Saleh & Agarwal, 2002; Tremellen, 2008).

Seminal oxidative stress arises when there is an imbalance between free radicals and antioxidants in semen. Free radicals are atoms or molecules that have unpaired electrons in their outer orbit making them chemically highly reactive and unstable. Production of free radicals are controlled by antioxidants within the system. Oxygen derivatives of free radicals are called reactive oxygen species (ROS) and are generated as natural by-products of cellular activity in the mitochondria where about 1-5% of the consumed oxygen is converted into ROS (Boveris & Chance, 1973; Chance et al., 1979). In addition, ROS can derive from exogenous sources including pollution, smoking and drugs (Phaniendra et al., 2015). In the event of ROS reacting with lipids that have numerous conjugated double-bonds, a process called lipid peroxidation (LPO) is initiated. Ultimately, this leads to significant membrane damages with decreased membrane fluidity and functionality (Riffo & Parraga, 1996; Sikka et al., 1995). LPO is propagated by a so-called 'radical chain reaction', in which an initial free radical will react with a double-bond in a lipid molecule to form a new lipid peroxide radical, which can in turn react with neighbouring double-bonds, continuing the process. Consequently, LPO can oxidise about 60% of the unsaturated fatty acids in the membrane resulting in significant cellular injury (Kothari et al., 2010; Sies, 1993).

Due to the extraordinary high amount of polyunsaturated fatty acids in sperm plasma membranes, the highly reduced cytoplasmic content, and consequently the low levels of cytoplasmic antioxidant enzymes available to detoxify ROS within the cell, sperm are

highly susceptible to damage by ROS (Henkel, 2011; Parks & Lynch, 1992). The high levels of polyunsaturated fatty acids in the sperm plasma membrane renders the lipid bilayer a likely target for ROS-mediated damage affecting membrane fluidity and interfering with the acrosome reaction and sperm-oocyte binding (Collodel et al., 2020). Oxidative damage has also been associated with reduced progressive sperm motility due to its effect on mitochondrial function (Saleh and Agarwal 2002; Aitken 1995).

While membrane damage can be caused by both intracellular and extracellular ROS, intracellular ROS can also cause damage to intracellular proteins and to DNA. DNA damage by ROS arises due to the susceptibility of the aromatic nucleobases to lose an electron (Kanvah et al., 2010). This one electron oxidation forms a radical cation, whose charge can migrate through the double-helix, resulting in irreversible oxidative damage at the site of cation acceptance (Peluso et al., 2019).

Spermatozoa do not have a base excision repair system post-spermiogenesis, resulting in an inability to repair DNA (González-Marín et al., 2012). However, sperm carrying DNA damage can still fertilise the oocyte with some DNA damage repair happening in the fertilised oocyte and during early embryonic development (González-Marín et al., 2012; Henkel et al., 2004; Musson et al., 2022). Ideally, the oocyte repairs the DNA damage prior to the first cleavage to improve chances of reproductive success (Genescà et al., 1992). If the sperm DNA damage exceeds the capability of the oocyte repair mechanism, abnormal embryo development, implantation failure or natural abortion, miscarriage (Ahmadi & Ng, 1999; Robinson et al., 2012) and increased risk of transgenerational genomic instability in the offspring can occur (Adiga et al., 2010).

The role of antioxidants is to counteract oxidative stress by scavenging excess oxidants and neutralising them to restore redox homeostasis (**Figure 28**.**a**) (He et al., 2017). Antioxidants can be endogenous in source such as superoxide dismutase and catalase or exogenous such as flavonoids, vitamins, and minerals. Although antioxidants are essential to maintain redox homeostasis, excessively high levels can result in reductive stress, a condition which is as harmful as oxidative stress (Bouayed and Bohn 2010; Lipinsky, 2002; Castagne et al., 1999; Henkel et al., 2019; Symeonidis et al., 2021).

Intracellular and extracellular ROS can be measured with a chemiluminescence method such the membrane-permeant luminol (5-Amino-2,3-dihydrophthalazine-1,4-dione) as a probe (Agarwal, Gupta, et al., 2016). Hydrogen peroxide oxidises luminol, in a dose-dependent manner, to produce the highly unstable molecule 3-aminophtalate, that upon returning to ground state (**Figure 28.b**), emits light at 425 nm can be detected by a luminometer (measured as relative light units/s/10^6 sperm) (Bedouhène et al., 2017; Benjamin et al., 2012).

A novel method of measuring oxidative stress is by determining the oxidation-reduction potential (ORP) as a measure of the relationship between oxidants and reductants (**Figure 28.c)** using the MiOXSYS analyser. Higher ORP values, present an imbalance in the activity of oxidants to antioxidants (Agarwal, Sharma, et al., 2016).

4.2. Aims

Both the ROS and ORP measurements are valid techniques for measuring oxidative stress levels, however, they are based on different principles. The objectives of this study were as follows: (a) to elucidate the correlation between oxidation-reduction potential (ORP) and reactive oxygen species (ROS), (b) to compare semen analysis

parameters across various combinations of ORP and ROS, and (c) to assess sperm DNA damage levels in relation to different combinations of ORP and ROS.

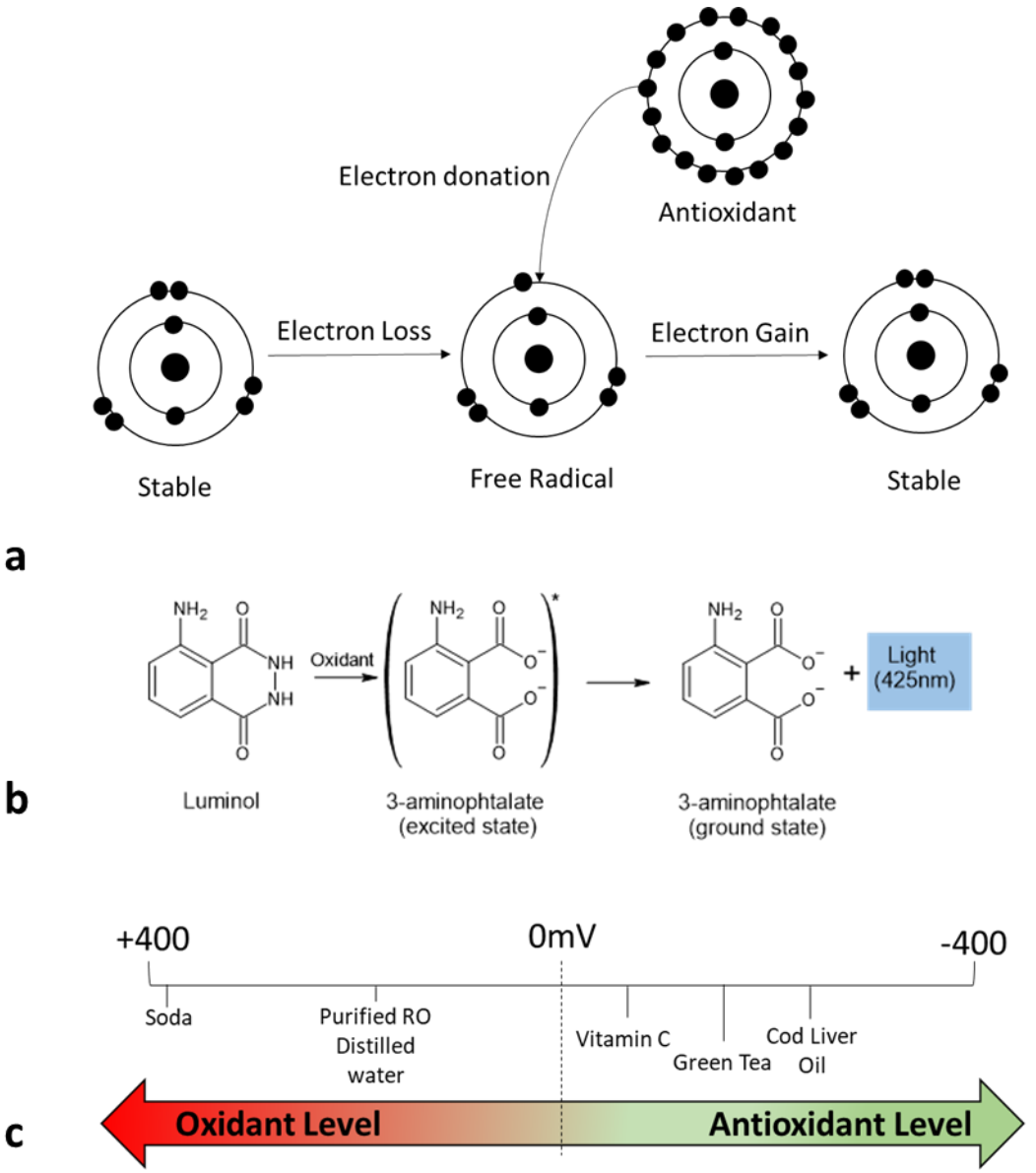


Figure 28. Illustration showing (a) the generation of a free radical from a stable state, and the return state of the stable state through electron donation from an antioxidant, b) the chemiluminescence observed through the oxidation of luminol by oxidants and c) an oxidation-reduction potential scale.

4.3. Results

A total of 1278 patients were included in this study, of which ROS was tested in 1255, ORP in 940 and SDF in 178 patients. Summary results are shown in **Table 12**.

Table 12. Summary statistics table.

	N	Minimum	Maximum	Mean	Median	SEM
DNA fragmentation testing (%)	178	4.00	57.00	16.62	13.50	0.80
Age (years)	1243	25.00	70.00	38.52	38.00	0.16
High DNA stainability	98	2.00	36.00	10.60	8.50	0.72
LeucoScreen	629	0.00	99.00	20.05	8.00	1.04
Leukocytes PMNL	220	0.00	30.00	1.07	0.20	0.20
ORP (mV)	913	-23.20	493.90	34.03	28.20	1.15
ORP (mV/10^6 sperm/mL)	1062	-5.68	153.29	3.64	0.90	0.33
% Normal Morphology	680	0.00	15.00	2.73	2.00	0.10
pH	681	6.40	10.00	8.46	8.40	0.01
Progressive Motility (%)	695	0.00	88.00	50.60	56.00	0.70
Round Cells (%)	1042	0.00	32.40	0.70	0.24	0.06
Sperm Concentration (10^6 sperm/mL)	1243	1.00	515.00	47.55	33.00	1.49
Total motility (%)	695	0.00	90.00	57.65	63.00	0.68
Vitality (%)	677	3.00	94.00	66.06	67.00	0.45

4.3.1. Correlation between ORP and ROS

The results show a significant correlation between ROS and ORP (r_s=0.33, R^2=0.148, p<0.001) (**Figure 29**).

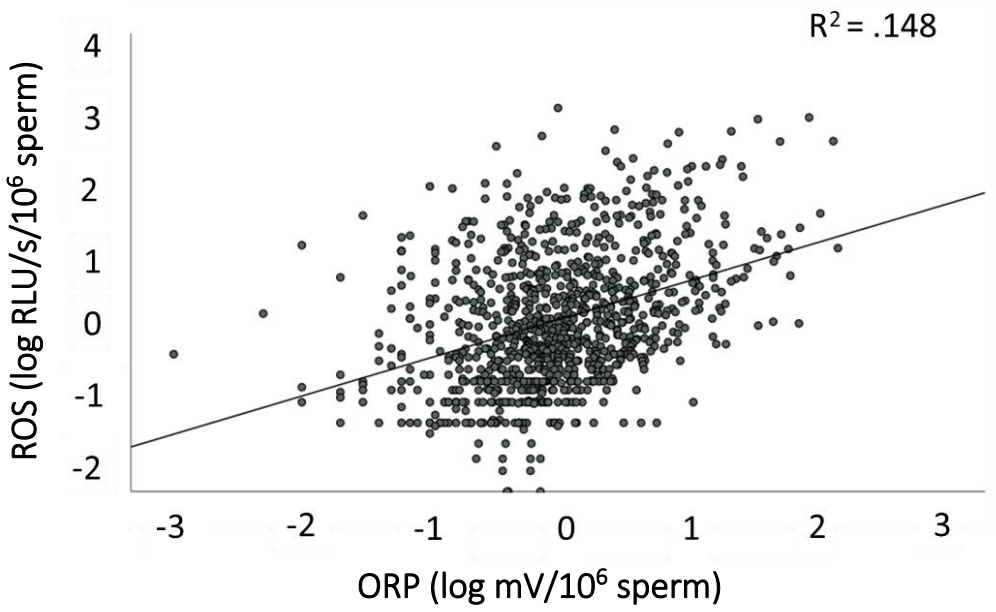
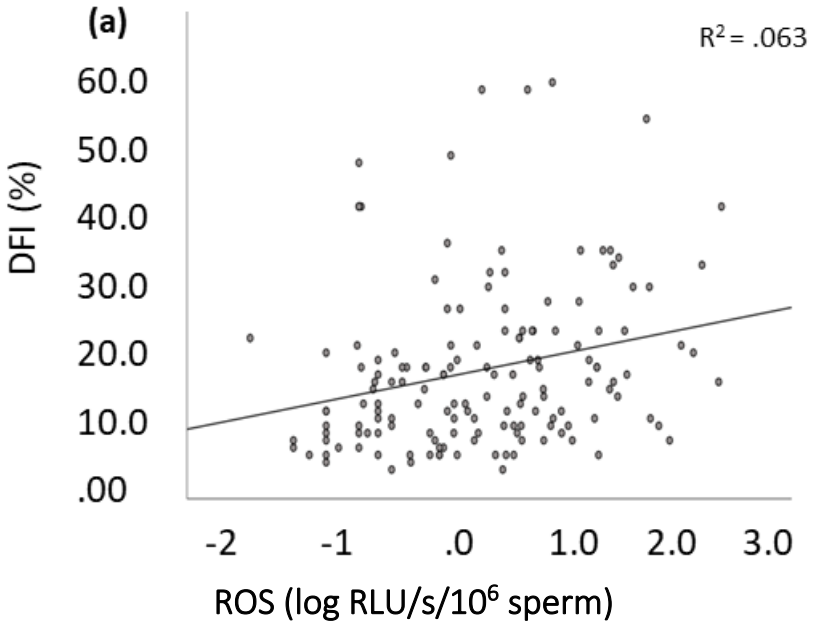
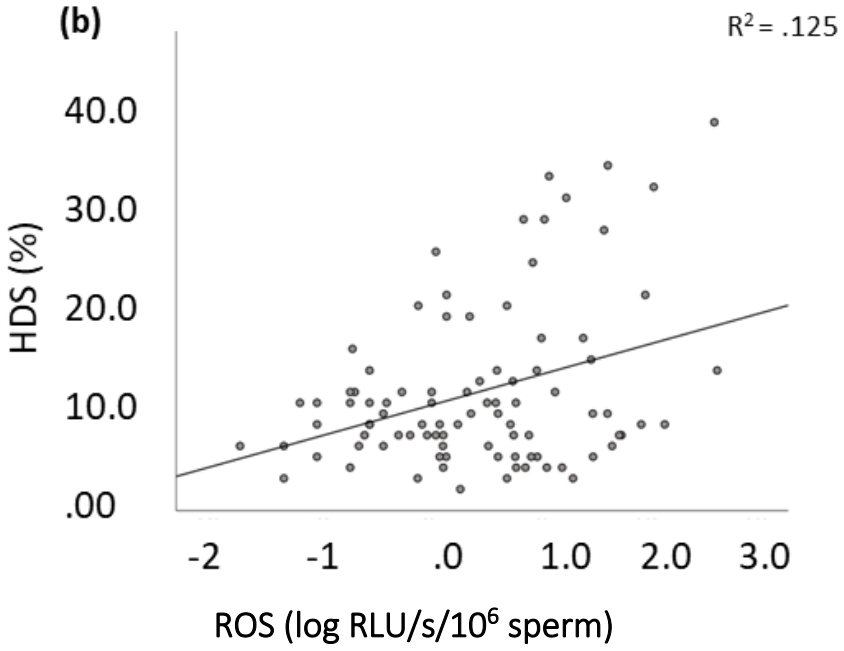


Figure 29. Scatter plot corresponding to the correlation between ROS (RLU/s/10^6 sperm) and ORP (mV/10^6 sperm) (in logarithmic scale).

4.3.2. Correlation between sperm DNA damage and ORP/ROS

The results show a weak but significant correlation between ROS and DFI (r_s=0.26, R^2=0.063, p=0.001), ROS and HDS (r_s=0.22, R^2=0.125, p=0.036) and DFI and ORP (r_s=0.27, R^2=0.101, p=<0.001). There is also a significant correlation between HDS and ORP (r_s=0.43, R^2=0.231, p=<0.001).


(a)
R² = .063
60.0
50.0
40.0
30.0
20.0
10.0
.00
DFI (%)
-2
-1
.0
1.0
2.0
3.0
ROS (log RLU/s/10⁶ sperm)


(b)
R² = .125
40.0
30.0
20.0
10.0
.00
HDS (%)
-2
-1
.0
1.0
2.0
3.0
ROS (log RLU/s/10⁶ sperm)

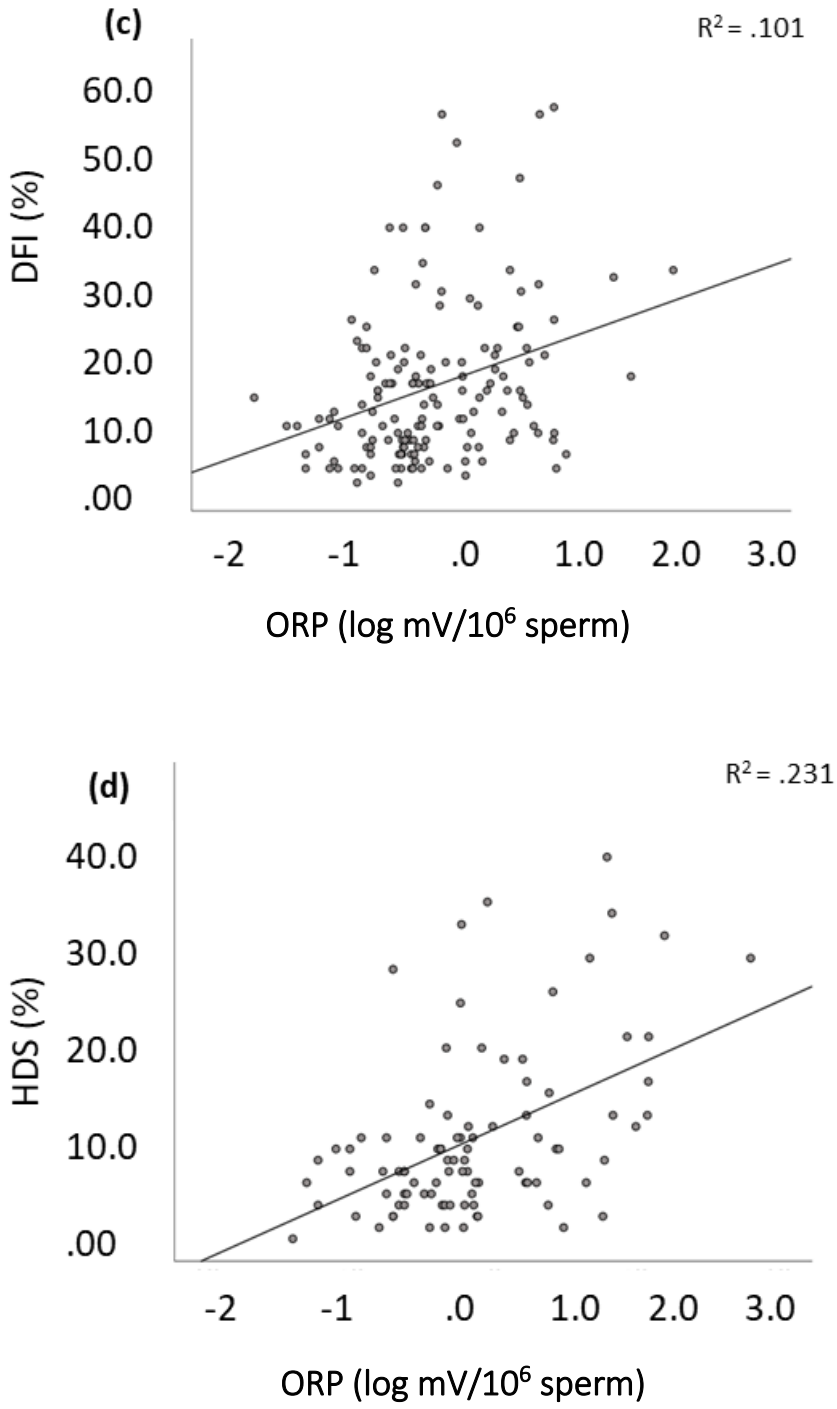


Figure 30. Scatter plots showing the linear relationships between (a) reactive oxygen species (ROS - RLU/s/10^6 sperm) and sperm DNA fragmentation index (% DFI), (b) reactive oxygen species (ROS - RLU/s/10^6 sperm) and immature spermatozoa (%HDS), (c) oxidation reduction potential (ORP – mV/10^6 sperm) and sperm DNA fragmentation index (% DFI), (d) oxidation reduction potential (ORP – mV/10^6 sperm) and immature spermatozoa (%HDS). ROS and ORP measurements are presented in logarithmic scale.

4.3.3. ORP and ROS measurements for normal semen parameters

The ROS and ORP assays were both validated and verified in-house at The Doctors Laboratory, London. The reference ranges determined by ROC analysis were ≤13.8 RLU/sec/10^6 sperm/mL (86% sensitivity; 86% specificity) for ROS and ≤1.4 mV/10^6 sperm/mL (76.4% sensitivity; 75.9% specificity) for ORP (Homa et al., 2019). 11.9% normozoospermic patients (patients presenting with normal sperm analysis result) had high ROS levels, while 15.2% normozoospermic patients had high ORP levels. This indicates that despite these patients having normal semen analysis results, they could still present with high oxidative stress measurements. Majority of patients (82.8%) that were oligozoospermic (patients presenting with a low sperm count) had high ORP levels, while 42.6% of oligozoospermic patients had high ROS levels. Almost half (45.4%) of oligozoospermic patients had high ORP but low ROS levels. The majority of asthenozoospermic patients (patients presenting with sperm having low motility) had high ORP (70.7%), while 38% had high ROS. 39.1% of asthenozoospermic patients had high ORP but low ROS measurements.

Table 13. % of normozoospermic, oligozoospermic and asthenozoospermic having high ROS (independent of ORP measurements) and high ORP (independent of ROS measurements).

	Normozoospermia (n=151)	**Oligozoospermia (n=163)**	**Asthenozoospermia (n=92)**	***p***
High ROS*	11.9% (18/151)	43.6% (71/163)	38.0% (35/92)	<.001
High ORP**	15.2% (23/151)	82.8% (135/163)	70.7% (65/92)	<.001

n is the number of patients.
*Significant difference between normo and oligo and between normo and astheno
**Significant difference between normo and oligo and between normo and astheno

4.3.4. Comparison of groups that fall above/below accepted thresholds for ORP and ROS

Samples were allocated into different groups according to whether they fell within or outside acceptable threshold limits (ORP: ≤1.4 mV/10^6 sperm/mL; ROS: ≤13.8 RLU/sec/10^6 sperm/mL). Samples were grouped as follows: group 1 (low ORP and low ROS); group 2 (low ORP and high ROS); group 3 (high ORP and low ROS) and group 4 (high ORP and high ROS). **Table 14** shows that DFI and HDS levels are significantly higher in group 3 and 4 compared to group 1. There was no statistically significant difference in (p=.796) age across groups: group 1: 39.1 ± 0.7, group 2: 39.4 ± 1.5, group 3: 38.1 ± 1.1; group 4: 41.4 ± 2.7.

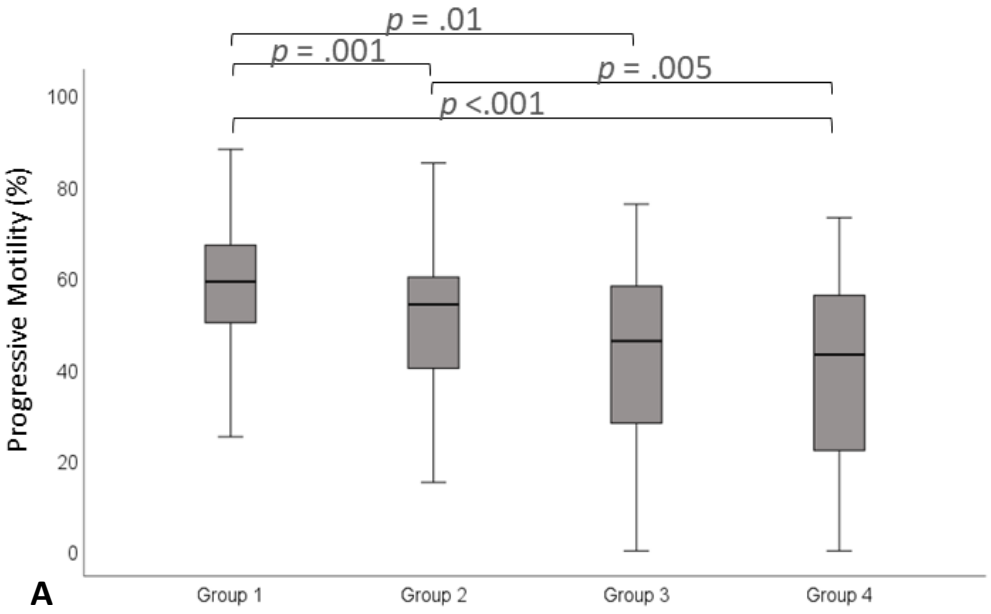
Table 14. Sperm DNA fragmentation (DFI) and high DNA stainability (HDS) for the different groups: group 1 (low ORP and low ROS); group 2 (low ORP and high ROS); group 3 (high ORP and low ROS) and group 4 (high ORP and high ROS).

	Group 1	Group 2	Group 3	Group 4	*p*
ROS (RLU/sec/10^6 sperm/mL)*	2.1 ± 0.1 (n=522)	126.3 ± 36.9 (n=114)	3.6 ± 0.2 (n=246)	236.6 ± 39.4 (n=135)	<.001
ORP (mV/10^6 sperm/mL) **	0.5 ± 0.02 (n=522)	0.4 ± 0.1 (n=114)	5.4 ± 0.5 (n=246)	14.4 ± 2.1 (n=135)	<.001
DFI (%)***	15.2 ± 1.1 (n=85)	19.5 ± 2.2 (n=19)	20.4 ± 2.1 (n=37)	23.1 ± 3.2 (n=15)	.002
HDS (%)***	8.2 ± 0.6 (n=53)	12.6 ± 2.6 (n=14)	13.7 ± 1.7 (n=22)	19.3 ± 4.7 (n=6)	.003

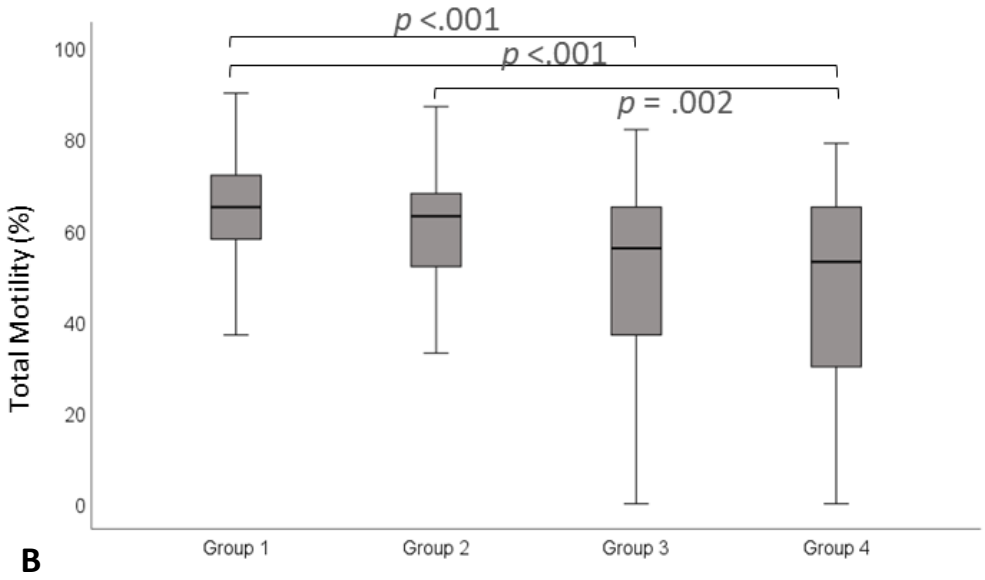
N is the number of patients, ± is standard error of mean.
* All ROS groups were significantly different to each other, apart from groups 2 and 4.
** All ORP groups are significantly different to each other, apart from groups 1 and 2 and groups 3 and 4.
*** Significant difference between group 1 and 3, and group 1 and 4 as assessed by Kruskal Wallis test.

4.3.5. Comparison of semen analysis parameters with different combinations of ROS and ORP

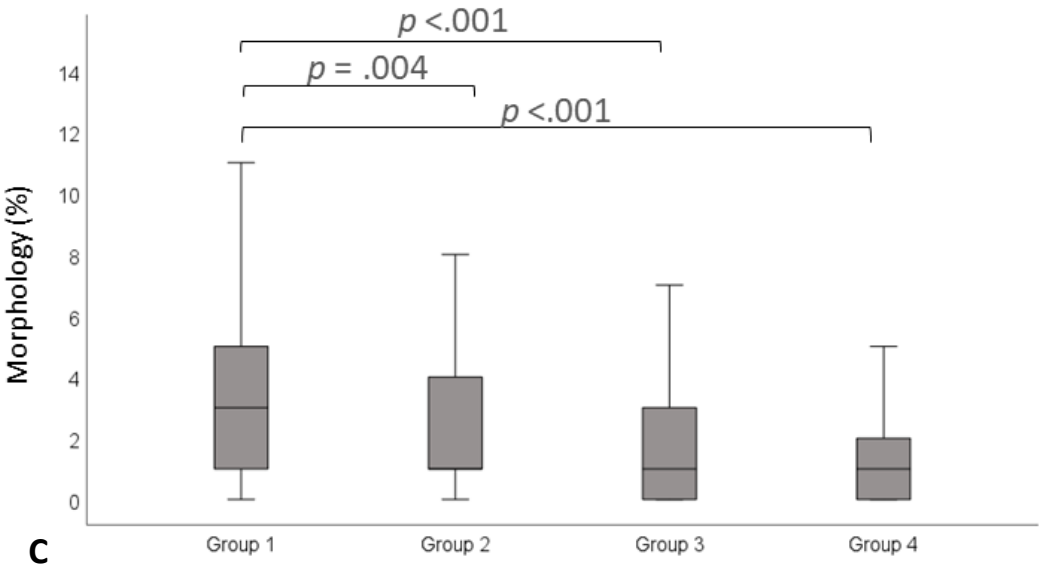
Results in **Figure 31** indicate that group 1 had the most overall best semen analysis parameters, while group 4 had the least optimal parameters. There was no overall significant difference in parameters between groups 2 and 3, however, group 3 had overall poorer semen analysis results compared to group 2. Interestingly, groups 3 and 4 had a significantly lower sperm concentration compared to groups 1 and 2. Leukocytospermia prevalence varied greatly between the groups: Group 1 - 0.4%; Group 2 – 16.7%; Group 3 – 0% and Group 4 – 11.9%, $p<.001$, having a significant difference between all the groups apart from between groups 2 and 4 and groups 1 and 3. Leukocyte counts were the highest in group 2, followed by group 4, indicating that high ROS, independent of ORP levels, could be markers for leukocytospermia.


p = .01
p = .001
p = .005
p <.001
Progressive Motility (%)
100
80
60
40
20
0
Group 1
Group 2
Group 3
Group 4

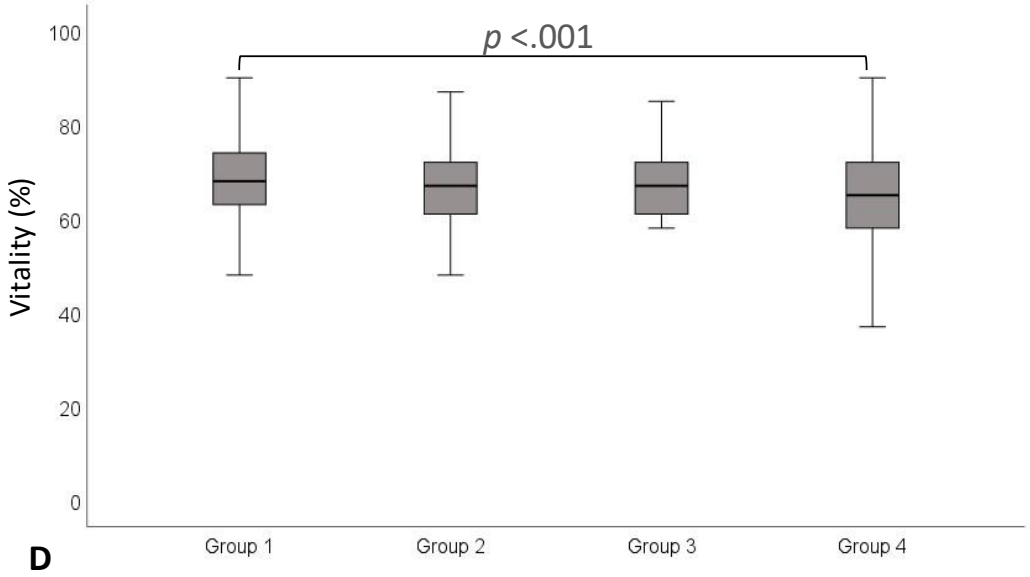
A


p <.001
p <.001
p = .002
Total Motility (%)
100
80
60
40
20
0
Group 1
Group 2
Group 3
Group 4

B


p <.001
p = .004
p <.001
Morphology (%)
14
12
10
8
6
4
2
0
Group 1
Group 2
Group 3
Group 4

C


p <.001
Vitality (%)
100
80
60
40
20
0
Group 1
Group 2
Group 3
Group 4

D

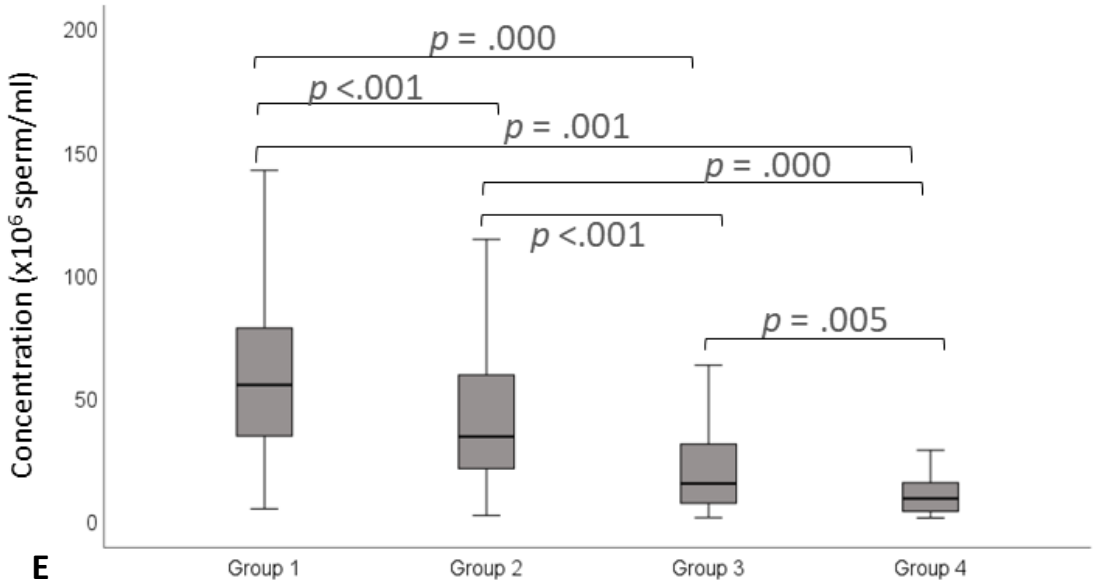


Figure 31. Box plots showing the variation of (A) progressive motility, (B) total motility, (C) morphology, (D) vitality and (E) sperm concentration for the different groups: group 1 (low ORP and low ROS); group 2 (low ORP and high ROS); group 3 (high ORP and low ROS) and group 4 (high ORP and high ROS). Box plots show the minimum, 25th, median, 75th, and maximum values. Differences between groups were investigated by the Mann-Whitney test. Significance was considered as *p*-value < 0.05.

4.4. Discussion

Seminal oxidative stress refers to an imbalance between the production of reactive oxygen species (ROS) and antioxidant levels in the semen. The balance between ROS and antioxidants can be analysed by taking oxidative-reductive potential (ORP) measurements. Several studies have analysed the correlations between seminal ROS levels and sperm parameters and between seminal ORP levels and sperm parameters (Agarwal et al., 2016; Sikka et al., 1995; Tremellen, 2008; Vessey et al., 2014). However, this is the first study to analyse how combinatory measurements of seminal ORP and seminal ROS correlate to different sperm parameters. There was a significant correlation between ORP and ROS, however, high ORP levels do not necessarily entail high ROS levels. Interestingly, in our study, 24% of the semen samples had high ORP but low ROS levels while 11% of the semen samples had low ORP but high ROS levels. Our retrospective study has demonstrated that semen samples having high levels of ORP and/or ROS levels had significantly lower sperm motility (progressive and total motility), sperm concentration and morphologically normal spermatozoa. Additionally, high levels of ORP and/or ROS were associated with increased levels of sperm DNA damage. Leukocytospermia levels were correlated to seminal ROS levels and not seminal ORP levels. Interestingly, there was a relatively high incidence rate of 16.7% of leukocytospermia in semen samples having high ROS and low ORP seminal levels, whereas there was a 0% leukocytospermia incidence rate when patients presented with low ROS and high ORP seminal levels.

Simultaneous testing of ORP and ROS levels allows different aspects of the oxidising and reducing environment to be tested. The ORP assay allows for the measurement of the

balance between oxidising and reducing agents (Agarwal, Sharma, et al., 2016) while the chemiluminescence assay allows for the measurement of total extracellular and intracellular ROS (Agarwal et al., 2006). A low ORP indicates a low oxidising environment which can help protect against oxidative damage and is therefore generally considered more beneficial for overall sperm quality, as demonstrated by a number of studies (Agarwal, Roychoudhury, et al., 2017; Agarwal, Sharma, et al., 2016). However, a low ORP value do not necessarily indicate low ROS levels. Our study showed that 11% (n=114) of semen samples had low ORP levels but high overall ROS levels. This could result from the presence of high levels of intracellular ROS as detected by the chemiluminescence assay that might not be detected by the MiOXYS® assays. Sources of elevated intracellular ROS include morphologically abnormal spermatozoa that have undergone impaired spermatogenesis, potentially resulting in incomplete removal of excess residual cytoplasm (Aitken & West, 1990; Gil-Guzman et al., 2001). Additionally, the presence of activated leukocytes will hold a high concentration of intracellular ROS (Homa et al., 2015; Li et al., 2020). The ROS produced by the leukocytes might trigger an antioxidant cascade to counterbalance the exogenous ROS, while maintaining a relatively high concentration of intracellular ROS (Gorrini et al., 2013). Therefore, this could explain why ORP levels does not correlate with leukocyte levels, as has been previously observed (Arafa et al., 2020), while ROS levels, as has been shown in a number of studies, are highly correlated to leukocyte levels (Aziz et al., 2004; Henkel et al., 2005; Homa et al., 2015). Consistent with this hypothesis, in our study the group having low ORP and high ROS levels had the highest incidence rate of leukocytospermia at 16.7%.

A high ORP value indicates a relatively high oxidising environment. This could be indicative of low levels of antioxidants and/or high levels of oxidising agents. 24% of the semen samples in this study had high ORP but low ROS levels. Interestingly, there was an observed lower overall sperm quality for the sperm exposed to a high ORP and low ROS environment compared to sperm exposed to a low ORP but high ROS environment, although both environments had a negative influence on overall sperm quality.

12% of patients that had normozoospermia had high ROS while 15% of normozoospermic patients had high ORP. Therefore, this suggests that patients diagnosed as having unexplained infertility, could have high oxidative stress levels, that will not be diagnosed unless these patients conduct seminal oxidative stress testing. This would help patient treatment pathway since these patients can then benefit from lifestyle changes to help reduce oxidative stress testing.

One major limitation of this study is that ORP and ROS were determined as being either high or low based thresholds established in previous literature (Agarwal et al., 2015, 2019). Modifying these thresholds levels will inherently change the results. Furthermore, it should be noted that the majority of published studies using MiOXSYS, the method employed for ORP assessment, prominently involve Professor Ashok Agarwal, who has affiliations with the company. This situation may potentially introduce a conflict of interest.

Measuring seminal oxidative stress using both ROS and ORP techniques can provide valuable information about the male fertility potential and the underlying mechanisms of infertility. Lifestyles factors including diet, smoking and environmental exposure to certain toxins can influence the seminal oxidative status. Therefore, upon understanding

the underlying cause of the male infertility, can lead to appropriate interventions aimed at reducing oxidative stress levels (if high) through dietary changes and antioxidant supplementation. Using the appropriate intervention could improve the potential strategy for improving sperm quality and fertility outcomes.

4.5. Conclusion

In conclusion, measuring seminal oxidative stress using both ROS and ORP techniques can provide valuable information about male fertility potential and the underlying mechanisms of infertility. Simultaneous testing of ORP and ROS levels allows for the assessment of different aspects of the oxidizing and reducing environment. Understanding the underlying cause of male infertility and implementing appropriate interventions to reduce oxidative stress levels, such as dietary changes and antioxidant supplementation, can improve sperm quality and fertility outcomes.

5. Chapter 5. Validation experiments for onsite sperm DNA damage testing (Acridine Orange based Flow-cytometric Testing – AOFT)

5.1. Introduction

Male infertility affects around 7% of all men and is defined as the male`s inability to make a fertile female pregnant after 12 months of unprotected intercourse (Krausz, 2011). Male fertility status is typically assessed using a standard semen analysis involving analysis of sperm concentration, motility, morphology, volume, and pH. The conventional semen analysis is an essential tool for the initial investigation of the male fertility status; however, it is not a test of fertility (Vasan, 2011). It does not provide information on the fertilizing capacity of a spermatozoon or information on the genetic integrity of the sperm. This results in a significant proportion of male infertility cases to remain unexplained, upon conventional assessment methods, leading to the perception that there is no cure for male factor infertility (Turner et al., 2020). Additionally, with the success and availability of ART and improved reproductive outcomes in cases of male infertility, has provided the idea that a thorough male evaluation is not necessary, reducing the male partner to a mere “sperm provider”, which disproportionately burdens women (Mehta et al., 2016). Despite the male partner still being often overlooked, research has very often shown that male factor infertility does have an adverse influence on ART outcomes (Nangia et al., 2011; Zheng et al., 2018; Zini et al., 2008).

With increased research showing that the levels of sperm DNA damage has an influence on ART outcomes including embryonic development and offspring health (Simon et al., 2014; Zheng et al., 2018; Zini & Libman, 2006), sperm DNA fragmentation (SDF) testing has emerged as an important marker for assessing male infertility status. High sperm DNA damage has been found to be associated with lower pregnancy rates following IUI (Bungum et al., 2004, 2007; Chen et al., 2019; Duran et al., 2002). Therefore, with IUI being often the first-line treatment option for many infertile couples, unknown presence of high seminal DNA fragmentation can reduce the chances of a successful outcome following fertility treatment. Additionally, increased levels of sperm DNA damage has also been indicated to influence IVF outcome, with lower fertilization rates, implantation rates and clinical pregnancy rates reported (Ribas-Maynou et al., 2021; Zhu et al., 2022).

There are several assays for measuring sperm DNA damage, and very often the term DNA fragmentation is used to refer to any type of chromatin damage including single and double-strand breaks. A widely used test is the sperm chromatin structure assay (SCSA) which is based on acid denaturation of the sperm DNA at the sites of single and double DNA strand breaks (Evenson, 2013). Another popular technique is the TUNEL assay which uses the terminal deoxynucleotidyl transferase enzyme to label the 3' free ends of the DNA (Sharma et al., 2010). Both techniques allow for sperm DNA damage levels to be measured using flow cytometers and thus by passing the use of more operator dependent microscopy techniques such as the Comet Assay.

5.2. Aims

The aim of this study was to validate sperm DNA fragmentation testing at the University of Kent. Due to the advantages of using flow cytometry to test sperm DNA damage

levels, a testing technique based on the sperm chromatin structure assay was validated at the University of Kent.

5.3. Results

Validation of a test requires ensuring its reliability, consistency, and ability to generate dependable results (clinical validation refers to evaluating performance and utility of a test in clinical practice, establishing links between results and patient diagnosis/treatment). Additionally, establishing that the test is not operator-dependent enhances result reliability, reproducibility, standardization, clinical utility, and supports quality control and accreditation efforts.

Semen samples were obtained from TDL Andrology (Wimpole Street, London), with one vial going for sperm DNA damage testing at SCSA Diagnostics in America and one vial going for sperm DNA damage testing at University of Kent. The DFI values obtained from the University of Kent were labelled as AOFT – Acridine Orange Flow Cytometric Testing. The results between the two centres were compared. TUNEL assay was additionally performed on 35 of the semen samples and the results obtained by the two sperm DNA damage assays (AOFT and TUNEL) were compared.

5.3.1. Summary statistics of parameters analysed

Table 15 shows a summary of the sperm DNA damage results for 123 patient semen samples. TUNEL was additionally performed on 35 semen samples.

Table 15. Summary statistics of the DFI levels as measured by AOFT, SCSA and TUNEL.

Test	n	Mean ± S.E.	Median	Range
AOFT DFI (University of Kent)	123	17.9% ± 1.4%	13%	2.6% – 89.2%
SCSA Diagnostics DFI	123	17.7% ± 1.4%	13%	3.0% - 87.1%
TUNEL DFI (University of Kent)	35	11.7% ± 1.3	9.6%	1.3% - 31.7%

5.3.2. Assessing inter-lab variation between University of Kent and SCSA Diagnostics

There was a high statistically significant Pearson correlation of .985 (<.001) between the testing done at the University of Kent and the testing performed at SCSA Diagnostics (**Figure 32**).

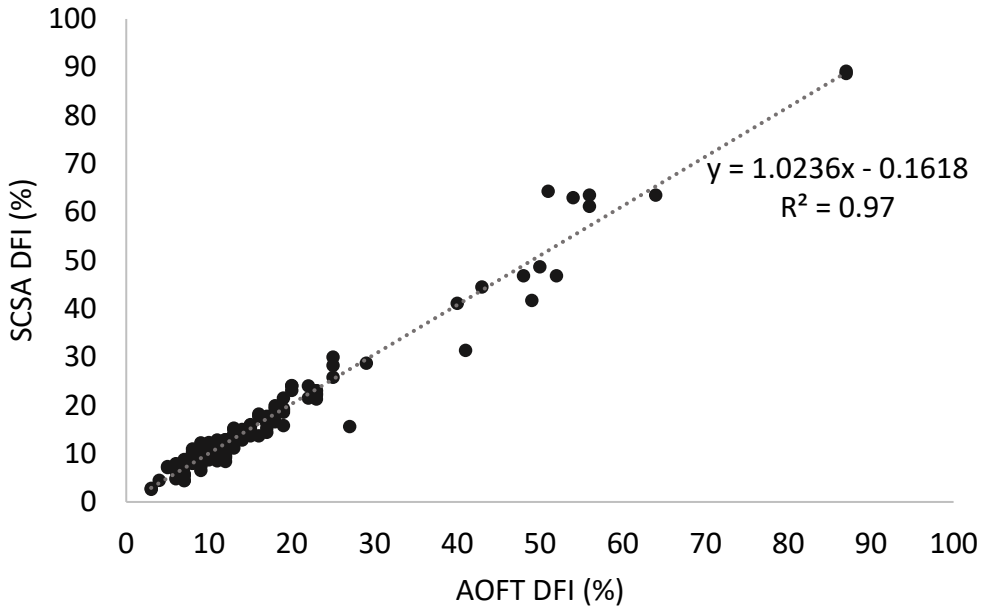


Figure 32. Relationship among the percentage of DFI between SCSA® test and AOFT by general linear model.

The Bland-Altman Plots allows for the assessment of agreement between testing done at University of Kent and testing performed at SCSA Diagnostics in America. It is an informative demonstration on comparing the two testing techniques. The solid horizontal line indicates the mean difference between two methods of measurement, and the dashed horizontal line indicates the line of agreement (mean difference ± 1.96 S.D.). The mean difference for the comparison of the two operators was -0.26 with limits of agreement set at -5.68 and 5.17. A total of 6 points (out of the 123 samples) were above the limits of agreement. The plot shows no slope indicating no proportional error.

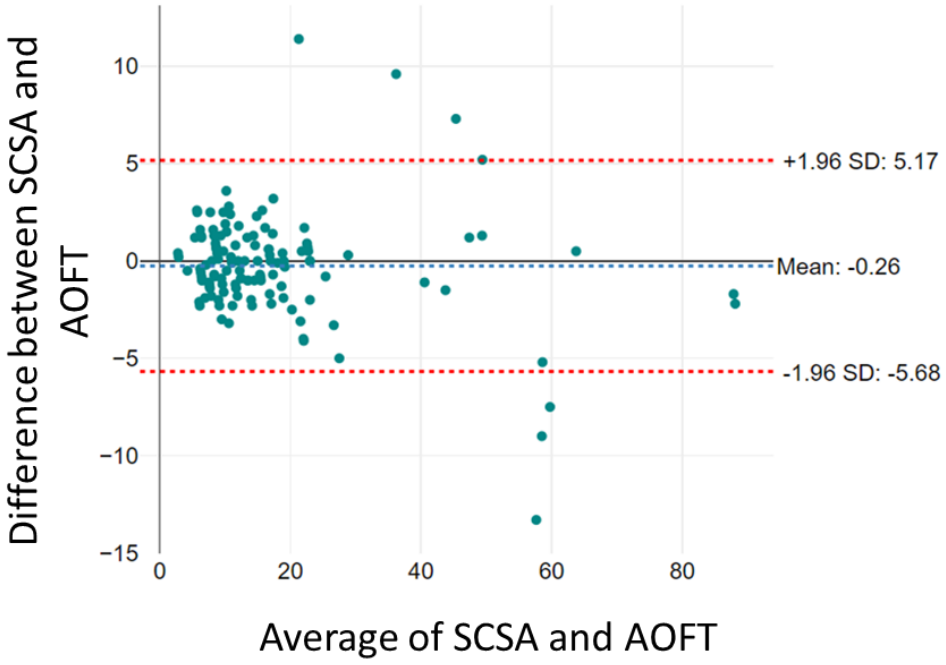


Figure 33. Bland-Altman Plot analysis of the two testing techniques: AOFT and SCSA® test.

5.3.3. Testing of dependence on experimental conditions of Acridine Orange Flow Cytometric Testing (AOFT)

There are three principle potential sources of variability: operator, consumables and hardware. As a preliminary test an analysis was done where all the above sources of variability were simultaneously assessed.

A total of 49 samples were used for assessing the AOFT dependence on the set experimental conditions. The DFI measurements obtained by three other operators (collectively referred to as Operator 2) were compared to the DFI measurements obtained by the principal investigator (the author of this thesis - Operator 1). Different acridine orange stocks were used between the operators, to ensure that same results are obtained regardless of whether a different batch of acridine orange is used, or a

different operator performed the result. This part of experiment was conducted in a blind fashion, whereby the operators did not share DFI results until the end of investigation. The principal investigator was using a BD Accuri C6 Plus flow cytometer while the other operator was using a BD Accuri C6 Plus with the BD CSampler Plus flow cytometer (i.e. a second instrument with the same basic hardware but including the additional optional auto-load attachment). Since there were no differences between the results there was no need for subsequent follow-up testing to identify specific sources of variability.

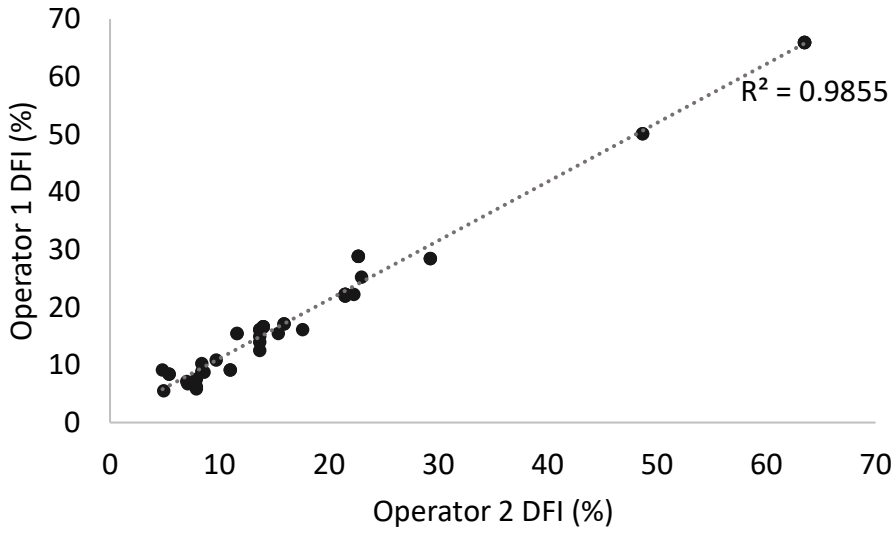


Figure 34. Relationship among the percentage of DFI between the two operators by general linear model.

There was a strong agreement between the results obtained from the different operators at varying experimental conditions, having a Pearson correlation of .993

(p<.001). The mean difference for the comparison of the two operators was -1.32 with limits of agreement set at -5.26 and 2.63. One point was below the limits of agreement.

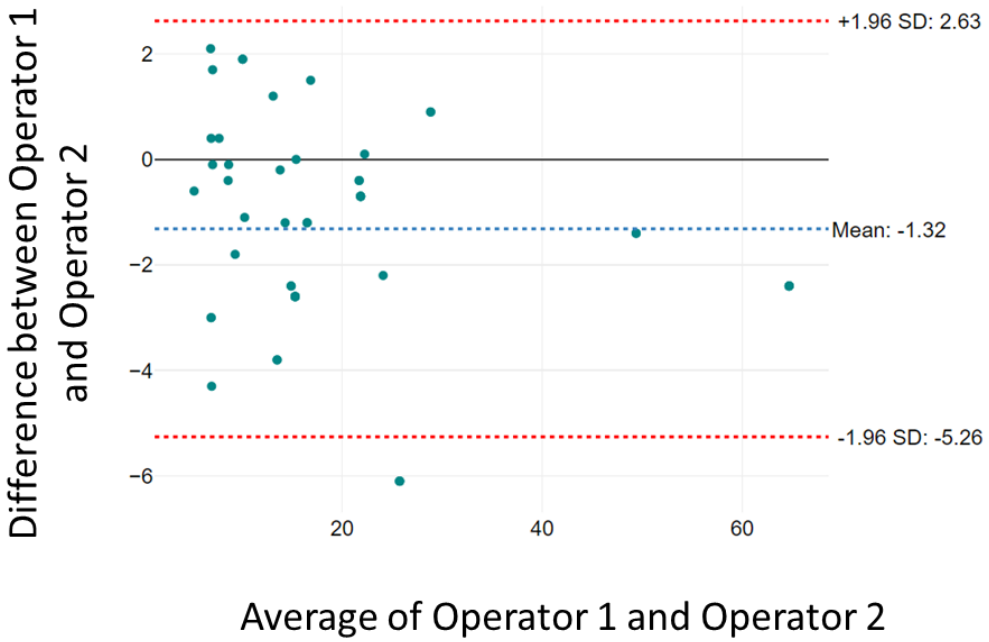


Figure 35. Bland Altman Plot for operator 2 vs operator 1.

5.3.4. Analysis of Acridine Orange Flow Cytometric Testing (AOFT) time sensitivity

To analyse time sensitivity of the AOFT assay, once the acridine orange was added to the sperm sample, readings of DFI over a range of time (0 seconds to 1800 seconds) were recorded. The 1st reading was 8.8% and last reading was 8.6%. The minimum DFI was 7.5% and maximum 9.8%. Average was 8.8%. The coefficient of variation was 7.15%. According to published SCSA protocol (Evenson, 2013), data should start being recorded after 3 minutes following acridine orange staining and data can be obtained within a

maximum of 7 minutes. However, our results have demonstrated that even after 30 minutes, DFI values remain relatively constant.

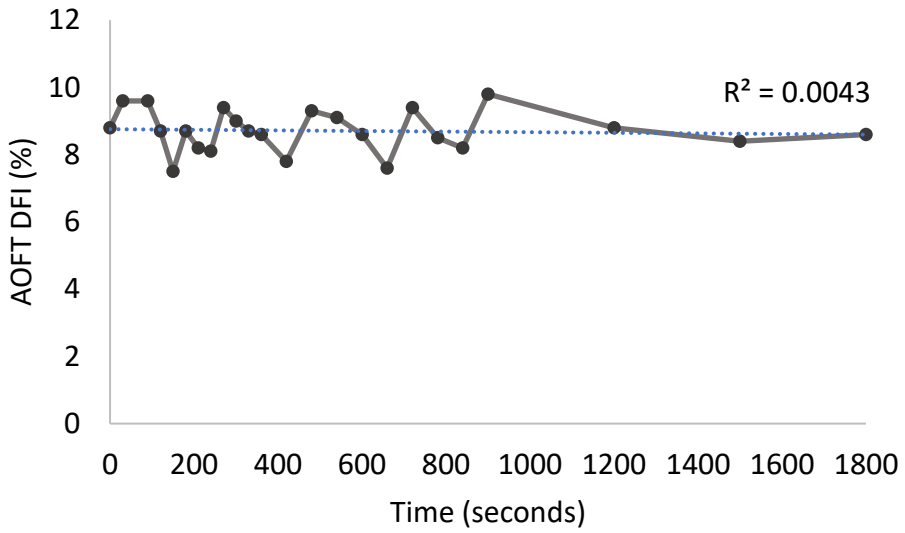


Figure 36. Variations and linear regression analysis of DFI levels over a range of time (0 seconds to 1800 seconds).

5.3.5. Acridine Orange Flow Cytometric Testing (AOFT) Repeatability

Analysis of repeatability of the sperm DNA damage assay was done by performing multiple sperm DNA damage testing on the same semen sample (40 times) to analyse the consistency of testing. AOFT repeatability was assessed using two different semen samples (sample 1 and sample 2). Average DFI (± S.D.) for sample 1 was 7.82% ± 0.85% and average DFI (± S.D.) for sample 2 was 9.36% ± 0.79%. The minimum DFI was 6.2% and maximum DFI was 9.9% for sample 1. The minimum DFI was 8.2% and maximum DFI was 11.57% for sample 2. The coefficients of variation were 10.8% for sample 1 and 8.57% for sample 2.

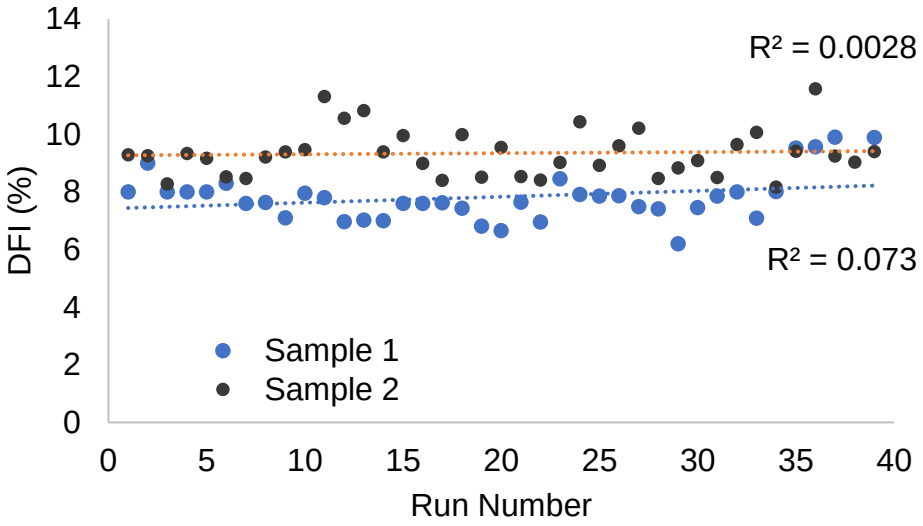


Figure 37. Analysis of the AOFT repeatability of two semen samples by general linear model.

5.3.6. Acridine Orange Flow Cytometric Testing (AOFT) vs TUNEL

Between the two assays a significant Pearson correlation of 0.693 (p<.001). was observed.

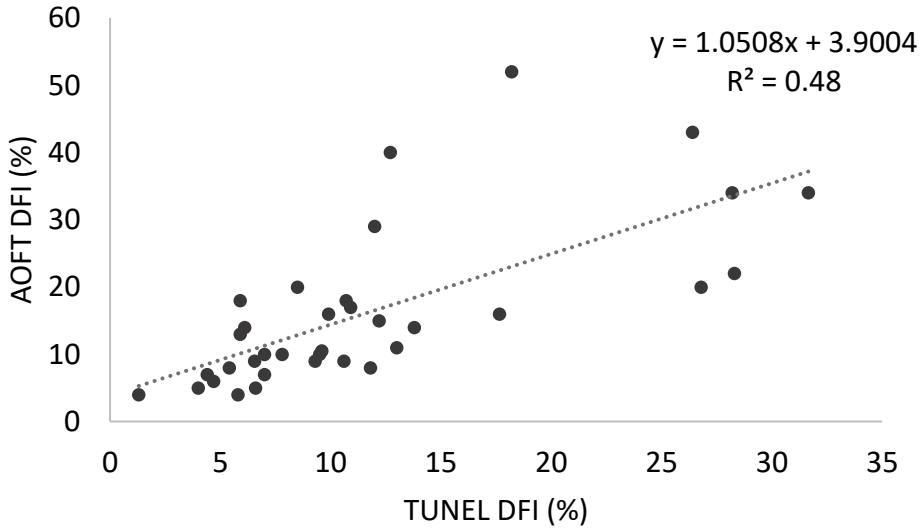


Figure 38. Relationship among the percentage of DFI between the two assays: AOFT and TUNEL, by general linear model.

5.4. Discussion

Sperm DNA damage testing is becoming increasingly used in the evaluation of male infertility. It allows for the assessment of the sperm genetic integrity, which can aid in clinical decision on treatment on infertile patients (Shamsi et al., 2011).

Results from the survey conducted in **Chapter 3** indicated that the two most popular sperm DNA damage screening tests that urologists in the UK refer to, were the Comet assay (32% of respondents) and the SCSA (28% of respondents). The Comet assay requires the use of the light microscope and analyses a total of 100 sperm cells (Hughes et al., 1997) while the SCSA using flow cytometric techniques allows measurement of over 5000 sperm cells in around 5 minutes (Evenson, 2022). Therefore, due to the benefits of reducing testing time, while increasing precision, a sperm DNA damage testing service based on the SCSA was chosen to be validated at the University of Kent. Currently, semen samples are typically shipped to America for SCSA testing, which can have negative implications namely high turnaround times and increased cost. Therefore, a UK based flow cytometric sperm DNA damage testing service has several benefits including (1) timely results allowing the clinics to make informed decisions promptly, (2) cost-effectiveness which can help in managing the overall cost of fertility treatment and (3) flexibility since clinicians can prioritize urgent cases and conduct testing according to their preferred timeline. Taking these benefits into consideration, an acridine orange-based flow cytometric test (AOFT) was validated in this study, for establishing a sperm DNA damage testing service.

The findings from this study have shown that the AOFT sperm DNA test is a valid test that is reliable and consistent. The method used for the AOFT is similar to that reported

previously (Evenson, 2013; Rex et al., 2019), however those assays were performed using flow cytometers of different brands/flow cytometer software from what was used in this study. Therefore, to ensure that the data obtained on our BD Accuri™ C6 flow cytometer using the BD Accuri™ C6 Software matches the data obtained on the Ortho Diagnostics L30 flow cytometer at SCSA Diagnostics in America using their proprietary software, SCSASoft, the % DFI results from both centers were compared. There was no significant difference among the % DFI measured on the human semen aliquots on the two different flow cytometers, and a high correlation 0f 0.99 was obtained between the two centers. Additionally, the AOFT assay shows a high degree of reliability and accuracy, having consistent % DFI results between replicates of semen samples. Appropriately trained operators obtained similar data to the principal operator, even when they the operators were using different acridine orange stock solutions to the principal operator, indicating a high repeatability of the assay.

Limitations in this study include no analysis on the DFI variability at varying ambient temperatures (although more tests are being done to generate this information) and only limited investigation of how DFI may vary depending on the period of time between semen thawing and sample analysis. Future work involves collaboration with clinics in Europe offering an acridine orange-based DNA damage screening service to set up a monthly external quality assessment. Additionally, in response the urologist survey (**Chapter 3**), a high proportion of respondents stated that it would be beneficial for a 'suite' of DNA fragmentation tests to be conducted on a semen sample, therefore, further validation tests of the TUNEL assay is currently in progress, to be able to provide it in conjunction to the AOFT. In particular, it will be interesting to compare outcome data from patients where AOFT/SCSA and TUNEL assays disagree on the level of sperm

DNA fragmentation. This will give an important indication of whether one is intrinsically better (i.e. more closely correlated to clinical outcome) than the other, or whether both are giving independent information with prognostic value.

5.5. Conclusion

In conclusion, sperm DNA damage testing is increasingly important in evaluating male infertility and guiding treatment decisions. To overcome limitations such as long turnaround times and high costs, a validated UK-based flow cytometric sperm DNA damage testing service using the acridine-orange flow cytometric test, AOFT, was established. The study confirmed the reliability and consistency of AOFT. Future research will focus on investigating DFI variability in different conditions, collaborating with European clinics for external quality assessment, and validating the TUNEL assay to offer a comprehensive suite of DNA fragmentation tests for enhanced prognostic value.

6. Chapter 6: Effect of microfluidic based separation on boar sperm parameters

6.1. Introduction

Assisted Reproductive Technologies (ARTs) are medical procedures used to treat infertility in both men and women. These procedures involve the manipulation of eggs, sperm, or embryos in a laboratory setting to increase the chances of a successful pregnancy. Over the years, there has been an increase in the understanding of the importance of sperm quality in ART success rates and offspring health (Sharma et al., 2015; Villani et al., 2022; Zini & Libman, 2006). This has resulted in increased pressure towards identifying sperm preparation techniques that select an optimal sperm population for treatment (Said & Land, 2011). The current techniques for sperm selection for the isolation of viable spermatozoa usually require several sperm washing steps and rely on separation based on density and/or sperm motility and are therefore not representative of what spermatozoa experience *in vivo*. Given the current trend in ART to emulate the natural selection process whenever possible (Leung et al., 2022), microfluidics has been suggested as an alternative, more gentle sperm preparation technique (Samuel et al., 2018).

Microfluidic sperm preparation is a cutting-edge technology that has enabled optimization of the selection and preparation of sperm exhibiting higher motility and lower DNA damage to improve ART success rate (Huang et al., 2023; Nosrati et al., 2017). The integration of microscale channels and chambers in microfluidic sperm sorting devices are designed to mimic the structure and function of the female reproductive

tract where billions of sperm can be processed in hours (Kim & Chun, 2020; Swain et al., 2013). The three-dimensional structures of the microfluidic devices help manipulation of the fluid flow pattern to help naturally select spermatozoa based on their ability to navigate narrow channels (Huang et al., 2023). Additionally, microfluidics is a gentler technique compared to swim-up and density gradient centrifugation since it reduces the requirement for laboratory interventions including centrifugation and washing techniques. Such sperm handing techniques can subject the sperm cells to exogenous shear forces which can result in increased levels of sperm DNA damage and/or seminal oxidative stress levels (Gualtieri et al., 2021).

Microfluidic based sperm separation has been shown to select spermatozoa of improved genomic integrity relative to more conventional techniques in human semen (Quinn et al., 2018; Shirota et al., 2016), however, such benefit in boar sperm has yet to be evaluated. Although the use of microfluidics for sperm preparation has been analysed using boar sperm, only sperm viability was assessed as it was a proof of concept study (Hamacher et al., 2020).

Boar semen is important for animal breeding and insemination programs, and there is a need to improve the sperm preparation techniques to maximise the chances of a successful fertilisation outcome and reduce the need for multiple inseminations (Morrell, 2011). Therefore, improving boar sperm parameters can help increase reproductive efficiency of breeding programs which can lead to more animals being produced in a relatively shorter amount of time resulting in economic benefits for the farmers and producers.

6.2. Aims

The aim of this chapter was to analyse whether the Zymot™ microfluidics device performs as good as the gold standard (density gradient centrifugation) for boar sperm preparation. This study also aims at analyzing sperm DNA damage levels following microfluidic sperm separation to assess how it compares to density gradient centrifugation.

6.3. Results

Two different sperm separation techniques were compared: microfluidic chip-based sperm separation (MCSS) and density gradient centrifugation (DGC). Sperm concentration, progressive and total motility, vitality, acrosome reactivity and sperm DNA fragmentation index were compared between the two different sperm separation techniques. A total of 20 boar semen samples were evaluated in this study. Semen samples in extender were also evaluated in this study to allow for comparison with the semen samples post sperm separation.

6.3.1. Basic semen parameters

When sperm concentration was compared in all the groups (MCSS, DGC and Ext), there was a significant difference between all the groups. The average sperm concentration following MCSS was significantly lower than sperm concentration following DGC (4.5 ± 3.2 x 10^6 sperm cells/mL vs 9.4 ± 4.0 x 10^6 sperm cells/mL; p = 0.031). There was an expected significant reduction in sperm concentration following both sperm separation techniques (Ext vs MCSS, $p < 0.001$; Ext vs DGC, p = 0.006) when compared to the pre-sperm preparation sample which had an average sperm concentration of 20.9 ± 12.5 x 10^6 sperm cells/mL.

There was a significant increase (groups were compared using the Kruskal-Wallis test) in progressive motility from 27.8 ± 17.3 % (Ext group) to 46.3 ± 16.0 % following MCSS (p = 0.01), and from 27.8 ± 17.3 % to 57.9 ± 21.2 % following DGC ($p < 0.001$) (Figure 39). There was a significant difference in total motility between DGC and MCSS (p = 0.03). There was a significant increase in total motility from 57.8 ± 25.0 % (Ext group) to 73.8

± 17.9% following MCSS (p = 0.02) and from 57.8 ± 25.0 % to 67.5 ± 18.4 % following DGC (p < 0.001) (Figure 39). No significant difference in total motility was present between DGC and MCSS (p = 0.13).

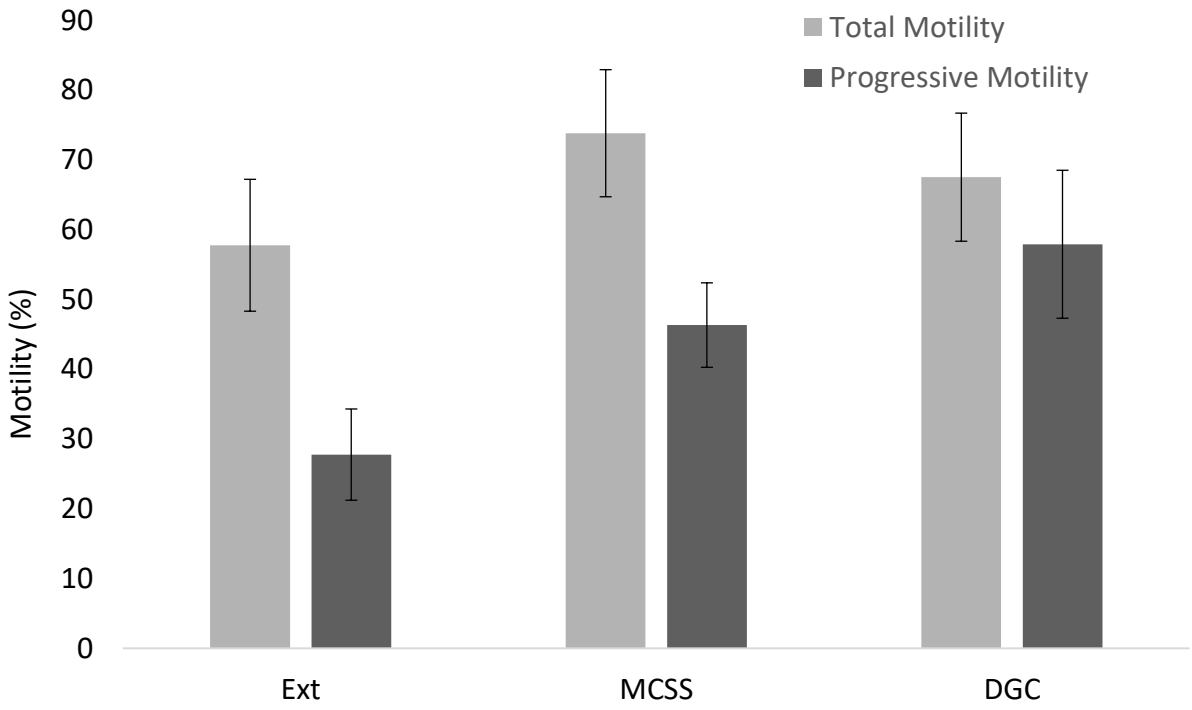


Figure 39. Mean and standard error of total motility and progressive motility for two sperm selection techniques (microfluidic chip-based sperm sorting - MCSS and density gradient centrifugation – DGC) and for sperm in extender group (pre-sperm selection). N=20 replicates for each group.

6.3.2. CASA Parameters

The iSperm computer assisted semen analysis (CASA) was used to analyse the patterns of sperm motility traits: curvilinear velocity (VCL), velocity average path (VAP), velocity straight line (VSL) across the different groups. The results showed that the VCL of the samples significantly increased when selected with DGC (87.9 ± 32.8 µm/s) compared to

the extender group (66.6 ± 13.2 µm/s; $p = 0.001$) and MCSS (68.1 ± 26.8 µm/s; $p = 0.028$), while MCSS did not differ significantly from the extender group. MCSS showed a significantly increased VAP (59.9 ± 25.3 µm/s) and VSL (49.3 ± 18.8 µm/s) compared to the extender group (52.9 ± 10.5 µm/s, $p = 0.01$ for VAP and 39.6±2.4 µm/s, $p = 0.031$ for VSL). There was a greater observed increase in VAP ($p < 0.001$) and VSL ($p = 0.002$) values in DGC group (75.2 ± 29.8 µm/s and 49.3 ± 18.8 µm/s for VAP and VSL, respectively) compared to the extender group.

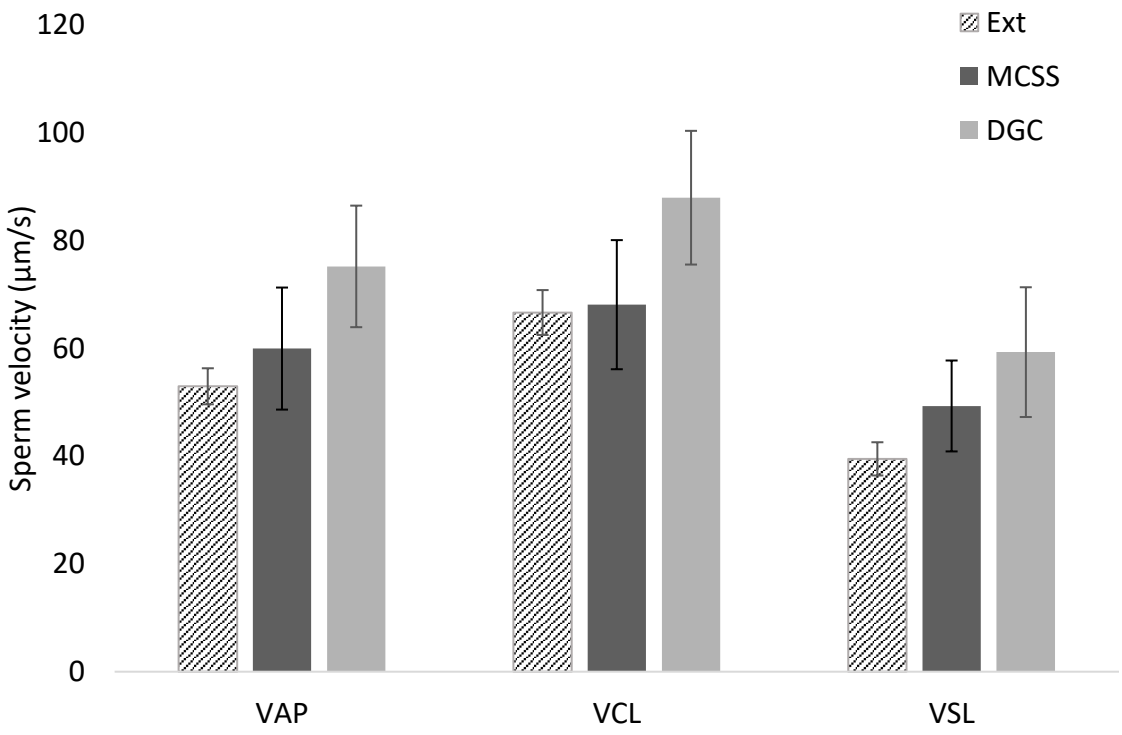


Figure 40. Mean and standard error of VAP, VCL and VSL for two sperm selection techniques (microfluidic chip-based sperm sorting - MCSS and density gradient centrifugation – DGC) and for sperm in extender group (pre-sperm selection). N=20 replicates for each group.

6.3.3. Vitality and acrosome reactivity

There was a significant decrease in % vitality following DGC (82.8 ± 7.7 %) when compared to the extender (97.1 ± 1.3 %; $p<0.01$) and the MCSS groups (95.3 ± 3.4 %; $p<0.01$). There was no significant difference in % vitality between MCSS and extender group. The % of acrosome reacted sperm following DGC (11.8 ± 20.8 %) was higher compared to extender (5.0 ± 6.4 %) and MCSS (5.0 ± 4.6 %) groups, however, it was not a significant increase ($p = 0.18$).

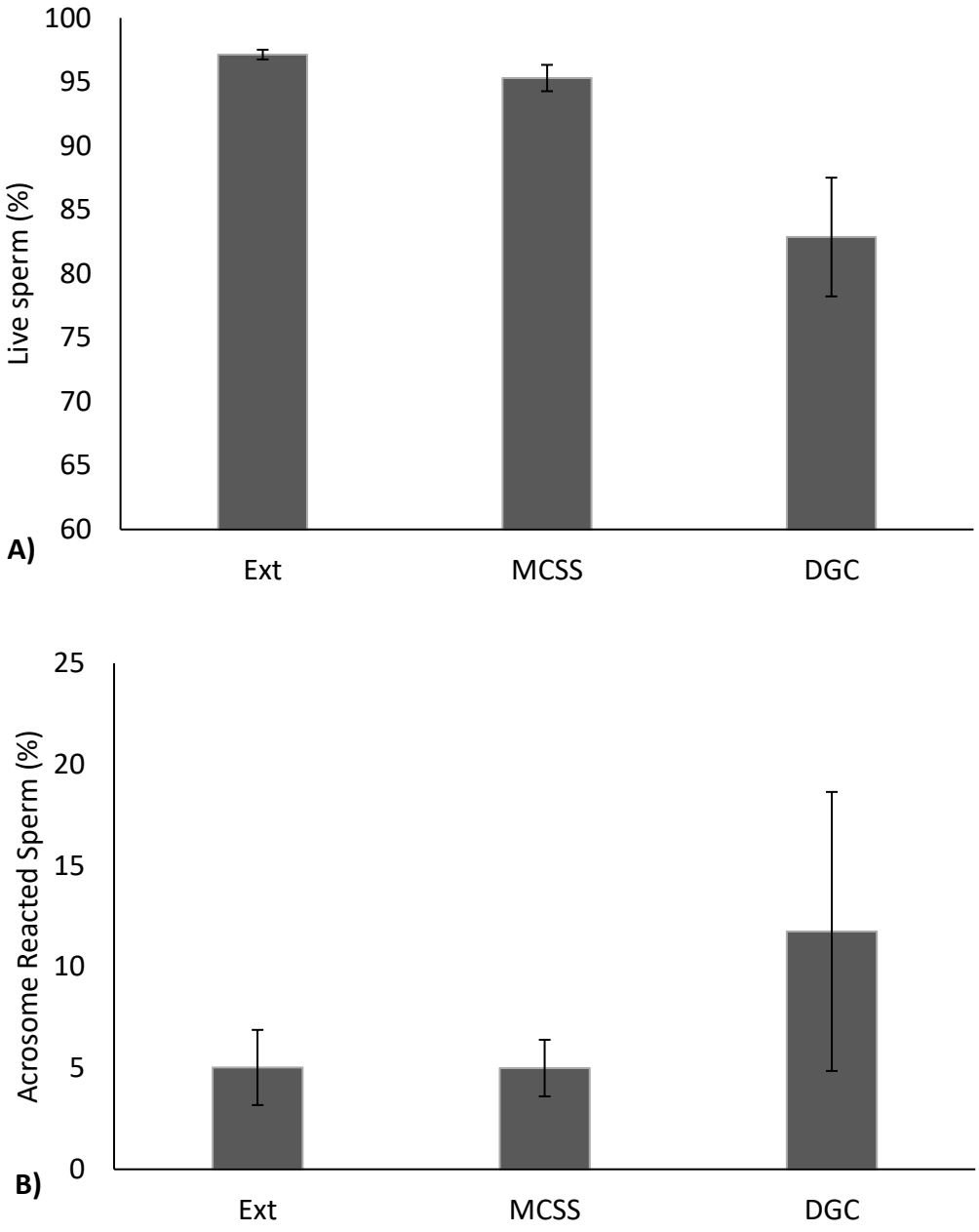


Figure 41. Mean and standard error of (A) % live sperm and (B) % acrosome reacted for two sperm selection techniques: microfluidic chip-based sperm sorting (MCSS) and density gradient centrifugation (DGC). Ext refers to sperm in extender (pre-sperm selection). N=20 replicates for each group.

6.3.4. Sperm DNA quality and Immature Spermatozoa

The % DFI was significantly reduced following both sperm separation techniques (2.4 ± 1.3 % Ext vs 0.7 ± 1.0 % MCSS, $p < 0.01$; 2.4 ± 1.3 % Ext vs 1.1 ± 0.8 % DGC $p < 0.0004$). There was no significant difference in % DFI between both sperm preparation techniques. MCSS has a significantly lower ($p < 0.0004$) number of immature spermatozoa (identified as high DNA staining cells – HDS) as measured by the acridine orange-based flow cytometric testing compared to extender group. %HDS was also reduced following DGC (1.7 ± 1.3 % DGC vs 2.3 ± 1.8 % Ext), albeit not significant.

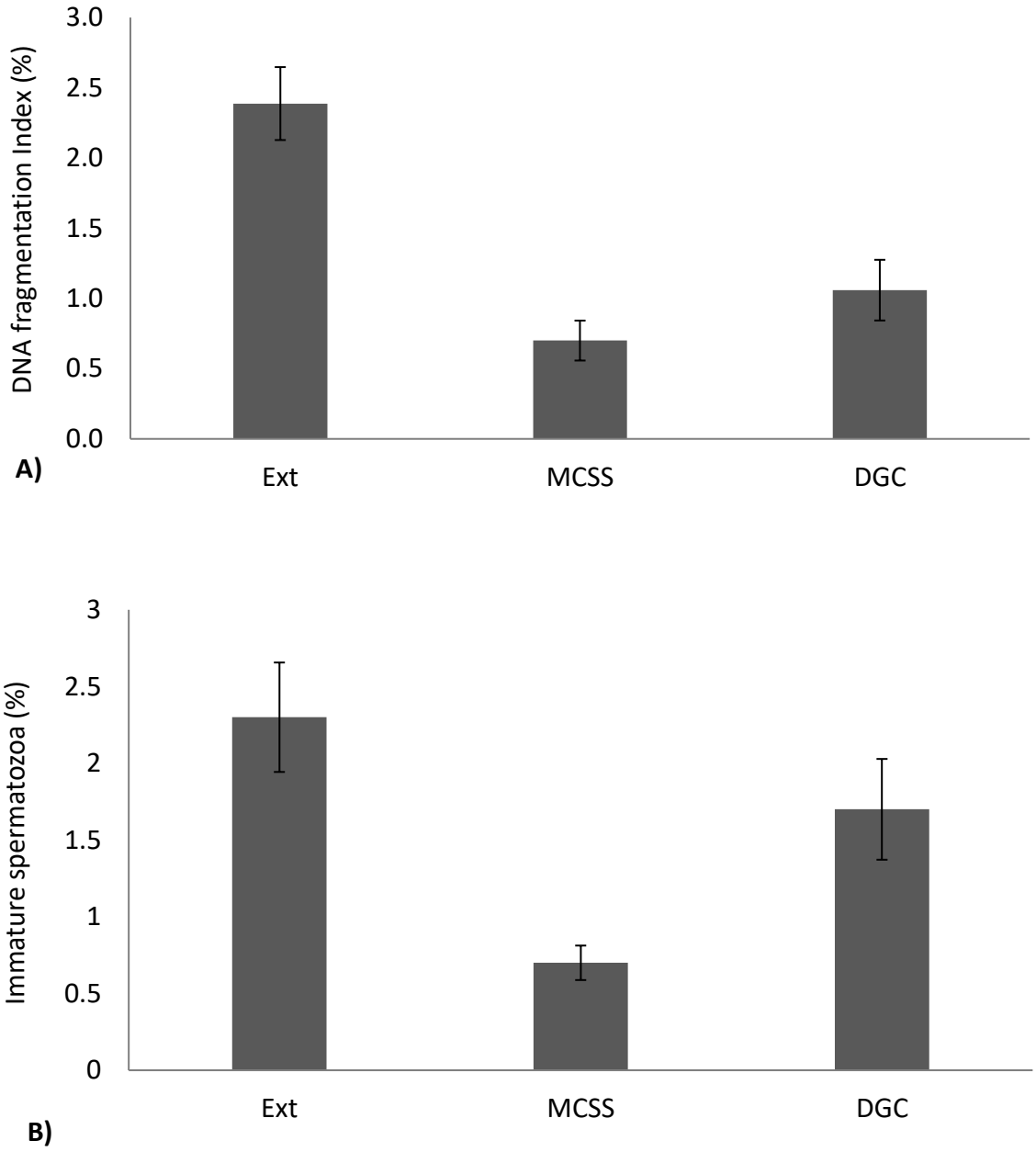


Figure 42. Effect of sperm preparation technique on % DNA fragmentation Index (DFI) and % of immature spermatozoa (High DNA stainability – HDS) for two sperm selection techniques: microfluidic chip-based sperm sorting (MCSS) and density gradient centrifugation (DGC). Ext refers to sperm in extender (pre-sperm selection). N=20 replicates for each group. Data is shown as % mean ± SEM.

6.3.5. Sperm DNA quality between the upper chamber and lower chamber of the microfluidic device

Following sperm extraction from the upper chamber of the microfluidic device, sperm was also extracted from the bottom chamber (the sperm that did not pass through the filter into the upper chamber). This was done in order to analyse the efficacy of the device in separating sperm having low DNA damage with sperm having higher DNA damage levels.

There was a significant difference in % DFI between the two chambers ($p < 0.05$) indicating that sperm having lower levels of DNA damage were more likely to pass through the microfluidic channels and be collected in the upper chamber.

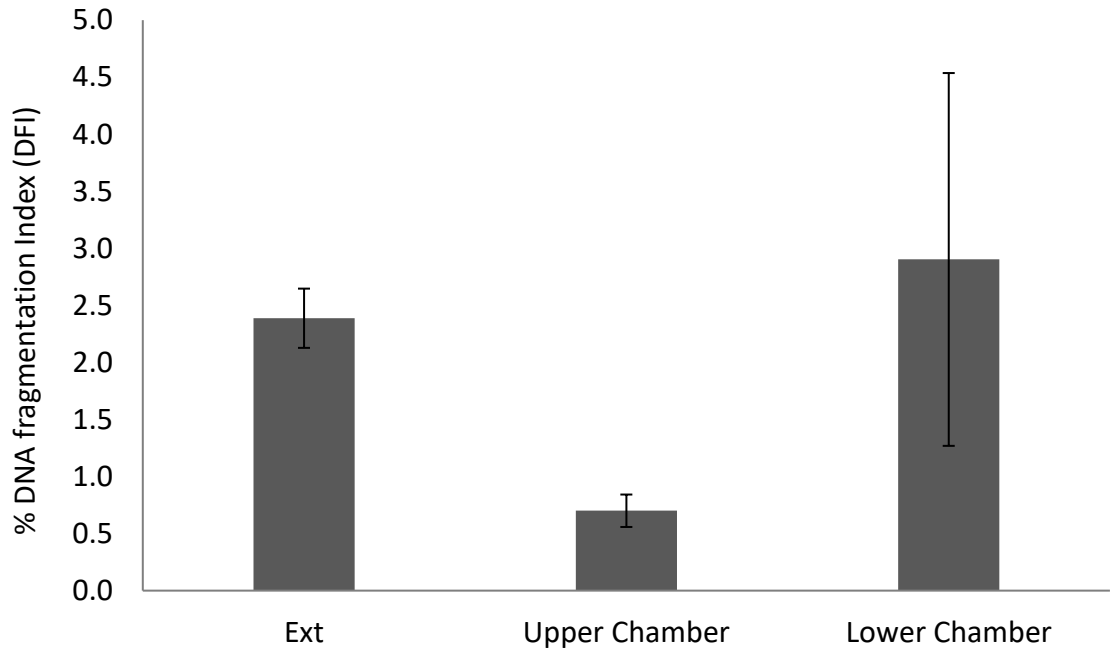


Figure 43. % DNA fragmentation Index (DFI) between lower chamber of microfluidics sperm separation device, upper chamber of the microfluidic sperm separation device and extender. N=20 replicates for each group. Data is shown as % mean ± SEM.

6.4. Discussion

Microfluidic devices for sperm preparation techniques are relatively new concepts and are still undergoing research and development to optimize their effectiveness. In the boar breeding industry, microfluidic-based sperm preparation has the potential to improve breeding outcomes by enhancing sperm quality and selection, thus potentially increasing fertilisation rates. Since the field of reproductive technology is moving towards 'a closer to nature' approach, reducing the requirement for centrifugation by utilizing microfluidics, helps to mimic more closely the natural selection process that occurs *in vivo* (Leung et al., 2022). Since the gold standard for boar sperm preparation is density gradient centrifugation, this study analysed whether microfluidics-based sperm separation is at least as good as the gold standard. The results obtained in this study have demonstrated that microfluidics obtained comparable results to density gradient centrifugation. Therefore, the benefits of utilizing microfluidics including reduced time and labor for sperm preparation and reduced centrifugation steps, warrants further studies to optimize and standardize microfluidics-based sperm preparation for boar ART.

The two sperm preparation techniques analysed in this study, DGC and MCSS, increased the overall progressive motility and total motility of the sample when compared to the pre-processed sample. DGC was associated with a higher overall progressive motility relative to MCSS. Interestingly, multiple published literature have reported higher progressive motility in MCSS when compared to DGC, although majority of the studies were conducted on human sperm samples (Gode et al., 2020; Guler et al., 2021; Quinn et al., 2018). A potential contribution factor to the observed lower progressive motility

in MCSS group relative to DGC, was that the sperm handling medium used in the microfluidics device was not optimized for microfluidics sperm sorting. The same sperm handling medium utilized for DGC was used for microfluidics. Therefore, future research should be directed towards analyzing different media for use with microfluidics.

Interestingly, DGC was associated with a lower number of viable spermatozoa relative to the MCSS and the pre-processed sample. Additionally, DGC had a higher number of acrosome reacted spermatozoa. Such observation could be due to the centrifugation process potentially inducing membrane instability and shortening the life-span of the spermatozoa (Coetzee et al., 1992; Hernández-Silva et al., 2021). This can result in premature acrosome reaction and preclude zona binding.

The results demonstrated in the study indicate both sperm preparation techniques selected spermatozoa having lower DNA fragmentation, however, MCSS was associated with the lowest DFI values (although not significant). Some studies have suggested that the centrifugation process can increase levels of reactive oxygen species in the media and can result in DNA damage (Zini et al., 2000). However, contradicting studies have showed that DGC does not increase ROS and DFI levels (Malvezzi et al., 2014; Takeshima et al., 2017). Our study demonstrates that there was no significant difference in DFI levels between DGC and MCSS, although DFI levels following MCSS were lower.

In terms of study limitations, it is important to note that the semen samples used for this study came from breeding boars, which have been selected for their optimal sperm parameters. Therefore, the DFI levels of these samples were already very low. Future work will be aimed at investigating poorer quality samples, to determine which sample preparation method is better at enriching healthy sperm from a mixed population of

cells. Additionally, it is important to note that these results are based on a limited sample size and further studies with larger sample sizes may be needed to confirm these findings. Nevertheless, the present study provides important insights into the effects of different sperm separation methods on the overall sperm quality.

There are still several challenges that need to be addressed before microfluidics can be widely adopted in the industry, such as the need for standardization and validation of the techniques, as well as the cost and scalability of the devices. Further challenges include the lack of standardized protocols for microfluidic based sperm preparation and the need for more data on their effectiveness. Overall microfluidic sperm preparation can allow for a promising technology for use in the boar breeding industry and is at least as good as the gold standard for sperm selection, density gradient centrifugation. Therefore, it could be a promising approach for improving success rates in pig ART, and in other species, including humans, if the results prove to generalize beyond the context studied here.

6.5. Conclusion

In conclusion, microfluidic devices offer a promising approach for sperm preparation in the boar breeding industry, with the potential to enhance breeding outcomes by improving sperm quality and selection. This study demonstrated that microfluidics-based sperm separation is comparable to the gold standard method of density gradient centrifugation. Nevertheless, challenges remain, including the standardization, validation, cost, scalability, and development of protocols for microfluidic-based sperm

preparation. Despite these challenges, microfluidics holds promise as a technology that can enhance success rates in pig assisted reproductive technologies.

7. Chapter 7: Assessing the safety of optical coherence tomography (OCT) broadband infrared sources on boar and human spermatozoa.

7.1. Introduction

Successful implantation following embryo transfer requires the synchronisation of both the embryo and the endometrium (Diedrich et al., 2007). A crucial step during embryo transfer is the selection of embryos (as determined by morphological and morphokinetic assessment) having the highest chance of attaining a successful pregnancy (Cummins et al., 1986; Gardner et al., 2000; Meseguer, Herrero, et al., 2011). Currently, the gold standard for embryo assessment is static observation of embryo development during specific time points using microscopy. Based on the morphological assessments, embryos are assigned a grade according to their predicted ability to implant (Gardner et al., 2000). However, using fixed time points for monitoring embryo development can result in key embryonic events being missed. To counteract this, time-lapse systems (TLS) have been developed to allow for continuous monitoring of embryos. The use of TLS in artificial reproduction technology (ART) clinics has paved way for further understanding of the morphological mechanisms of fertilisation and embryo morphokinetics (Castellò et al., 2016; Kovacs, 2014). Since TLS requires the periodic exposure of embryos to visible light which is an unnatural stress to embryos, clinical safety was established and TLS were validated for clinical use (Kirkegaard et al., 2012; Nakahara et al., 2010).

In recent years, the use of TLS with morphokinetic monitoring has enabled several embryo selection algorithms to be developed with the aim of identifying the ideal morphokinetic parameters for a successful outcome (Chamayou et al., 2013; Cruz et al., 2012; Dal Canto et al., 2012; Petersen et al., 2016; Storr et al., 2018). This has resulted in the generation of a wealth of embryo morphokinetic data based on the 2-dimensional (2-D) time-lapse images produced. Although, multiple iterations of TLS have been developed to optimise embryo selection, it is ultimately limited by the inability to see beyond the external surface anatomy of embryos.

3-Dimensional (3-D) live imaging of embryos using optical coherence tomography (OCT) could allow for analysis of 3D embryo volumes with high resolution depth profiling and this could provide additional information for selecting the optimal embryo for transfer. Adopting this technique could help identify potential defects in embryos which would otherwise be unobservable through more conventional analysis.

OCT is an imaging method utilising low coherence interferometry to produce 3-D cross-sectional images (Popescu et al., 2011). OCT was initially commercialized for optical tomography (Fercher et al., 1993) and is now considered a standard of care for retinal imagining technique (Sampson et al., 2022); however, over recent years, OCT has been demonstrated as a fundamental research tool for real time, non-invasive *in-vivo* and *ex-vivo* imaging (Alam & Poddar, 2022; Cernat et al., 2012; Holmes, 2009; Sepehr et al., 2008).

OCT employs the use of a broadband, low coherence optical source to produce a beam which is divided between a sample arm and a reference arm (**Figure 44**) (Aumann et al., 2019; Popescu et al., 2011). The interference between the sample and reference beams

are coupled and detected to produce a reflectivity profile in depth of the embryo. 3D volumes are then acquired using the galvanometer scanners that operate in X and Y axes to provide structural and functional information on embryos (Raghunathan et al., 2016).

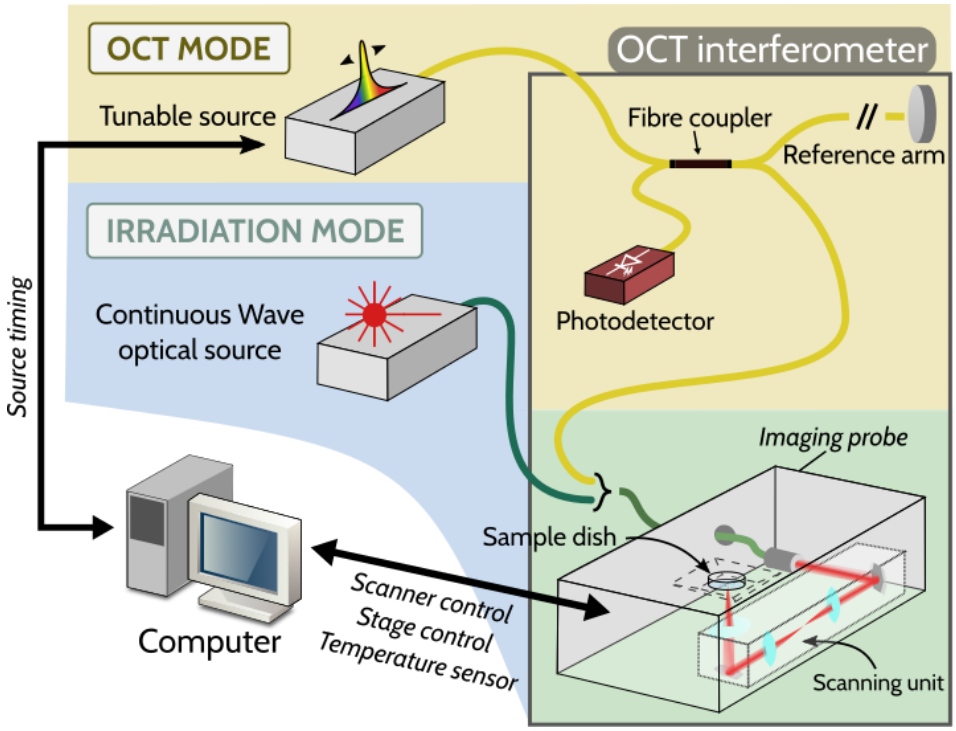


Figure 44. Diagram of optical set up (used with permission from Optics Group at University of Kent).

Numerous studies have demonstrated the potential of OCT as an exploratory tool within developmental biology for imaging of live cells (Burton et al., 2015; Garcia-Martin et al., 2014; Jenkins et al., 2007; Umezu et al., 2022; Wang et al., 2022; Wang & Larin, 2015). Additionally, it could pave way to the inclusion of key novel biomarkers for grading of gametes and embryos during the selection process. However, despite the demonstrated benefits of OCT in developmental biology, it is vital to test the safety of the optical

sources on gametes especially since spermatozoa are particularly prone to DNA damage owing to the limited DNA repair ability post-spermiogenesis (García-Rodríguez et al., 2018). Although low levels of unrepaired sperm DNA damage levels can be repaired in zygote following fertilisation, if sperm DNA damage exceeds oocyte repair mechanism, there is an increased risk of implantation failure, miscarriage, abnormal foetal development, and birth defects in offspring (González-Marín et al., 2012; Vasilyeva et al., 2020; Wyck et al., 2018).

7.2. Aims

The main aim of this study was to experimentally validate the safety of OCT broadband infrared sources (at circa 1077 nm) on boar and human spermatozoa by simulating by simulating three different imaging protocols. The proposed testing of OCT based imaging is intended for eventual use in embryology. This study not only provides valuable information on the safety and benefits of OCT based imaging, but it is also the first systemic study analyzing the genotoxic impact of broadband infrared on boar and human sperm.

7.3. Results

The irradiation protocol for boar semen samples involved 3 different scanning protocols, referred to as protocol A, B and C. In protocol A, the time for scan of the entire field of view was kept unchanged but the overall time of acquisition was varied from 20 to 60 seconds. As higher overall acquisition time increases the theoretical image signal-to-noise ratio, an exposure time of 60 seconds (maximum time tested in protocol A) was used in protocol B, however varying scanning frequencies were assessed. Higher resolution results from the capacity of a lower scanning frequency to collect more points per scan. In comparison to scanning at higher frequencies, the dose across each point increases at lower imaging rates, as the laser beam moves over a given area at a slower pace.

Protocol C simulated different OCT scanning protocols that are commonly used for functional characterization of dynamic samples and was termed OCT angiography (OCT-A). Higher number of scans (galvanometric scans) results in improved spatial resolution and enhanced data acquisition. For protocols A, B and C, the control samples were boar sperm in extender that were not exposed to any of the described scanning protocols.

The irradiation protocol for human semen samples was performed with a constant 300x300 sampling density and 60 seconds exposure time. The semen samples were separated into three groups: 0 mW (control), 3 mW power and 20 mW power at 1077 nm. By comparing the outcomes at different power levels, the most suitable energy intensity that ensures reliable scanning results without compromising sperm quality could be established.

7.3.1. Protocol A: Effects of varying overall time of acquisition (with constant time to scan) on sperm parameters

Exposure of boar spermatozoa in a commercial extender to varying overall exposure times (20 to 60 seconds) resulted in no significant change in the tested sperm parameters (progressive motility, total motility, vitality, acrosome reactivity and sperm DNA damage).

With regards to the sperm kinetics (**Figure 45.A**) parameters, the control showed a similar VAP (Control: 50.5 ± 2.2 µm/s; 20 seconds: 45.3 ± 2.2 µm/s; 40 seconds: 34.0 ± 6.7 µm/s; 60 seconds: 42.5 ± 1.8), VSL (Control: 43.0 ± 2.3 µm/s; 20 seconds: 36.3 ± 1.5 µm/s; 40 seconds: 29.0 ± 6.0 µm/s; 60 seconds: 44.3 ± 11.2) and VCL (Control: 57.7 ± 4.0 µm/s; 20 seconds: 52.3 ± 5.2 µm/s; 40 seconds: 37.5 ± 6.8 µm/s; 60 seconds: 48.7 ± 3.2) for the different acquisition times (20 seconds, 40 seconds and 60 seconds).

Table 16. Boar sperm parameters subjected to varying overall exposure time at constant time to scan.

Exposure Time (seconds)	Progressive motility (%)	Total motility (%)	Vitality (%)	Acrosome Intact (%)	DFI (%)
0 (Control)	31.0 ± 16.6	48.2 ± 19.8	75.8 ± 10.5	73.3 ± 10.6	2.9 ± 0.4
20	14.7 ± 3.2	42.7 ± 11.9	90.4 ± 4.8	88.5 ± 4.5	2.3 ± 0.3
40	24.7 ± 18.7	35.3 ± 16.8	89.9 ± 4.6	87.5 ± 4.5	2.6 ± 0.3
60	23.7 ± 18.0	34.8 ± 14.3	84.7 ± 7.1	82.4 ± 7.3	2.6 ± 0.3
p value	.901	.921	.487	.615	.745

Values represent the average ± standard error. This experiment was repeated 3 times using 3 different boar ejaculates.

7.3.2. Protocol B: Boar sperm parameters subjected to varying scanning frequency at constant overall time of acquisition (60 seconds)

Exposure of boar spermatozoa in a commercial extender to varying scanning frequency, at time of acquisition being 60 seconds, resulted in no significant change in the tested sperm parameters (progressive motility, total motility, vitality, acrosome reactivity and sperm DNA damage).

With regards to the sperm kinetics (**Figure 45.B**) parameters, the control showed a similar VAP (Control: 52.0 ± 1.7 µm/s; 300x300: 46.0 ± 1.9 µm/s; 500x500: 47.4 ± 1.5 µm/s; 2000x2000: 45.6 ± 1.6 µm/s), VSL (Control: 42.7 ± 2.9 µm/s; 300x300: 37.9 ± 2.6 µm/s; 500x500: 37.3 ± 1.9 µm/s; 2000x2000: 36.8 ± 1.0 µm/s) and VCL (Control: 55.8 ± 1.6 µm/s; 300x300: 50.9 ± 2.0 µm/s; 500x500: 52.0 ± 1.3 µm/s; 2000x2000: 50.0 ± 1.7 µm/s) for the varying scanning frequencies.

Table 17. Boar sperm parameters subjected to varying scanning frequency at constant overall time of acquisition (60 seconds).

Imaging Rate	Progressive motility (%)	Total motility (%)	Vitality (%)	Acrosome Intact (%)	DFI (%)
300x300	24.4±5.1	61.3±11.2	93.3±1.7	91.7±2.0	2.7±0.5
500x500	24.3±4.6	62.8±9.7	93.5±1.9	92.1±2.1	2.5±0.4
2000x2000	21.5±2.9	60±8.5	93.4±1.7	92.1±2.0	2.2±0.3
Control	30.9±3.9	64.5±6.8	94.3±1.8	92.6±2.1	2.6±0.6
p value	.464	.982	.982	.992	.907

Values represent the average ± standard error. This experiment was repeated 5 times using 5 different boar ejaculates.

7.3.3. Protocol C: Boar sperm parameters subjected to varying optical coherence tomography (OCT) scanning protocol

Exposure of boar spermatozoa in a commercial extender to varying OCT scanning protocols (5 N, 10 N and 20 N) resulted in no significant change in the tested sperm parameters (progressive motility, total motility, vitality, acrosome reactivity and sperm DNA damage).

With regards to the sperm kinetics (**Figure 45.C**) parameters, the control showed a similar VAP (Control: 42.0 ± 4.8 µm/s; 5 N: 44.2 ± 1.0 µm/s; 10 N: 49.0 ± 2.0 µm/s; 20 N: 45.0 ± 5.1 µm/s), VSL (Control: 33.7 ± 3.4 µm/s; 5 N: 36.3 ± 1.9 µm/s; 10 N: 41.0 ± 2.3 µm/s; 20 N : 38.7 ± 2.9 µm/s) and VCL (Control: 46.8 ± 5.7 µm/s; 5 N: 49.2 ± 0.3 µm/s; 10 N:55.5 ± 1.8 µm/s; 20 N: 48.5 ± 6.1 µm/s) for the varying scanning protocols.

Table 18. Boar sperm parameters subjected to varying optical coherence tomography (OCT) scanning protocol.

OCT-A	Progressive motility (%)	Total motility (%)	Vitality (%)	Acrosome Intact (%)	DFI (%)
5 N	13.3 ± 0.7	39.8 ± 0.8	90.8 ± 0.3	88.2 ± 0.7	2.0 ± 0.3
10 N	13 ± 2.6	32.7 ± 4.1	91.1 ± 3.8	89.1 ± 4.8	1.9 ± 0.3
20 N	9.3 ± 1.7	24.0 ± 5.5	91.6 ± 2.1	89.2 ± 2.7	2.1 ± 0.2
Control	11.3 ± 3.9	32.2 ± 6.4	93.0 ± 2.3	90.3 ± 2.8	2.0 ± 0.2
p value	.676	.207	.931	.972	.989

Values represent the average ± standard error. This experiment was repeated 3 times using 3 different boar ejaculates.
N represents the number of times the galvanometric scanner scans the same line.

7.3.4. Human sperm parameters following varied irradiation power.

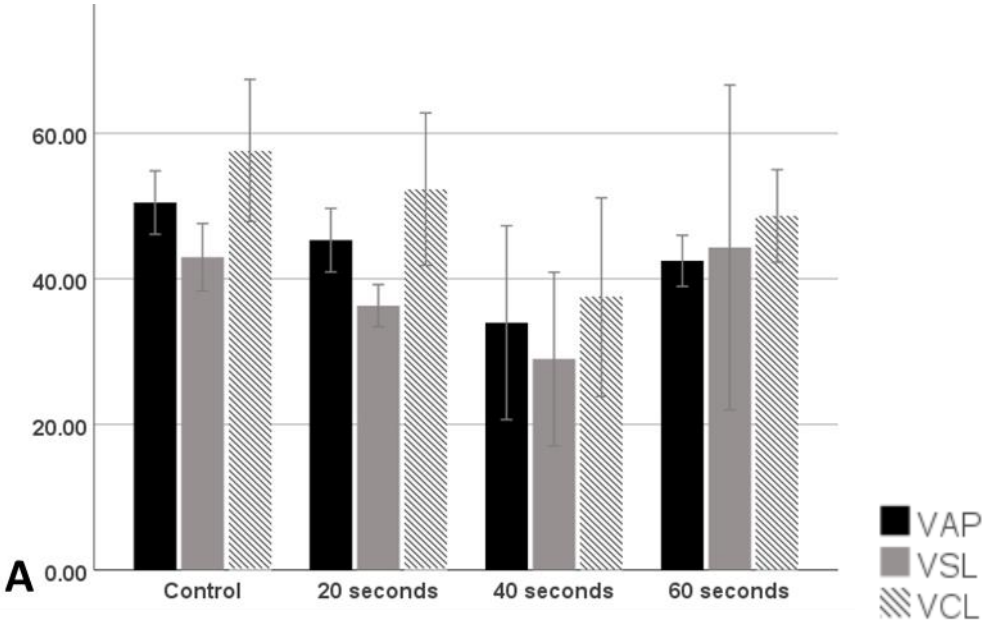
Exposure of human semen samples at 300 x 300 sampling density for 60 seconds at varied power (3 mW and 20 mW at 1077 nm) resulted in no significant change in the tested parameters across the different groups.

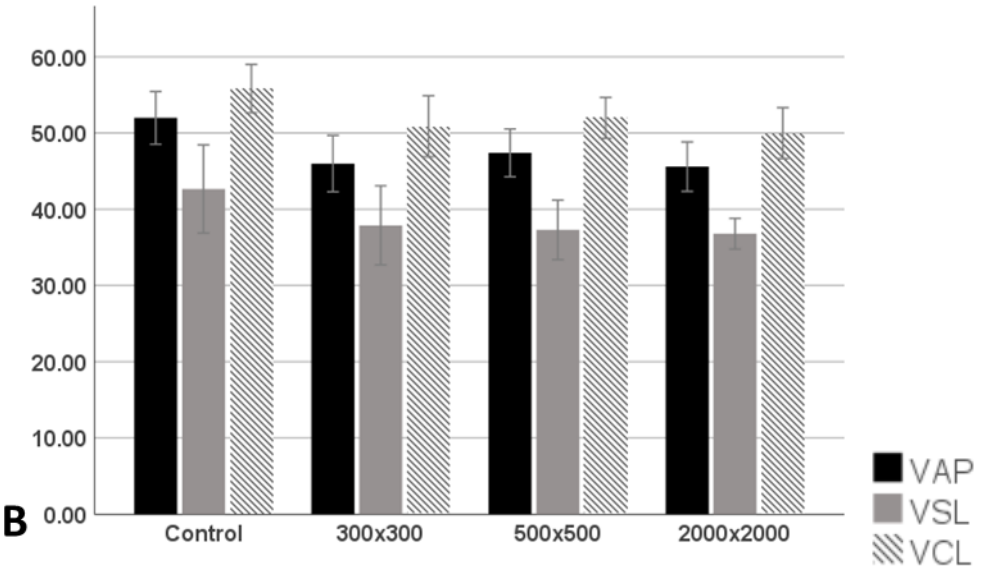
With regards to the sperm kinetics (**Figure 45.D**) parameters, the control showed a similar VAP (Control: 22.8 ± 1.6 µm/s; 3 mW: 24.1 ± 1.1 µm/s; 20 mW: 21.4 ± 2.0 µm/s), VSL (Control: 18.5 ± 1.6 µm/s; 3 mW: 18.6 ± 1.0 µm/s; 20 mW: 16.4 ± 1.8 µm/s) and VCL (Control: 35.7 ± 4.5 µm/s; 3 mW: 35.1± 2.8 µm/s; 20 mW: 32.6 ± 3.6 µm/s) for the two different irradiation powers (3 mW and 20 mW) tested.

Table 19. Human sperm parameters following varying irradiation power.

	Progressive motility (%)	Total motility (%)	Vitality (%)	DFI (%)
Control	12.0 ± 2.2	51.7 ± 3.8	64.7 ± 5.8	28.5 ± 6.6
3 mW	11.6 ± 2.3	51 ± 4.8	63.7 ± 5.3	27.6 ± 6.9
20 mW	11.9 ± 2.3	46.3 ± 5.3	67.0 ± 6.6	28.5 ± 7.5
p value	.992	.671	.921	.995

Values represent the average ± standard error. This experiment was repeated using 6 different human ejaculates from 3 donors.


A
60.00
40.00
20.00
0.00
Control
20 seconds
40 seconds
60 seconds
VAP
VSL
VCL


B
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Control
300x300
500x500
2000x2000
VAP
VSL
VCL

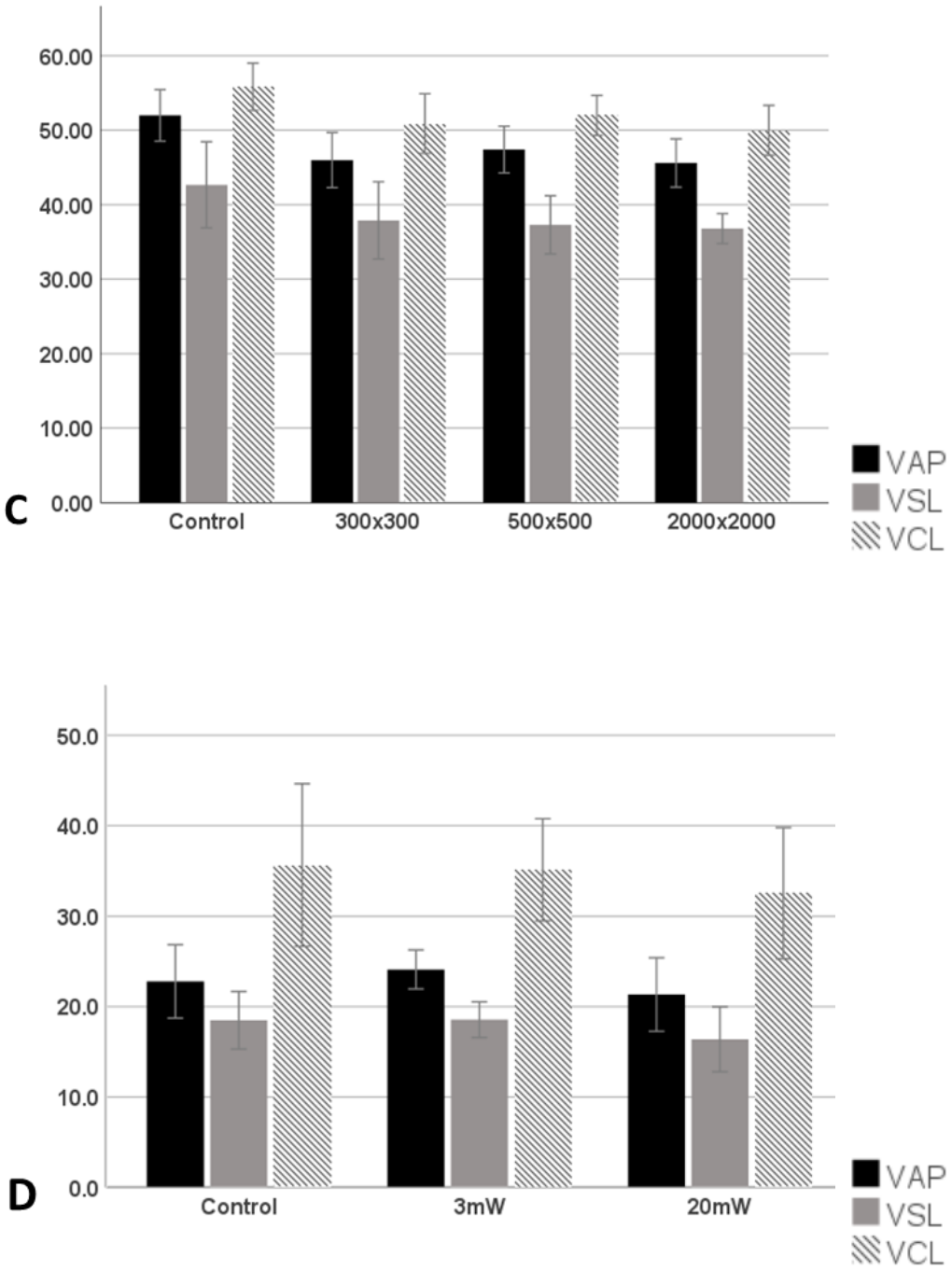


Figure 45. Boar and human sperm motility parameters when subjected to varying conditions: A) boar sperm subjected to varied overall time of acquisition, b) boar sperm subjected to varied scanning frequency, c) boar sperm subjected to varying scanning protocol and d) human sperm irradiated with two different optical powers.

7.4. Discussion

Optical coherence tomography (OCT) is an imaging method utilising low coherence interferometry to produce 3-Dimensional (3-D) cross-sectional images (Popescu et al., 2011). OCT imaging provides high resolution depth profiling and has the potential of analyzing 3-D embryo volumes providing additional information for selecting the optimal embryo for transfer. The inclusion of an OCT technology merged with traditional time-lapse systems could help not only improve embryo selection but also allow for the identification of potential embryonic defects that might not be otherwise observable. Additionally, the in-depth OCT imaging could allow for visualization of subcellular structures in oocytes and help provide a guide to the position of the microinjection needles during ICSI procedures, potentially increasing safety and accuracy of ICSI. However, OCT requires the exposure of gametes/embryos to infrared irradiation, therefore, before analyzing the potential benefits of OCT in a clinical setting, the safety of the OCT based imaging (broadband infrared sources at c.1077 nm) needs to be assessed. In this feasibility study, analysis of boar and human sperm parameters following exposure to varying irradiation conditions, were assessed. Our results suggest that there is little effect on sperm parameters from exposure following broadband IR infrared irradiation. The proposed testing of OCT based imaging is intended for eventual use in embryology. This study not only provides valuable information on the safety and benefits of OCT based imaging, but it is also the first systemic study analyzing the genotoxic impact of broadband infrared on boar and human sperm.

Although multiple studies have analysed the effects of infra-red exposure on sperm parameters, the outcome of the results are diverse, largely due to varied IR wavelengths

and intensity used in the different studies. Firestone et al. conducted a study on the effects of infrared exposure at 905 nm on sperm motility and sperm DNA damage. Their findings showed improved sperm motility and no significant change in sperm DNA damage levels following infrared irradiation (Firestone et al., 2011). Another study, by Preece et al., irradiated the sperm at 633 nm and also showed increased sperm motility following irradiation (Preece et al., 2017). Additionally, the infrared irradiation did not induce significant levels of oxidative stress, nor did it increase levels of double-strand DNA breaks as measured by testing for γH2AX. The increased sperm motility following infrared irradiation could be due to the photonic energy in red light being absorbed by the mitochondrial cytochrome-c oxidase boosting ATP production and subsequently improving sperm motility (Breitbart et al., 1996; Karu & Kolyakov, 2005).

The ability of OCT to provide high-resolution imaging without the need for physical contact with the sample has made it a useful exploratory tool in developmental biology research. This imaging method has been utilized in various non-mammalian model systems to capture live structural embryonic images with the primary focus on embryonic vasculature system (Garita et al., 2011; Kagemann et al., 2008; Mariampillai et al., 2007; Rugonyi et al., 2008). Larina et al. utilized *in utero* imaging methods to acquire images of the embryonic eye during different embryo developmental stages (Larina et al., 2012). Another study, by the same group, focused on live imaging of mouse embryos *in utero* using OCT from 12.5 to 18.5 days post coitus (Syed et al., 2011).

Although multiple studies have focused on *in vitro* and *in vivo* use of OCT during embryogenesis, very few studies have focused on the safety of an OCT system. A study conducted by Takae et al. analysed the safety of OCT examination on mouse ovarian

tissue. They demonstrated that OCT did not affect reproductive outcomes (including fertilisation rate and blastocyst rate) nor was it associated with increased birth defect rates (Takae et al., 2017). In addition, their research was able to report primordial follicle detection on unfixed ovaries indicating its potential clinical application in assessing ovarian reserve.

For OCT to be a viable imaging tool for use with traditional time-lapse system, it is necessary to show that the laser irradiation does not induce damage to the gametes. DNA damage in sperm has been associated with reduced fertilisation rates, increased chances of miscarriage, reduced embryo quality and reduced offspring health (Henkel et al., 2004; Ribas-Maynou & Benet, 2019; Zhu et al., 2022; Zini et al., 2008). Our study demonstrates that boar sperm exposed to broadband infrared at 1077 nm at varying exposure time, imaging rates and sampling density had no significant effect on sperm DNA damage. The optimal OCT parameters for use on human sperm were selected based on the results from the boar sperm samples (300x300 sampling density at 60 seconds exposure time). Two varying power levels were tested on the human sperm (3mW and 20mW), however, even at the high-power level of 20mW, there was no effect on sperm parameters including sperm DNA damage levels. It is worth noting that the human semen samples used in this study had moderate DFI levels, therefore analysis of more human semen samples having varying levels of sperm DNA damage levels is required.

The acrosome reaction is also a critical process for successful fertilization (Hirohashi & Yanagimachi, 2018; Tello-Mora et al., 2018), and the finding that infrared illumination does not impact the proportion of boar sperm undergoing acrosome reaction suggests

that the treatment does not compromise the structural or functional integrity of sperm membrane confirming the preserved fertilization potential of spermatozoa. A limitation to this is that the acrosome integrity was only assessed in boar spermatozoa, and not human spermatozoa, although this will be assessed in future studies. However, a study conducted by Singer *et al.* on human sperm indicated that there was no effect on sperm acrosome reactivity following irradiation at 940 nm (Singer et al., 1991).

Although OCT images of oocytes were taken, the fertilisation capacity of irradiation sperm and oocytes were not assessed. Therefore, the results from this feasibility study warrants further investigation in analyzing the fertilisation and embryo development potential following gamete and embryo irradiation for developing a more comprehensive embryo imaging technology. Additional further testing will involve the use of an integrated time-lapse system utilizing additional OCT technology to optimize embryo monitoring and selection.

7.5. Conclusion

To conclude, the use of OCT for imaging embryos can allow for non-invasive imaging of embryos and has the ability of providing valuable information about the timing and sequencing of key embryo developments. All irradiated samples showed no significant difference when compared to their matched manipulation control. This provides valuable information for those considering the development of OCT based imaging systems.

8. Chapter 8: General Discussion and Future Prospects

8.1. Overview

Studies have shown that sperm DNA damage is correlated to reproductive success rates (Aitken et al., 2010; Robinson et al., 2012; Zheng et al., 2018; Zhu et al., 2022). Therefore, due to emerging evidence on the links between sperm DNA damage and reproductive outcomes, the 6th edition of the WHO Manual for the Laboratory Examination and Processing of Human Semen, has included sperm DNA damage testing, as an extended examination, to guide the clinical characterisation of fertile or infertile men (World Health Organization, 2021).

However, substantial open questions remain regarding the degree to which academic understanding of sperm quality biomarkers has been (or may in future be) translated to clinical practice. Obstacles to test uptake include a lack of clinical awareness of which tests are available, genuine scientific uncertainties as to the interrelationships between these tests and clinical outcomes, and logistical / cost barriers to test implementation. Additionally, for some aspects of sperm function there is as yet no validated test for clinical use, and thus future basic research is needed to develop appropriate methods to assay these.

The objectives of this thesis were therefore: (1) to assess the clinical understanding and demand for existing sperm function tests in the UK and abroad amongst urologists; (2) to analyse the links between ROS and ORP in semen samples and their effect on sperm DNA fragmentation; (3) to characterise the SCSA/AOFT test in our own laboratory and generate supporting data to enable offering it as a service; (4) and (5) to test novel

methods of remedying the consequences of sperm DNA damage either by sorting of sperm or by improved characterization of post-fertilization embryos.

Having achieved these aims, accordingly, **Chapters 3-5** of the thesis provide a platform for improved diagnostic testing of UK patients undergoing fertility investigations, grounded in an understanding both of clinical need and of how different measurements of oxidative stress relate to sperm DNA damage. The following two chapters, **Chapters 6-7**, provide data to support the development for two advanced treatment methods: improved processing of semen samples by microfluidic separation, and improved imaging of fertilization and early embryological events by OCT.

8.2. Understanding and meeting clinical needs for sperm testing in the UK

While obstetrician-gynaecologists are typically the first physicians that couples present to for a fertility evaluation, urologists play a key role in the diagnosis and management of male factor infertility issues. Therefore, a survey was conducted targeting urologists practicing in different geographical regions, to analyse perceptions towards advanced semen analysis tests. Overall, results from the survey showed strong agreement amongst the participants as to what they consider to be clinically relevant parameters in a basic semen analysis. There was also a clear desire to use more advanced testing where required, with 58% of urologists offering sperm DNA damage testing to their patients, 17% offering sperm aneuploid testing and 22% offering seminal oxidative stress testing (however, it is essential to recognize that the perceived unmet need for these tests may primarily be voiced by specific groups, some of which have a vested

interest due to their involvement in the development or marketing of such tests). A significant number of urologists indicated that they would like to offer these tests but were unable (or unwilling) to do so. This indicates a clear unmet need in this area, with the key barriers to use being scientific uncertainties on the value of testing, and the cost/availability of reliable validated tests. Test unfamiliarity was a key factor in relation to oxidative stress testing, while cost and availability were the key factors in relation to sperm DNA damage testing, despite a strong consensus in favour of carrying out this test. Hence, we designed projects (**Chapters 4 - 5**) to address these problems.

In relation to the scientific uncertainties surrounding oxidative stress testing, a correlational study was carried out to clarify the relationship between ORP, ROS and seminal parameters (including sperm DNA damage). Both ROS and ORP assays are valid techniques for measuring seminal oxidative stress levels, however, they provide different information on the overall seminal oxidative status. In the retrospective study, combinatory measurements of seminal ORP levels and seminal ROS levels were assessed and correlated to different sperm parameters. High ORP levels does not necessarily equate to high ROS levels, and vice versa. 24% of semen samples had high ORP levels but low ROS levels, while 11% of the semen samples had low ORP levels but high ROS levels. High ORP and/or ROS levels were associated with overall poorer sperm parameters.

The highest incidence of leukocytospermia was found in patients that had high ROS and low ORP seminal levels. This is almost certainly due to the fact that the ROS measurement includes *both* intra- and extra-cellular ROS, while ORP measures the net balance of *extracellular* ROS and antioxidants. The ROS measurement will therefore

include ROS found within the leukocytes themselves, while ORP will only measure leukocyte-derived ROS that has been released into the seminal plasma. Additionally, 12% of patients that were normozoospermic had high ROS while 15% of normozoospermic patients had high ORP. Therefore, this suggests that patients diagnosed as having unexplained infertility could have high oxidative stress levels, that would remain undiagnosed unless these patients undergo advanced sperm testing.

When patients exhibit inconsistent or discordant results between seminal oxidation-reduction potential (ORP) and reactive oxygen species (ROS) measurements, it poses a challenge in determining the appropriate course of action. In such cases, it becomes difficult to fully comprehend the underlying factors contributing to the oxidative stress levels and make informed decisions regarding treatment options more challenging. Therefore, further work is required to design a systematic approach that can assist in understanding the treatment pathway for patients with discordant ORP and ROS results.

In relation to the uncertainties surrounding sperm DNA damage testing, the urologists' responses indicate they believe single and double strand breaks are the most important DNA damage to measure, while other types of DNA damage are less significant to test for. If this perception is correct, it would be beneficial to develop more targeted tests specifically designed for detecting single and double strand breaks. If, however this is not correct, this indicates a necessity for further outreach and education to educate clinicians about the significance of other types of DNA damage. With male oxidative stress infertility being increasingly appreciated as a cause of male infertility (Elbardisi et al., 2020; Wright et al., 2014), and the strong correlations we found in **Chapter 4**, I favour

the latter viewpoint, since oxidative damage not only leads to single and double strand breaks but to multiple types of lesions (Nowicka-Bauer & Nixon, 2020).

The survey (**Chapter 3**) also highlighted a need for more cost effective and readily available tests for measuring sperm DNA damage. Providing an in-house UK based flow cytometric sperm DNA damage testing service could help overcome the current obstacles to this provision, allowing timely results to be produced and aiding clinicians to make informed decisions promptly. Based on the need for a low-cost, fast turnaround assay that measures multiple sources of DNA damage, the acridine orange-based flow cytometric test (AOFT) was validated for use in future studies and for eventual commercial service provision (**Chapter 5**). The findings from this study have shown that the AOFT is a valid test that is reliable and consistent.

As 59% of respondents in the survey said that it will be useful to provide a "suite" of different DNA fragmentation tests, TUNEL is also in process of validation, with the intent of being offered in conjunction to the AOFT. The combination of multiple DNA fragmentation tests can potentially offer independent and complementary information about sperm quality and its impact on fertility outcomes. This can improve the prognostic value of the testing process and aid in predicting the success of fertility treatments. However, integrating multiple DNA fragmentation tests results can introduce complexities in interpretation. Discordant results, between different assays, may require further investigation and discussions among healthcare professionals, to understand their implications and guide treatment decisions effectively. This may increase the complexity and time required for result analysis and decision-making processes.

Future work involves determining in house AOFT (and TUNEL) threshold values based on sperm parameters and reproductive outcomes, to improve diagnostic accuracy. Establishing in-house sperm DNA damage threshold values fosters a culture of continuous quality improvement. By regularly monitoring and reassessing the correlation between AOFT (and TUNEL) results and reproductive outcomes, threshold values can be refined over time. This ongoing evaluation helps to ensure that the thresholds remain relevant and reflective of the latest evidence and clinical practice, improving the overall quality and reliability of the AOFT (and TUNEL) testing process.

8.3. Trialing methodologies to remedy sperm DNA damage

For patients exhibiting high levels of sperm DNA damage, lifestyle modifications including reduced alcohol intake, stopping smoking and improved diet, can help improve sperm quality. Apart from lifestyle changes, sperm preparation techniques can also help reduce sperm DNA damage levels present in a semen sample. Microfluidic based sperm preparation has emerged as an alternative sperm preparation technique, that allows for sperm to be naturally selected based on motility, morphology and DNA integrity (Nosrati et al., 2017). As density gradient centrifugation (DGC) is the gold standard for boar sperm preparation, sperm parameters following DGC and microfluidics chip-based sperm sorting (MCSS) were compared. Both DGC and MCSS significantly reduced boar sperm DNA damage levels, with the lowest sperm DNA damage levels obtained following microfluidics sperm preparation. The results from this study indicate that microfluidic sperm preparation could allow for a promising technology and could help improve the success rates of pig ART. However, further work is needed on selecting

optimal sperm handling medium to be placed in the outlet port of the microfluidics device.

Additionally, further research was conducted at the University of Kent, in collaboration with another PhD student (Maria Serrano Albal), to compare fertilisation outcomes following DGC and MCSS. MCSS showed improved blastocyst quality relative to DGC (although not statistically significant), likely due to reduced sperm DNA damage levels associated with MCSS (**Figure 46**).

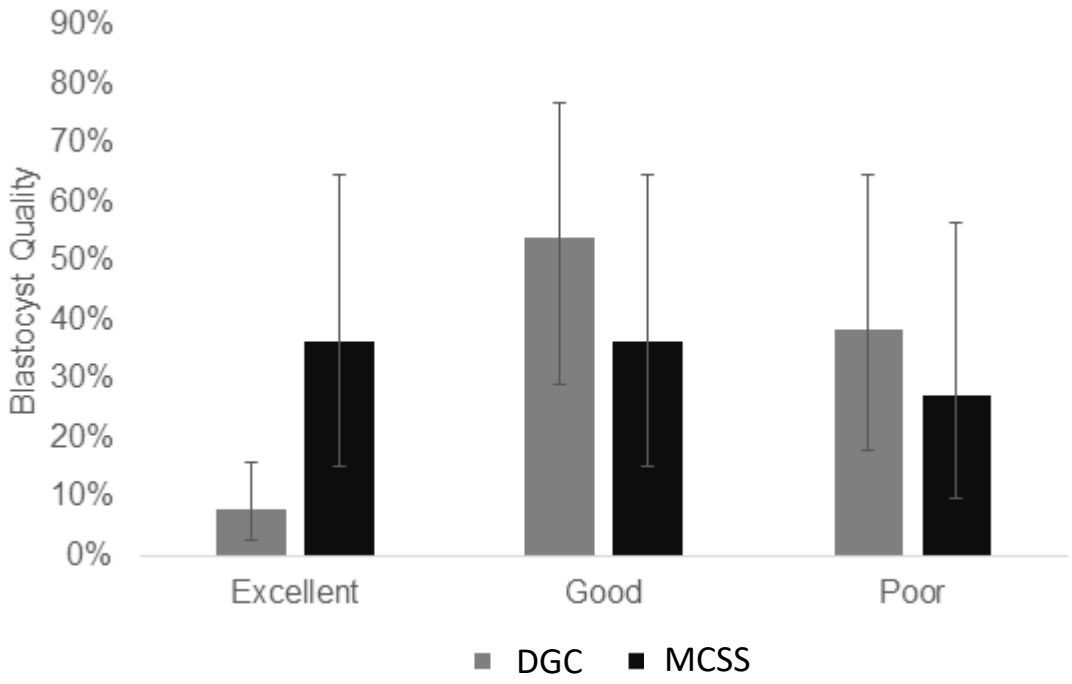


Figure 46. Effect of both sperm selection methods on blastocyst quality (Serrano-Albal *et al.*, 2023). Error bars show the 95% confidence interval (N=48). Groups did not differ ($p > 0.05$).

Sperm DNA quality has adverse influence on embryonic outcomes; therefore, it is imperative that genotoxic effects of any new technology, with the intention to be used in fertility clinics, is comprehensively assessed. Optical coherence tomography (OCT) is an imaging method that utilises low coherence interferometry, to provide high resolution depth profiles. This has the potential of analyzing 3-D embryo volumes, providing additional information for selecting optimal embryo for transfer. The use of OCT could prove to have extensive benefits as an add on technology to traditional time-lapse systems. As OCT requires the exposure of gametes/embryos to infrared irradiation, before analyzing the potential benefits of OCT on embryos in a clinical setting, the safety of the OCT based imaging (broadband infrared sources at c.1077 nm) was investigated.

No significant effect on sperm parameters following broadband infrared irradiation employed by the OCT system was observed. Subsequentially, following the safety assessment of OCT on spermatozoa, oocyte imaging was undertaken by the University of Kent Optics Group. OCT stacks of porcine oocytes were taken for imaging (**Figure 47**) and cumulus movement, following addition of spermatozoa, could be visually observed. Future work involves analysing the oocyte fertilization potential following irradiation.

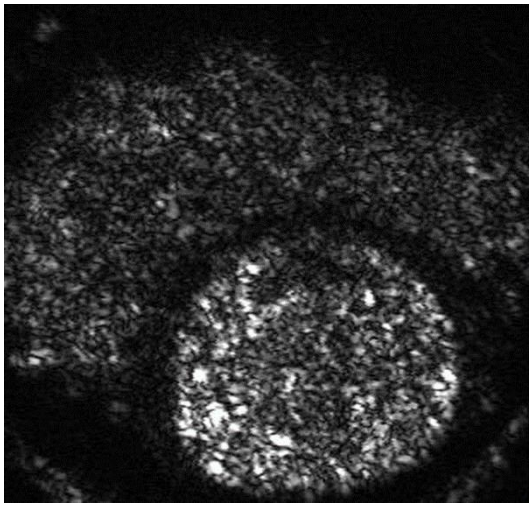


Figure 47. A slice extraction of an OCT image of in vitro matured porcine cumulus-oocyte complex at a specific time at one volume obtained from an OCT depth time-lapse analysis (obtained with permission from the University of Kent Optics Group).

8.4. Future Prospects

In the preceding sections I have highlighted specific areas where further research is needed. As we develop and improve the provision of advanced sperm testing in the UK, I envisage the following projects:

A) Ongoing survey analysis to assess whether the urologist perspectives on advanced sperm testing evolves over time. Furthermore, future survey should include questions designed to gather insights from urologists on their preferences for specific tests during a male fertility evaluation. By incorporating these questions, the study aims to bridge the gap between clinicians and scientists, fostering a better understanding of urologists' needs and preferences in the field of male fertility assessment.

B) Collaborate with clinicians to move beyond simple correlative work and make the link between sperm DNA damage and reproductive outcomes. The data generated from this collaboration will not only contribute to a better understanding of the relationship between sperm DNA damage and reproductive success but will also aid in the establishment of clinically relevant thresholds. This valuable information will guide clinicians in making informed decisions regarding treatment options and interventions, ultimately improving patient care in the field of reproductive medicine.

C) Further optimisations with the microfluidics sperm separation including optimisation of the media used. Refining the composition and properties of the media, can improve reproductive outcomes. Exploring different formulations and components of the media can help create an ideal environment for sperm processing, ensuring optimal sperm quality and functionality. Furthermore, it would be valuable to assess the usefulness of microfluidics sperm separation devices on poorer quality semen samples. By testing the performance of the devices on samples with lower sperm quality parameters, such as reduced motility or abnormal morphology, the efficacy of microfluidics sperm separation in challenging scenarios can be evaluated. This assessment will provide valuable insights into the device's ability to select the most viable and functional sperm from suboptimal semen samples, potentially expanding the range of patients who can benefit from this technology.

D) Integration of OCT with time-lapse system for more in depth morphokinetic monitoring of embryos. Furthermore, it would be valuable to investigate the potential influence of infra-red irradiation on oocyte fertilization capacity and embryonic development. By conducting controlled experiments and assessing the effects of infra-

red irradiation on various aspects of the fertilization process, such as sperm-oocyte interactions, fertilization rates, and subsequent embryonic development, we can gain insights into the impact of this technology on embryo selection and reproductive outcomes.

9. References

Abid, S. N., Richardson, T. E., Powell, H. M., Jaichander, P., Chaudhary, J., Chapman, K. M., & Hamra, F. K. (2014). A-Single Spermatogonia Heterogeneity and Cell Cycles Synchronize with Rat Seminiferous Epithelium Stages VIII–IX. *Biology of Reproduction*, *90*(2), 32. https://doi.org/10.1095/biolreprod.113.113555

Adiga, S. K., Upadhya, D., Kalthur, G., Sadashiva, S. R. B., & Kumar, P. (2010). Transgenerational changes in somatic and germ line genetic integrity of first-generation offspring derived from the DNA damaged sperm. *Fertility and Sterility*, *93*(8), 2486–2490. https://doi.org/10.1016/j.fertnstert.2009.06.015

Afanasieva, K. S., Zazhytskaya, M. O., & Sivolob, A. V. (2009). Mechanisms of DNA exit during neutral and alkaline comet assay. *Cytology and Genetics*, *43*(6), 367–370. https://doi.org/10.3103/S0095452709060012

Agarwal, A., Ahmad, G., & Sharma, R. (2015). Reference values of reactive oxygen species in seminal ejaculates using chemiluminescence assay. *Journal of Assisted Reproduction and Genetics*, *32*(12), 1721–1729. https://doi.org/10.1007/s10815-015-0584-1

Agarwal, A., Cho, C.-L., Esteves, S. C., & Majzoub, A. (2017). Current limitation and future perspective of sperm DNA fragmentation tests. *Translational Andrology and Urology*, *6*(Suppl 4), Article Suppl 4. https://doi.org/10.21037/tau.2017.05.11

Agarwal, A., Gupta, S., & Sharma, R. (2016). Reactive Oxygen Species (ROS) Measurement. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological Evaluation of Male Infertility: A Laboratory Guide* (pp. 155–163). Springer International Publishing. https://doi.org/10.1007/978-3-319-26797-5_21

Agarwal, A., & Majzoub, A. (2017). Role of Antioxidants in Assisted Reproductive Techniques. *The World Journal of Men's Health*, *35*(2), 77–93. https://doi.org/10.5534/wjmh.2017.35.2.77

Agarwal, A., Panner Selvam, M. K., & Ambar, R. F. (2021). Validation of LensHooke® X1 PRO and Computer-Assisted Semen Analyzer Compared with Laboratory-Based Manual Semen Analysis. *The World Journal of Men's Health*, *39*(3), 496–505. https://doi.org/10.5534/wjmh.200185

Agarwal, A., Panner Selvam, M. K., Arafa, M., Okada, H., Homa, S., Killeen, A., Balaban, B., Saleh, R., Armagan, A., Roychoudhury, S., & Sikka, S. (2019). Multi-center evaluation of oxidation-reduction potential by the MiOXSYS in males with abnormal semen. *Asian Journal of Andrology*, *21*(6), 565–569. https://doi.org/10.4103/aja.aja_5_19

Agarwal, A., Roychoudhury, S., Sharma, R., Gupta, S., Majzoub, A., & Sabanegh, E. (2017). Diagnostic application of oxidation-reduction potential assay for measurement of oxidative stress: Clinical utility in male factor infertility. *Reproductive BioMedicine Online*, *34*(1), 48–57. https://doi.org/10.1016/j.rbmo.2016.10.008

Agarwal, A., Sharma, R. K., Nallella, K. P., Thomas, A. J., Alvarez, J. G., & Sikka, S. C. (2006). Reactive oxygen species as an independent marker of male factor infertility. *Fertility and Sterility*, *86*(4), 878–885. https://doi.org/10.1016/j.fertnstert.2006.02.111

Agarwal, A., Sharma, R., Roychoudhury, S., Plessis, S. D., & Sabanegh, E. (2016). MiOXSYS: A novel method of measuring oxidation reduction potential in semen

and seminal plasma. *Fertility and Sterility*, *106*(3), 566-573.e10. https://doi.org/10.1016/j.fertnstert.2016.05.013

Agarwal, A., Virk, G., Ong, C., & du Plessis, S. S. (2014). Effect of Oxidative Stress on Male Reproduction. *The World Journal of Men's Health*, *32*(1), 1–17. https://doi.org/10.5534/wjmh.2014.32.1.1

Ahmadi, A., & Ng, S. C. (1999). Fertilizing ability of DNA-damaged spermatozoa. *The Journal of Experimental Zoology*, *284*(6), 696–704. https://doi.org/10.1002/(sici)1097-010x(19991101)284:6<696::aid-jez11>3.0.co;2-e

Ahmadi, S., Bashiri, R., Ghadiri-Anari, A., & Nadjarzadeh, A. (2016). Antioxidant supplements and semen parameters: An evidence based review. *International Journal of Reproductive Biomedicine*, *14*(12), 729–736.

Aitken, R. J. (1995). Free radicals, lipid peroxidation and sperm function. *Reproduction, Fertility, and Development*, *7*(4), 659–668. https://doi.org/10.1071/rd9950659

Aitken, R. J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Molecular Reproduction and Development*, *84*(10), 1039–1052. https://doi.org/10.1002/mrd.22871

Aitken, R. J., De Iuliis, G. N., Finnie, J. M., Hedges, A., & McLachlan, R. I. (2010). Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: Development of diagnostic criteria. *Human Reproduction*, *25*(10), 2415–2426. https://doi.org/10.1093/humrep/deq214

Aitken, R. J., & Koppers, A. J. (2011). Apoptosis and DNA damage in human spermatozoa. *Asian Journal of Andrology*, *13*(1), 36–42. https://doi.org/10.1038/aja.2010.68

Aitken, R. J., & West, K. M. (1990). Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated on Percoll gradients. *International Journal of Andrology*, *13*(6), 433–451. https://doi.org/10.1111/j.1365-2605.1990.tb01051.x

Ajina, T., Ammar, O., Haouas, Z., Sallem, A., Ezzi, L., Grissa, I., Sakly, W., Jlali, A., & Mehdi, M. (2017). Assessment of human sperm DNA integrity using two cytochemical tests: Acridine orange test and toluidine blue assay. *Andrologia*, *49*(10), e12765. https://doi.org/10.1111/and.12765

Aktan, G., Doğru-Abbasoğlu, S., Küçükgergin, C., Kadıoğlu, A., Özdemirler-Erata, G., & Koçak-Toker, N. (2013). Mystery of idiopathic male infertility: Is oxidative stress an actual risk? *Fertility and Sterility*, *99*(5), 1211–1215. https://doi.org/10.1016/j.fertnstert.2012.11.045

Alam, Z., & Poddar, R. (2022). An in-vivo depth-resolved imaging of developing zebrafish microstructure and microvasculature using swept-source optical coherence tomography angiography. *Optics and Lasers in Engineering*, *156*, 107087. https://doi.org/10.1016/j.optlaseng.2022.107087

Appasamy, M., Muttukrishna, S., Pizzey, A. R., Ozturk, O., Groome, N. P., Serhal, P., & Jauniaux, E. (2007). Relationship between male reproductive hormones, sperm DNA damage and markers of oxidative stress in infertility. *Reproductive Biomedicine Online*, *14*(2), 159–165. https://doi.org/10.1016/s1472-6483(10)60783-3

Arafa, M., Henkel, R., Agarwal, A., Robert, K., Finelli, R., Majzoub, A., & ElBardisi, H. (2020). Seminal oxidation–reduction potential levels are not influenced by the

presence of leucocytospermia. *Andrologia*, *52*(7), e13609. https://doi.org/10.1111/and.13609

Aumann, S., Donner, S., Fischer, J., & Müller, F. (2019). Optical Coherence Tomography (OCT): Principle and Technical Realization. In J. F. Bille (Ed.), *High Resolution Imaging in Microscopy and Ophthalmology: New Frontiers in Biomedical Optics* (pp. 59–85). Springer International Publishing. https://doi.org/10.1007/978-3-030-16638-0_3

Aziz, N., Saleh, R. A., Sharma, R. K., Lewis-Jones, I., Esfandiari, N., Thomas, A. J., & Agarwal, A. (2004). Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertility and Sterility*, *81*(2), 349–354. https://doi.org/10.1016/j.fertnstert.2003.06.026

Bakos, H. W., Henshaw, R. C., Mitchell, M., & Lane, M. (2011). Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertility and Sterility*, *95*(5), 1700–1704. https://doi.org/10.1016/j.fertnstert.2010.11.044

Balhorn, R. (2007). The protamine family of sperm nuclear proteins. *Genome Biology*, *8*(9), 227. https://doi.org/10.1186/gb-2007-8-9-227

Baskaran, S., Agarwal, A., Panner Selvam, M. K., Finelli, R., Robert, K. A., Iovine, C., Pushparaj, P. N., Samanta, L., Harlev, A., & Henkel, R. (2019). Tracking research trends and hotspots in sperm DNA fragmentation testing for the evaluation of male infertility: A scientometric analysis. *Reproductive Biology and Endocrinology*, *17*(1), 1–13. https://doi.org/10.1186/s12958-019-0550-3

Bedouhène, S., Moulti-Mati, F., Hurtado-Nedelec, M., Dang, P. M.-C., & El-Benna, J. (2017). Luminol-amplified chemiluminescence detects mainly superoxide anion produced by human neutrophils. *American Journal of Blood Research*, *7*(4), 41–48.

Bellastella, G., Carotenuto, R., Caiazzo, F., Longo, M., Cirillo, P., Scappaticcio, L., Carbone, C., Arcaniolo, D., Maiorino, M. I., & Esposito, K. (2022). Varicocele: An Endocrinological Perspective. *Frontiers in Reproductive Health*, *4*, 863695. https://doi.org/10.3389/frph.2022.863695

Benjamin, D., Sharma, R. K., Moazzam, A., & Agarwal, A. (2012). Methods for the Detection of ROS in Human Sperm Samples. In A. Agarwal, R. J. Aitken, & J. G. Alvarez (Eds.), *Studies on Men's Health and Fertility* (pp. 257–273). Humana Press. https://doi.org/10.1007/978-1-61779-776-7_13

Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P.-C., Nicolas, A., & Forterre, P. (1997). An atypical topoisomerase II from archaea with implications for meiotic recombination. *Nature*, *386*(6623), Article 6623. https://doi.org/10.1038/386414a0

Bianchi, P. G., Manicardi, G. C., Bizzaro, D., Bianchi, U., & Sakkas, D. (1993). Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biology of Reproduction*, *49*(5), 1083–1088. https://doi.org/10.1095/biolreprod49.5.1083

Boitrelle, F., Shah, R., Saleh, R., Henkel, R., Kandil, H., Chung, E., Vogiatzi, P., Zini, A., Arafa, M., & Agarwal, A. (2021). The Sixth Edition of the WHO Manual for Human Semen Analysis: A Critical Review and SWOT Analysis. *Life*, *11*(12), 1368. https://doi.org/10.3390/life11121368

Bouayed, J., & Bohn, T. (2010). Exogenous antioxidants—Double-edged swords in cellular redox state. *Oxidative Medicine and Cellular Longevity*, *3*(4), 228–237. https://doi.org/10.4161/oxim.3.4.12858

Boué, J., Bou A, null, & Lazar, P. (1975). Retrospective and prospective epidemiological studies of 1500 karyotyped spontaneous human abortions. *Teratology*, *12*(1), 11–26. https://doi.org/10.1002/tera.1420120103

Boveris, A., & Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochemical Journal*, *134*(3), 707–716.

Bray, I., & Gunnell, D. (2006). Advanced paternal age: How old is too old? *Journal of Epidemiology and Community Health*, *60*(10), 851–853. https://doi.org/10.1136/jech.2005.045179

Breitbart, H., Levinshal, T., Cohen, N., Friedmann, H., & Lubart, R. (1996). Changes in calcium transport in mammalian sperm mitochondria and plasma membrane irradiated at 633 nm (HeNe laser). *Journal of Photochemistry and Photobiology. B, Biology*, *34*(2–3), 117–121. https://doi.org/10.1016/1011-1344(95)07281-0

Brewer, L. R. (2011). Deciphering the structure of DNA toroids. *Integrative Biology*, *3*(5), 540–547. https://doi.org/10.1039/c0ib00128g

Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., & Giwercman, A. (2007). Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Human Reproduction*, *22*(1), 174–179. https://doi.org/10.1093/humrep/del326

Bungum, M., Humaidan, P., Spano, M., Jepson, K., Bungum, L., & Giwercman, A. (2004). The predictive value of sperm chromatin structure assay (SCSA) parameters for

the outcome of intrauterine insemination, IVF and ICSI. *Human Reproduction (Oxford, England)*, *19*(6), 1401–1408. https://doi.org/10.1093/humrep/deh280

Burden, F., Ellis, P. J. I., & Farré, M. (2023). A shared "vulnerability code" underpins varying sources of DNA damage throughout paternal germline transmission in mouse. *Nucleic Acids Research*, *51*(5), 2319–2332. https://doi.org/10.1093/nar/gkad089

Burton, J. C., Wang, S., Stewart, C. A., Behringer, R. R., & Larina, I. V. (2015). High-resolution three-dimensional in vivo imaging of mouse oviduct using optical coherence tomography. *Biomedical Optics Express*, *6*(7), 2713–2723. https://doi.org/10.1364/BOE.6.002713

Campbell, M. J., Lotti, F., Baldi, E., Schlatt, S., Festin, M. P. R., Björndahl, L., Toskin, I., & Barratt, C. L. R. (2021). Distribution of semen examination results 2020 – A follow up of data collated for the WHO semen analysis manual 2010. *Andrology*, *9*(3), 817–822. https://doi.org/10.1111/andr.12983

Caroppo, E., & Dattilo, M. (2022). Sperm redox biology challenges the role of antioxidants as a treatment for male factor infertility. *F&S Reviews*, *3*(1), 90–104. https://doi.org/10.1016/j.xfnr.2021.12.001

Castellò, D., Motato, Y., Basile, N., Remohí, J., Espejo-Catena, M., & Meseguer, M. (2016). How much have we learned from time-lapse in clinical IVF? *MHR: Basic Science of Reproductive Medici*, *22*. https://doi.org/10.1093/molehr/gaw056

Cernat, R., Tatla, T. S., Pang, J., Tadrous, P. J., Bradu, A., Dobre, G., Gelikonov, G., Gelikonov, V., & Podoleanu, A. Gh. (2012). Dual instrument for in vivo and ex vivo OCT imaging in an ENT department. *Biomedical Optics Express*, *3*(12), 3346–3356. https://doi.org/10.1364/BOE.3.003346

Chamayou, S., Patrizio, P., Storaci, G., Tomaselli, V., Alecci, C., Ragolia, C., Crescenzo, C., & Guglielmino, A. (2013). The use of morphokinetic parameters to select all embryos with full capacity to implant. *Journal of Assisted Reproduction and Genetics*, *30*(5), 703–710. https://doi.org/10.1007/s10815-013-9992-2

Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, *59*(3), 527–605. https://doi.org/10.1152/physrev.1979.59.3.527

Chen, Q., Zhao, J.-Y., Xue, X., & Zhu, G.-X. (2019). The association between sperm DNA fragmentation and reproductive outcomes following intrauterine insemination, a meta analysis. *Reproductive Toxicology*, *86*, 50–55. https://doi.org/10.1016/j.reprotox.2019.03.004

Cheng, C. Y., Wong, E. W., Lie, P. P., Li, M. W., Mruk, D. D., Yan, H. H., Mok, K.-W., Mannu, J., Mathur, P. P., Lui, W., Lee, W. M., Bonanomi, M., & Silvestrini, B. (2011). Regulation of blood-testis barrier dynamics by desmosome, gap junction, hemidesmosome and polarity proteins. *Spermatogenesis*, *1*(2), 105–115. https://doi.org/10.4161/spmg.1.2.15745

Chua, S. C., Yovich, S. J., Hinchliffe, P. M., & Yovich, J. L. (2023). The Sperm DNA Fragmentation Assay with SDF Level Less Than 15% Provides a Useful Prediction for Clinical Pregnancy and Live Birth for Women Aged under 40 Years. *Journal of Personalized Medicine*, *13*(7), Article 7. https://doi.org/10.3390/jpm13071079

Coetzee, K., Franken, D. R., Kruger, T. F., & Lombard, C. J. (1992). Effect of multiple centrifugations on the evaluation of the acrosome reaction in human spermatozoa. *Andrologia*, *24*(6), 331–334. https://doi.org/10.1111/j.1439-0272.1992.tb02664.x

Collodel, G., Castellini, C., Lee, J. C.-Y., & Signorini, C. (2020). Relevance of Fatty Acids to Sperm Maturation and Quality. *Oxidative Medicine and Cellular Longevity*, *2020*, 7038124. https://doi.org/10.1155/2020/7038124

Comar, V. A., Petersen, C. G., Mauri, A. L., Mattila, M., Vagnini, L. D., Renzi, A., Petersen, B., Nicoletti, A., Dieamant, F., Oliveira, J. B. A., Baruffi, R. L. R., & Franco Jr., J. G. (2017). Influence of the abstinence period on human sperm quality: Analysis of 2,458 semen samples. *JBRA Assisted Reproduction*, *21*(4), 306–312. https://doi.org/10.5935/1518-0557.20170052

Cruz, M., Garrido, N., Herrero, J., Pérez-Cano, I., Muñoz, M., & Meseguer, M. (2012). Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reproductive Biomedicine Online*, *25*(4), 371–381. https://doi.org/10.1016/j.rbmo.2012.06.017

CUI, X., JING, X., WU, X., WANG, Z., & LI, Q. (2016). Potential effect of smoking on semen quality through DNA damage and the downregulation of Chk1 in sperm. *Molecular Medicine Reports*, *14*(1), 753–761. https://doi.org/10.3892/mmr.2016.5318

Cummins, J. M., Breen, T. M., Harrison, K. L., Shaw, J. M., Wilson, L. M., & Hennessey, J. F. (1986). A formula for scoring human embryo growth rates in in vitro fertilization: Its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *Journal of in Vitro Fertilization and Embryo Transfer: IVF*, *3*(5), 284–295. https://doi.org/10.1007/BF01133388

Dal Canto, M., Coticchio, G., Mignini Renzini, M., De Ponti, E., Novara, P. V., Brambillasca, F., Comi, R., & Fadini, R. (2012). Cleavage kinetics analysis of human embryos

predicts development to blastocyst and implantation. *Reproductive BioMedicine Online*, *25*(5), 474–480. https://doi.org/10.1016/j.rbmo.2012.07.016

Dawson, E. B., Harris, W. A., Teter, M. C., & Powell, L. C. (1992). Effect of ascorbic acid supplementation on the sperm quality of smokers. *Fertility and Sterility*, *58*(5), 1034–1039.

De Iuliis, G. N., Thomson, L. K., Mitchell, L. A., Finnie, J. M., Koppers, A. J., Hedges, A., Nixon, B., & Aitken, R. J. (2009). DNA Damage in Human Spermatozoa Is Highly Correlated with the Efficiency of Chromatin Remodeling and the Formation of 8-Hydroxy-2'-Deoxyguanosine, a Marker of Oxidative Stress1. *Biology of Reproduction*, *81*(3), 517–524. https://doi.org/10.1095/biolreprod.109.076836

De Kretser, D. M. (1989). Illustrated Pathology of Human Spermatogenesis A. F. Holstein, E. C. Roosen-Runge, C. Schirren. *Andrologia*, *21*(3), 197–197. https://doi.org/10.1111/j.1439-0272.1989.tb02394.x

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., & Gagnon, C. (1997). Reactive oxygen species and sperm physiology. *Reviews of Reproduction*, *2*(1), 48–54. https://doi.org/10.1530/ror.0.0020048

de Macedo, M. P., Glanzner, W. G., Gutierrez, K., & Bordignon, V. (2021). Chromatin role in early programming of embryos. *Animal Frontiers: The Review Magazine of Animal Agriculture*, *11*(6), 57–65. https://doi.org/10.1093/af/vfab054

Derijck, A., van der Heijden, G., Giele, M., Philippens, M., & de Boer, P. (2008). DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. *Human Molecular Genetics*, *17*(13), 1922–1937. https://doi.org/10.1093/hmg/ddn090

Diedrich, K., Fauser, B. C. J. M., Devroey, P., Griesinger, G., & on behalf of the Evian Annual Reproduction (EVAR) Workshop Group. (2007). The role of the endometrium and embryo in human implantation. *Human Reproduction Update*, *13*(4), 365–377. https://doi.org/10.1093/humupd/dmm011

Domínguez-Fandos, D., Camejo, M. I., Ballescà, J. L., & Oliva, R. (2007). Human sperm DNA fragmentation: Correlation of TUNEL results as assessed by flow cytometry and optical microscopy. *Cytometry. Part A: The Journal of the International Society for Analytical Cytology*, *71*(12), 1011–1018. https://doi.org/10.1002/cyto.a.20484

Dunkel, L., Hirvonen, V., & Erkkilä, K. (1997). Clinical aspects of male germ cell apoptosis during testis development and spermatogenesis. *Cell Death & Differentiation*, *4*(3), 171–179. https://doi.org/10.1038/sj.cdd.4400234

Durairajanayagam, D. (2017). Commentary: The value of testing sperm DNA fragmentation in infertile men. *Translational Andrology and Urology*, *6*(Suppl 4), S678–S680. https://doi.org/10.21037/tau.2017.03.42

Duran, E. H., Morshedi, M., Taylor, S., & Oehninger, S. (2002). Sperm DNA quality predicts intrauterine insemination outcome: A prospective cohort study. *Human Reproduction (Oxford, England)*, *17*(12), 3122–3128. https://doi.org/10.1093/humrep/17.12.3122

Eggert-Kruse, W., Rohr, G., Kerbel, H., Schwalbach, B., Demirakca, T., Klinga, K., Tilgen, W., & Runnebaum, B. (1996). The Acridine Orange test: A clinically relevant screening method for sperm quality during infertility investigation? *Human Reproduction (Oxford, England)*, *11*(4), 784–789. https://doi.org/10.1093/oxfordjournals.humrep.a019255

Erkek, S., Hisano, M., Liang, C.-Y., Gill, M., Murr, R., Dieker, J., Schübeler, D., Vlag, J. van der, Stadler, M. B., & Peters, A. H. F. M. (2013). Molecular determinants of nucleosome retention at CpG-rich sequences in mouse spermatozoa. *Nature Structural & Molecular Biology*, *20*(7), Article 7. https://doi.org/10.1038/nsmb.2599

Esteves, S. C., Sánchez-Martín, F., Sánchez-Martín, P., Schneider, D. T., & Gosálvez, J. (2015). Comparison of reproductive outcome in oligozoospermic men with high sperm DNA fragmentation undergoing intracytoplasmic sperm injection with ejaculated and testicular sperm. *Fertility and Sterility*, *104*(6), 1398–1405. https://doi.org/10.1016/j.fertnstert.2015.08.028

Esteves, S. C., Zini, A., Coward, R. M., Evenson, D. P., Gosálvez, J., Lewis, S. E. M., Sharma, R., & Humaidan, P. (2021). Sperm DNA fragmentation testing: Summary evidence and clinical practice recommendations. *Andrologia*, *53*(2), e13874. https://doi.org/10.1111/and.13874

Evenson, D. P. (2013). Sperm Chromatin Structure Assay (SCSA®). In D. T. Carrell & K. I. Aston (Eds.), *Spermatogenesis: Methods and Protocols* (pp. 147–164). Humana Press. https://doi.org/10.1007/978-1-62703-038-0_14

Evenson, D. P. (2016). The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. *Animal Reproduction Science*, *169*, 56–75. https://doi.org/10.1016/j.anireprosci.2016.01.017

Evenson, D. P. (2022). Sperm Chromatin Structure Assay (SCSA®) for Fertility Assessment. *Current Protocols*, *2*(8), e508. https://doi.org/10.1002/cpz1.508

Evenson, D. P., Larson, K. L., & Jost, L. K. (2002). Sperm Chromatin Structure Assay: Its Clinical Use for Detecting Sperm DNA Fragmentation in Male Infertility and Comparisons With Other Techniques. *Journal of Andrology*, *23*(1), 25–43. https://doi.org/10.1002/j.1939-4640.2002.tb02599.x

Fathi Najafi, T., Latifnejad Roudsari, R., Namvar, F., Ghavami Ghanbarabadi, V., Hadizadeh Talasaz, Z., & Esmaeli, M. (2015). Air Pollution and Quality of Sperm: A Meta-Analysis. *Iranian Red Crescent Medical Journal*, *17*(4), e26930. https://doi.org/10.5812/ircmj.17(4)2015.26930

Fercher, A. F., Hitzenberger, C. K., Drexler, W., Kamp, G., & Sattmann, H. (1993). In Vivo Optical Coherence Tomography. *American Journal of Ophthalmology*, *116*(1), 113–114. https://doi.org/10.1016/S0002-9394(14)71762-3

Fernández, J. L., Johnston, S., & Gosálvez, J. (2018). Sperm Chromatin Dispersion (SCD) Assay. In A. Zini & A. Agarwal (Eds.), *A Clinician's Guide to Sperm DNA and Chromatin Damage* (pp. 137–152). Springer International Publishing. https://doi.org/10.1007/978-3-319-71815-6_8

Fernández, J. L., Muriel, L., Rivero, M. T., Goyanes, V., Vazquez, R., & Alvarez, J. G. (2003). The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. *Journal of Andrology*, *24*(1), 59–66. https://doi.org/10.1002/j.1939-4640.2003.tb02641.x

Fernández-Gonzalez, R., Moreira, P. N., Pérez-Crespo, M., Sánchez-Martín, M., Ramirez, M. A., Pericuesta, E., Bilbao, A., Bermejo-Alvarez, P., Hourcade, J. de D., Fonseca, F. R. de, & Gutiérrez-Adán, A. (2008). Long-Term Effects of Mouse Intracytoplasmic Sperm Injection with DNA-Fragmented Sperm on Health and

Behavior of Adult Offspring1. *Biology of Reproduction*, *78*(4), 761–772. https://doi.org/10.1095/biolreprod.107.065623

Firestone, R., Esfandiari, N., Moskovtsev, S., Burstein, E., Videna, G., Librach, C., Bentov, Y., & Casper, R. (2011). The Effects of Low-Level Laser Light Exposure on Sperm Motion Characteristics and DNA Damage. *Journal of Andrology*, *33*, 469–473. https://doi.org/10.2164/jandrol.111.013458

Fraga, C. G., Motchnik, P. A., Shigenaga, M. K., Helbock, H. J., Jacob, R. A., & Ames, B. N. (1991). Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(24), 11003–11006.

Garcia-Martin, E., Rodriguez-Mena, D., Satue, M., Almarcegui, C., Dolz, I., Alarcia, R., Seral, M., Polo, V., Larrosa, J. M., & Pablo, L. E. (2014). Electrophysiology and Optical Coherence Tomography to Evaluate Parkinson Disease Severity. *Investigative Ophthalmology & Visual Science*, *55*(2), 696–705. https://doi.org/10.1167/iovs.13-13062

García-Rodríguez, A., Gosálvez, J., Agarwal, A., Roy, R., & Johnston, S. (2018). DNA Damage and Repair in Human Reproductive Cells. *International Journal of Molecular Sciences*, *20*(1), 31. https://doi.org/10.3390/ijms20010031

Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., & Schoolcraft, W. B. (2000). Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertility and Sterility*, *73*(6), 1155–1158. https://doi.org/10.1016/s0015-0282(00)00518-5

Garolla, A., Torino, M., Sartini, B., Cosci, I., Patassini, C., Carraro, U., & Foresta, C. (2013). Seminal and molecular evidence that sauna exposure affects human

spermatogenesis. *Human Reproduction (Oxford, England)*, *28*(4), 877–885. https://doi.org/10.1093/humrep/det020

Gat, Y., Zukerman, Z., Chakraborty, J., & Gornish, M. (2005). Varicocele, hypoxia and male infertility. Fluid Mechanics analysis of the impaired testicular venous drainage system. *Human Reproduction (Oxford, England)*, *20*(9), 2614–2619. https://doi.org/10.1093/humrep/dei089

Gavrilov, L. A., & Gavrilova, N. S. (2001). Human Longevity and Parental Age at Conception. In J.-M. Robine, T. B. L. Kirkwood, & M. Allard (Eds.), *Sex and Longevity: Sexuality, Gender, Reproduction, Parenthood* (pp. 7–31). Springer. https://doi.org/10.1007/978-3-642-59558-5_2

Gawecka, J. E., Marh, J., Ortega, M., Yamauchi, Y., Ward, M. A., & Ward, W. S. (2013). Mouse Zygotes Respond to Severe Sperm DNA Damage by Delaying Paternal DNA Replication and Embryonic Development. *PLOS ONE*, *8*(2), e56385. https://doi.org/10.1371/journal.pone.0056385

Genescà, A., Caballín, M. R., Miró, R., Benet, J., Germà, J. R., & Egozcue, J. (1992). Repair of human sperm chromosome aberrations in the hamster egg. *Human Genetics*, *89*(2), 181–186. https://doi.org/10.1007/BF00217120

Gerton, J. L., & Hawley, R. S. (2005). Homologous chromosome interactions in meiosis: Diversity amidst conservation. *Nature Reviews Genetics*, *6*(6), Article 6. https://doi.org/10.1038/nrg1614

Gil-Guzman, E., Ollero, M., Lopez, M. C., Sharma, R. K., Alvarez, J. G., Thomas, A. J., Jr, & Agarwal, A. (2001). Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Human Reproduction*, *16*(9), 1922–1930. https://doi.org/10.1093/humrep/16.9.1922

Gode, F., Gürbüz, A. S., Tamer, B., Pala, I., & Isik, A. Z. (2020). The Effects of Microfluidic Sperm Sorting, Density Gradient and Swim-up Methods on Semen Oxidation Reduction Potential. *Urology Journal*, *17*(4), 397–401. https://doi.org/10.22037/uj.v0i0.5639

Goldstein, M., & Eid, J. F. (1989). Elevation of intratesticular and scrotal skin surface temperature in men with varicocele. *The Journal of Urology*, *142*(3), 743–745. https://doi.org/10.1016/s0022-5347(17)38874-2

González-Marín, C., Gosálvez, J., & Roy, R. (2012a). Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. *International Journal of Molecular Sciences*, *13*(11), 14026–14052. https://doi.org/10.3390/ijms131114026

González-Marín, C., Gosálvez, J., & Roy, R. (2012b). Types, Causes, Detection and Repair of DNA Fragmentation in Animal and Human Sperm Cells. *International Journal of Molecular Sciences*, *13*(11), 14026–14052. https://doi.org/10.3390/ijms131114026

Gorczyca, W., Traganos, F., Jesionowska, H., & Darzynkiewicz, Z. (1993). Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: Analogy to apoptosis of somatic cells. *Experimental Cell Research*, *207*(1), 202–205. https://doi.org/10.1006/excr.1993.1182

Gorrini, C., Harris, I. S., & Mak, T. W. (2013). Modulation of oxidative stress as an anticancer strategy. *Nature Reviews Drug Discovery*, *12*(12), Article 12. https://doi.org/10.1038/nrd4002

Gosalvez, Jaime, Fernandez, Jose-Luis, Yaniz, Jesus, de la Casa, Moises, Lopez-Fernandez, Carmen, & Johnston, Stephen. (2015). A Comparison of Sperm DNA Damage in the Neat Ejaculate of Sperm Donors and Males Presenting for their Initial Seminogram. *Austin J Reprod Med Infertil*. https://austinpublishinggroup.com/reproductive-medicine/fulltext/ajrm-v2-id1014.php

Griswold, M. D. (2016). Spermatogenesis: The Commitment to Meiosis. *Physiological Reviews*, *96*(1), 1–17. https://doi.org/10.1152/physrev.00013.2015

Gualtieri, R., Kalthur, G., Barbato, V., Longobardi, S., Di Rella, F., Adiga, S. K., & Talevi, R. (2021). Sperm Oxidative Stress during In Vitro Manipulation and Its Effects on Sperm Function and Embryo Development. *Antioxidants*, *10*(7), 1025. https://doi.org/10.3390/antiox10071025

Guiraldelli, M. F., Felberg, A., Almeida, L. P., Parikh, A., Castro, R. O. de, & Pezza, R. J. (2018). SHOC1 is a ERCC4-(HhH)2-like protein, integral to the formation of crossover recombination intermediates during mammalian meiosis. *PLOS Genetics*, *14*(5), e1007381. https://doi.org/10.1371/journal.pgen.1007381

Guler, C., Melil, S., Ozekici, U., Donmez Cakil, Y., Selam, B., & Cincik, M. (2021). Sperm Selection and Embryo Development: A Comparison of the Density Gradient Centrifugation and Microfluidic Chip Sperm Preparation Methods in Patients with Astheno-Teratozoospermia. *Life*, *11*(9), Article 9. https://doi.org/10.3390/life11090933

Haddock, L., Gordon, S., Lewis, S. E. M., Larsen, P., Shehata, A., & Shehata, H. (2021). Sperm DNA fragmentation is a novel biomarker for early pregnancy loss.

Reproductive BioMedicine Online, *42*(1), 175–184. https://doi.org/10.1016/j.rbmo.2020.09.016

Haglund, K., Nezis, I. P., & Stenmark, H. (2011). Structure and functions of stable intercellular bridges formed by incomplete cytokinesis during development. *Communicative & Integrative Biology*, *4*(1), 1–9. https://doi.org/10.4161/cib.4.1.13550

Hajizadeh Maleki, B., & Tartibian, B. (2015). Long-term Low-to-Intensive Cycling Training: Impact on Semen Parameters and Seminal Cytokines. *Clinical Journal of Sport Medicine: Official Journal of the Canadian Academy of Sport Medicine*, *25*(6), 535–540. https://doi.org/10.1097/JSM.0000000000000122

Hamacher, T., Berendsen, J. T. W., Kruit, S. A., Broekhuijse, M. L. W. J., & Segerink, L. I. (2020). Effect of microfluidic processing on the viability of boar and bull spermatozoa. *Biomicrofluidics*, *14*(4), 044111. https://doi.org/10.1063/5.0013919

Handel, M. A., & Schimenti, J. C. (2010). Genetics of mammalian meiosis: Regulation, dynamics and impact on fertility. *Nature Reviews Genetics*, *11*(2), Article 2. https://doi.org/10.1038/nrg2723

Harlev, A., Agarwal, A., Gunes, S. O., Shetty, A., & du Plessis, S. S. (2015). Smoking and Male Infertility: An Evidence-Based Review. *The World Journal of Men's Health*, *33*(3), 143–160. https://doi.org/10.5534/wjmh.2015.33.3.143

He, L., He, T., Farrar, S., Ji, L., Liu, T., & Ma, X. (2017). Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species. *Cellular Physiology and Biochemistry*, *44*(2), 532–553. https://doi.org/10.1159/000485089

Health, T. L. G. (2022). Infertility—Why the silence? *The Lancet Global Health*, *10*(6), e773. https://doi.org/10.1016/S2214-109X(22)00215-7

Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Gips, H., Schill, W.-B., & Kruger, T. F. (2004). Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertility and Sterility*, *81*(4), 965–972. https://doi.org/10.1016/j.fertnstert.2003.09.044

Henkel, R., Kierspel, E., Stalf, T., Mehnert, C., Menkveld, R., Tinneberg, H.-R., Schill, W.-B., & Kruger, T. F. (2005). Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. *Fertility and Sterility*, *83*(3), 635–642. https://doi.org/10.1016/j.fertnstert.2004.11.022

Henkel, R. R. (2011). Leukocytes and oxidative stress: Dilemma for sperm function and male fertility. *Asian Journal of Andrology*, *13*(1), 43–52. https://doi.org/10.1038/aja.2010.76

Hernández-Silva, G., López-Torres, A. S., Maldonado-Rosas, I., Mata-Martínez, E., Larrea, F., Torres-Flores, V., Treviño, C. L., & Chirinos, M. (2021). Effects of Semen Processing on Sperm Function: Differences between Swim-Up and Density Gradient Centrifugation. *The World Journal of Men's Health*, *39*(4), 740–749. https://doi.org/10.5534/wjmh.200115

Hirohashi, N., & Yanagimachi, R. (2018). Sperm acrosome reaction: Its site and role in fertilization†. *Biology of Reproduction*, *99*(1), 127–133. https://doi.org/10.1093/biolre/ioy045

Hofmann, M.-C., & McBeath, E. (2022). Sertoli Cell-Germ Cell Interactions Within the Niche: Paracrine and Juxtacrine Molecular Communications. *Frontiers in*

Endocrinology, *13*. https://www.frontiersin.org/articles/10.3389/fendo.2022.897062

Holmes, J. (2009). In vivo real-time optical coherence tomography imaging of Drosophila for cardiovascular research. *Nature Methods*, *6*(10), Article 10. https://doi.org/10.1038/nmeth.f.270

Holstein, A.-F., Schulze, W., & Davidoff, M. (2003). Understanding spermatogenesis is a prerequisite for treatment. *Reproductive Biology and Endocrinology : RB&E*, *1*, 107. https://doi.org/10.1186/1477-7827-1-107

Homa, S. T., Vassiliou, A. M., Stone, J., Killeen, A. P., Dawkins, A., Xie, J., Gould, F., & Ramsay, J. W. A. (2019). A Comparison Between Two Assays for Measuring Seminal Oxidative Stress and their Relationship with Sperm DNA Fragmentation and Semen Parameters. *Genes*, *10*(3), Article 3. https://doi.org/10.3390/genes10030236

Homa, S. T., Vessey, W., Perez-Miranda, A., Riyait, T., & Agarwal, A. (2015). Reactive Oxygen Species (ROS) in human semen: Determination of a reference range. *Journal of Assisted Reproduction and Genetics*, *32*(5), 757–764. https://doi.org/10.1007/s10815-015-0454-x

Horta, F., Catt, S., Ramachandran, P., Vollenhoven, B., & Temple-Smith, P. (2020). Female ageing affects the DNA repair capacity of oocytes in IVF using a controlled model of sperm DNA damage in mice. *Human Reproduction (Oxford, England)*, *35*(3), 529–544. https://doi.org/10.1093/humrep/dez308

Hoshi, K., Katayose, H., Yanagida, K., Kimura, Y., & Sato, A. (1996). The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability

of human sperm. *Fertility and Sterility*, *66*(4), 634–639. https://doi.org/10.1016/S0015-0282(16)58581-1

Huang, J., Chen, H., Li, N., & Zhao, Y. (2023). Emerging microfluidic technologies for sperm sorting. *Engineered Regeneration*, *4*(2), 161–169. https://doi.org/10.1016/j.engreg.2023.02.001

Hubbard, R. E., Andrew, M. K., & Rockwood, K. (2009). Effect of parental age at birth on the accumulation of deficits, frailty and survival in older adults. *Age and Ageing*, *38*(4), 380–385. https://doi.org/10.1093/ageing/afp035

Hughes, C. M., Lewis, S. E. M., McKelvey-Martin, V. J., & Thompson, W. (1997). Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *374*(2), 261–268. https://doi.org/10.1016/S0027-5107(96)00241-2

Hull, M. G., Glazener, C. M., Kelly, N. J., Conway, D. I., Foster, P. A., Hinton, R. A., Coulson, C., Lambert, P. A., Watt, E. M., & Desai, K. M. (1985). Population study of causes, treatment, and outcome of infertility. *British Medical Journal (Clinical Research Ed.)*, *291*(6510), 1693–1697.

Ickowicz, D., Finkelstein, M., & Breitbart, H. (2012). Mechanism of sperm capacitation and the acrosome reaction: Role of protein kinases. *Asian Journal of Andrology*, *14*(6), 816–821. https://doi.org/10.1038/aja.2012.81

Ilić, B. S., Kolarević, A., Kocić, G., & Šmelcerović, A. (2018). Ascorbic acid as DNase I inhibitor in prevention of male infertility. *Biochemical and Biophysical Research Communications*, *498*(4), 1073–1077. https://doi.org/10.1016/j.bbrc.2018.03.120

Jenkins, M. W., Chughtai, O. Q., Basavanhally, A. N., Watanabe, M., & Rollins, A. M. (2007). In vivo gated 4D imaging of the embryonic heart using optical coherence tomography. *Journal of Biomedical Optics*, *12*(3), 030505. https://doi.org/10.1117/1.2747208

Johnson, L., Neaves, W. B., Barnard, J. J., Keillor, G. E., Brown, S. W., & Yanagimachi, R. (1999). A comparative morphological study of human germ cells in vitro or in situ within seminiferous tubules. *Biology of Reproduction*, *61*(4), 927–934. https://doi.org/10.1095/biolreprod61.4.927

Johnson, L., Petty, C. S., & Neaves, W. B. (1983). Further quantification of human spermatogenesis: Germ cell loss during postprophase of meiosis and its relationship to daily sperm production. *Biology of Reproduction*, *29*(1), 207–215. https://doi.org/10.1095/biolreprod29.1.207

Kanamori, K., Ahmad, S. M., Shin, C. S., Hamid, A., & Lutfy, K. (2022). Identification of 5-Hydroxycotinine in the Plasma of Nicotine-Treated Mice: Implications for Cotinine Metabolism and Disposition in Vivo. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *50*(12), 1454–1463. https://doi.org/10.1124/dmd.122.001059

Kanvah, S., Joseph, J., Schuster, G. B., Barnett, R. N., Cleveland, C. L., & Landman, U. (2010). Oxidation of DNA: Damage to Nucleobases. *Accounts of Chemical Research*, *43*(2), 280–287. https://doi.org/10.1021/ar900175a

Karu, T. I., & Kolyakov, S. F. (2005). Exact action spectra for cellular responses relevant to phototherapy. *Photomedicine and Laser Surgery*, *23*(4), 355–361. https://doi.org/10.1089/pho.2005.23.355

Keeney, S., Giroux, C. N., & Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell*, *88*(3), 375–384. https://doi.org/10.1016/s0092-8674(00)81876-0

Keltz, J., Zapantis, A., Jindal, S. K., Lieman, H. J., Santoro, N., & Polotsky, A. J. (2010). Overweight Men: Clinical pregnancy after ART is decreased in IVF but not in ICSI cycles. *Journal of Assisted Reproduction and Genetics*, *27*(9–10), 539–544. https://doi.org/10.1007/s10815-010-9439-y

Khandwala, Y. S., Zhang, C. A., Lu, Y., & Eisenberg, M. L. (2017). The age of fathers in the USA is rising: An analysis of 168 867 480 births from 1972 to 2015. *Human Reproduction*, *32*(10), 2110–2116. https://doi.org/10.1093/humrep/dex267

Khanehzad, M., Abbaszadeh, R., Holakuyee, M., Modarressi, M. H., & Nourashrafeddin, S. M. (2021). FSH regulates RA signaling to commit spermatogonia into differentiation pathway and meiosis. *Reproductive Biology and Endocrinology*, *19*(1), 4. https://doi.org/10.1186/s12958-020-00686-w

Kim, S. M., Kim, S. K., Jee, B. C., & Kim, S. H. (2019). Effect of Sperm DNA Fragmentation on Embryo Quality in Normal Responder Women in In Vitro Fertilization and Intracytoplasmic Sperm Injection. *Yonsei Medical Journal*, *60*(5), 461–466. https://doi.org/10.3349/ymj.2019.60.5.461

Kim, Y., & Chun, K. (2020). New disposable microfluidic chip without evaporation effect for semen analysis in clinics and homes. *Microsystem Technologies*, *26*(2), 647–655. https://doi.org/10.1007/s00542-019-04527-8

Kirkegaard, K., Hindkjaer, J. J., Grøndahl, M. L., Kesmodel, U. S., & Ingerslev, H. J. (2012). A randomized clinical trial comparing embryo culture in a conventional incubator

with a time-lapse incubator. *Journal of Assisted Reproduction and Genetics*, *29*(6), 565–572. https://doi.org/10.1007/s10815-012-9750-x

Kostereva, N., & Hofmann, M.-C. (2008). Regulation of the Spermatogonial Stem Cell Niche. *Reproduction in Domestic Animals = Zuchthygiene*, *43*(Suppl 2), 386–392. https://doi.org/10.1111/j.1439-0531.2008.01189.x

Kothari, S., Thompson, A., Agarwal, A., & du Plessis, S. S. (2010). Free radicals: Their beneficial and detrimental effects on sperm function. *Indian Journal of Experimental Biology*, *48*(5), 425–435.

Kovacs, P. (2014). Embryo selection: The role of time-lapse monitoring. *Reproductive Biology and Endocrinology*, *12*(1), 124. https://doi.org/10.1186/1477-7827-12-124

Kowalczyk, A. (2021). The Role of the Natural Antioxidant Mechanism in Sperm Cells. *Reproductive Sciences*, *29*(5), 1387–1394. https://doi.org/10.1007/s43032-021-00795-w

Krausz, C. (2011). Male infertility: Pathogenesis and clinical diagnosis. *Best Practice & Research. Clinical Endocrinology & Metabolism*, *25*(2), 271–285. https://doi.org/10.1016/j.beem.2010.08.006

Krausz, C., Brannigan, R. E., & Sigman, M. (2015). Subspecialty training in andrology. *Fertility and Sterility*, *104*(1), 12–15. https://doi.org/10.1016/j.fertnstert.2015.04.038

Krokan, H. E., & Bjørås, M. (2013). Base Excision Repair. *Cold Spring Harbor Perspectives in Biology*, *5*(4), a012583. https://doi.org/10.1101/cshperspect.a012583

Krzastek, S. C., Farhi, J., Gray, M., & Smith, R. P. (2020). Impact of environmental toxin exposure on male fertility potential. *Translational Andrology and Urology*, *9*(6), 2797–2813. https://doi.org/10.21037/tau-20-685

Kulaksiz, D., Toprak, T., Tokat, E., Yilmaz, M., Ramazanoglu, M. A., Garayev, A., Sulukaya, M., Degirmentepe, R. B., Allahverdiyev, E., Gul, M., & Verit, A. (2022). Sperm concentration and semen volume increase after smoking cessation in infertile men. *International Journal of Impotence Research*, *34*(6), Article 6. https://doi.org/10.1038/s41443-022-00605-0

Kumar, M., Kumar, K., Jain, S., Hassan, T., & Dada, R. (2013). Novel insights into the genetic and epigenetic paternal contribution to the human embryo. *Clinics*, *68*, 5–14. https://doi.org/10.6061/clinics/2013(Sup01)02

Kumar, N., & Singh, A. K. (2015). Trends of male factor infertility, an important cause of infertility: A review of literature. *Journal of Human Reproductive Sciences*, *8*(4), 191–196. https://doi.org/10.4103/0974-1208.170370

Kuroda, S., Usui, K., Sanjo, H., Takeshima, T., Kawahara, T., Uemura, H., & Yumura, Y. (2020). Genetic disorders and male infertility. *Reproductive Medicine and Biology*, *19*(4), 314–322. https://doi.org/10.1002/rmb2.12336

Lalinde-Acevedo, P. C., Mayorga-Torres, B. J. M., Agarwal, A., du Plessis, S. S., Ahmad, G., Cadavid, Á. P., & Cardona Maya, W. D. (2017). Physically Active Men Show Better Semen Parameters than Their Sedentary Counterparts. *International Journal of Fertility & Sterility*, *11*(3), 156–165.

Larina, I. V., Larin, K. V., Justice, M. J., & Dickinson, M. E. (2011). Optical Coherence Tomography for live imaging of mammalian development. *Current Opinion in*

Genetics & Development, *21*(5), 579–584. https://doi.org/10.1016/j.gde.2011.09.004

Larina, I. V., Syed, S. H., Sudheendran, N., Overbeek, P. A., Dickinson, M. E., & Larin, K. V. (2012). Optical coherence tomography for live phenotypic analysis of embryonic ocular structures in mouse models. *Journal of Biomedical Optics*, *17*(8), 081410. https://doi.org/10.1117/1.JBO.17.8.081410

Le, M. T., Nguyen, T. A. T., Nguyen, H. T. T., Nguyen, T. T. T., Nguyen, V. T., Le, D. D., Nguyen, V. Q. H., & Cao, N. T. (2019). Does sperm DNA fragmentation correlate with semen parameters? *Reproductive Medicine and Biology*, *18*(4), 390–396. https://doi.org/10.1002/rmb2.12297

Lehti, M. S., & Sironen, A. (2017). Formation and function of sperm tail structures in association with sperm motility defects†. *Biology of Reproduction*, *97*(4), 522–536. https://doi.org/10.1093/biolre/iox096

Leung, E. T. Y., Lee, C.-L., Tian, X., Lam, K. K. W., Li, R. H. W., Ng, E. H. Y., Yeung, W. S. B., & Chiu, P. C. N. (2022). Simulating nature in sperm selection for assisted reproduction. *Nature Reviews. Urology*, *19*(1), 16–36. https://doi.org/10.1038/s41585-021-00530-9

Lévesque, D., Veilleux, S., Caron, N., & Boissonneault, G. (1998). Architectural DNA-binding properties of the spermatidal transition proteins 1 and 2. *Biochemical and Biophysical Research Communications*, *252*(3), 602–609. https://doi.org/10.1006/bbrc.1998.9687

Li, K., Yang, X., & Wu, T. (2022). The Effect of Antioxidants on Sperm Quality Parameters and Pregnancy Rates for Idiopathic Male Infertility: A Network Meta-Analysis of

Randomized Controlled Trials. *Frontiers in Endocrinology*, *13*. https://www.frontiersin.org/articles/10.3389/fendo.2022.810242

Li, X., Ni, M., Xing, S., Yu, Y., Zhou, Y., Yang, S., Li, H., Zhu, R., & Han, M. (2020). Reactive Oxygen Species Secreted by Leukocytes in Semen Induce Self-Expression of Interleukin-6 and Affect Sperm Quality. *American Journal of Men's Health*, *14*(5), 1557988320970053. https://doi.org/10.1177/1557988320970053

Li, Z., Zhou, Y., Liu, R., Lin, H., Liu, W., Xiao, W., & Lin, Q. (2012). Effects of semen processing on the generation of reactive oxygen species and mitochondrial membrane potential of human spermatozoa. *Andrologia*, *44*(3), 157–163. https://doi.org/10.1111/j.1439-0272.2010.01123.x

Ligny, W. de, Smits, R. M., Mackenzie-Proctor, R., Jordan, V., Fleischer, K., Bruin, J. P. de, & Showell, M. G. (2022). Antioxidants for male subfertility. *Cochrane Database of Systematic Reviews*, *5*. https://doi.org/10.1002/14651858.CD007411.pub5

López, G., Lafuente, R., Checa, M. A., Carreras, R., & Brassesco, M. (2013). Diagnostic value of sperm DNA fragmentation and sperm high-magnification for predicting outcome of assisted reproduction treatment. *Asian Journal of Andrology*, *15*(6), 790–794. https://doi.org/10.1038/aja.2013.81

Majzoub, A., Agarwal, A., Cho, C. L., & Esteves, S. C. (2017). Sperm DNA fragmentation testing: A cross sectional survey on current practices of fertility specialists. *Translational Andrology and Urology*, *6*(Suppl 4), S710–S719. https://doi.org/10.21037/tau.2017.06.21

Malić Vončina, S., Stenqvist, A., Bungum, M., Schyman, T., & Giwercman, A. (2021). Sperm DNA fragmentation index and cumulative live birth rate in a cohort of

2,713 couples undergoing assisted reproduction treatment. *Fertility and Sterility*, *116*(6), 1483–1490. https://doi.org/10.1016/j.fertnstert.2021.06.049

Malvezzi, H., Sharma, R., Agarwal, A., Abuzenadah, A. M., & Abu-Elmagd, M. (2014). Sperm quality after density gradient centrifugation with three commercially available media: A controlled trial. *Reproductive Biology and Endocrinology: RB&E*, *12*, 121. https://doi.org/10.1186/1477-7827-12-121

Mannucci, A., Argento, F. R., Fini, E., Coccia, M. E., Taddei, N., Becatti, M., & Fiorillo, C. (2022). The Impact of Oxidative Stress in Male Infertility. *Frontiers in Molecular Biosciences*, *8*, 799294. https://doi.org/10.3389/fmolb.2021.799294

Marcon, L., & Boissonneault, G. (2004). Transient DNA Strand Breaks During Mouse and Human Spermiogenesis:New Insights in Stage Specificity and Link to Chromatin Remodeling1. *Biology of Reproduction*, *70*(4), 910–918. https://doi.org/10.1095/biolreprod.103.022541

Martínez-Holguín, E., Lledó-García, E., Rebollo-Román, Á., González-García, J., Jara-Rascón, J., & Hernández-Fernández, C. (2020). Antioxidants to Improve Sperm Quality. In D. Mortimer, G. Kovacs, & R. J. Aitken (Eds.), *Male and Sperm Factors that Maximize IVF Success* (pp. 106–120). Cambridge University Press. https://doi.org/10.1017/9781108762571.009

Martire, S., & Banaszynski, L. A. (2020). The roles of histone variants in fine-tuning chromatin organization and function. *Nature Reviews. Molecular Cell Biology*, *21*(9), 522–541. https://doi.org/10.1038/s41580-020-0262-8

Mayeur, A., Ahdad, N., Hesters, L., Brisset, S., Romana, S., Tosca, L., Tachdjian, G., & Frydman, N. (2019). Chromosomal translocations and semen quality: A study on

144 male translocation carriers. *Reproductive BioMedicine Online*, *38*(1), 46–55. https://doi.org/10.1016/j.rbmo.2018.10.003

Meacham, R. B., Hellerstein, D. K., & Lipshultz, L. I. (1993). Evaluation and treatment of ejaculatory duct obstruction in the infertile male. *Fertility and Sterility*, *59*(2), 393–397. https://doi.org/10.1016/S0015-0282(16)55683-0

Mehta, A. (2017). Pros and cons of sperm DNA fragmentation testing: Weighing the evidence. *Translational Andrology and Urology*, *6*(Suppl 4), S453–S454. https://doi.org/10.21037/tau.2017.03.71

Mehta, A., Bolyakov, A., Schlegel, P. N., & Paduch, D. A. (2015). Higher pregnancy rates using testicular sperm in men with severe oligospermia. *Fertility and Sterility*, *104*(6), 1382–1387. https://doi.org/10.1016/j.fertnstert.2015.08.008

Mehta, A., Nangia, A. K., Dupree, J. M., & Smith, J. F. (2016). Limitations and barriers in access to care for male factor infertility. *Fertility and Sterility*, *105*(5), 1128–1137. https://doi.org/10.1016/j.fertnstert.2016.03.023

Meistrich, M., Mohapatra, B., Shirley, C., & Zhao, M. (2003). Roles of transition nuclear proteins in spermiogenesis. *Chromosoma*, *111*, 483–488. https://doi.org/10.1007/s00412-002-0227-z

Menezo, Y., Clement, P., & Amar, E. (2017). Evaluation of sperm DNA structure, fragmentation and decondensation: An essential tool in the assessment of male infertility. *Translational Andrology and Urology*, *6*(Suppl 4), Article Suppl 4. https://doi.org/10.21037/tau.2017.03.11

Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J. G., Westphal, H., Saarma, M., & Sariola, H. (2000). Regulation of Cell Fate Decision of

Undifferentiated Spermatogonia by GDNF. *Science*, *287*(5457), 1489–1493. https://doi.org/10.1126/science.287.5457.1489

Meseguer, M., Herrero, J., Tejera, A., Hilligsøe, K. M., Ramsing, N. B., & Remohí, J. (2011). The use of morphokinetics as a predictor of embryo implantation†. *Human Reproduction*, *26*(10), 2658–2671. https://doi.org/10.1093/humrep/der256

Meseguer, M., Santiso, R., Garrido, N., García-Herrero, S., Remohí, J., & Fernandez, J. L. (2011). Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertility and Sterility*, *95*(1), 124–128. https://doi.org/10.1016/j.fertnstert.2010.05.055

Middelkamp, S., van Tol, H. T. A., Spierings, D. C. J., Boymans, S., Guryev, V., Roelen, B. A. J., Lansdorp, P. M., Cuppen, E., & Kuijk, E. W. (2020). Sperm DNA damage causes genomic instability in early embryonic development. *Science Advances*, *6*(16), eaaz7602. https://doi.org/10.1126/sciadv.aaz7602

Miller, D., Brinkworth, M., & Iles, D. (2010). Paternal DNA packaging in spermatozoa: More than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction*, *139*(2), 287–301. https://doi.org/10.1530/REP-09-0281

Mitchell, L. A., De Iuliis, G. N., & Aitken, R. J. (2011). The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: Development of an improved methodology. *International Journal of Andrology*, *34*(1), 2–13. https://doi.org/10.1111/j.1365-2605.2009.01042.x

Morgan, W. A. (1995). DNA single-strand breakage in mammalian cells induced by redox cycling quinones in the absence of oxidative stress. *Journal of Biochemical Toxicology*, *10*(4), 227–232. https://doi.org/10.1002/jbt.2570100407

Moritz, L., & Hammoud, S. S. (2022). The Art of Packaging the Sperm Genome: Molecular and Structural Basis of the Histone-To-Protamine Exchange. *Frontiers in Endocrinology*, *13*. https://www.frontiersin.org/articles/10.3389/fendo.2022.895502

Morrell, J. M. (2011). Artificial Insemination: Current and Future Trends. In *Artificial Insemination in Farm Animals*. IntechOpen. https://doi.org/10.5772/17943

Moskovtsev, S. I., Willis, J., White, J., & Mullen, J. B. M. (2009). Sperm DNA Damage: Correlation to Severity of Semen Abnormalities. *Urology*, *74*(4), 789–793. https://doi.org/10.1016/j.urology.2009.05.043

Murakami, H., & Keeney, S. (2008). Regulating the formation of DNA double-strand breaks in meiosis. *Genes & Development*, *22*(3), 286–292. https://doi.org/10.1101/gad.1642308

Musson, R., Gąsior, Ł., Bisogno, S., & Ptak, G. E. (2022). DNA damage in preimplantation embryos and gametes: Specification, clinical relevance and repair strategies. *Human Reproduction Update*, *28*(3), 376–399. https://doi.org/10.1093/humupd/dmab046

Nakahara, T., Iwase, A., Goto, M., Harata, T., Suzuki, M., Ienaga, M., Kobayashi, H., Takikawa, S., Manabe, S., Kikkawa, F., & Ando, H. (2010). Evaluation of the safety of time-lapse observations for human embryos. *Journal of Assisted Reproduction and Genetics*, *27*(2–3), 93–96. https://doi.org/10.1007/s10815-010-9385-8

Nallella, K. P., Sharma, R. K., Aziz, N., & Agarwal, A. (2006). Significance of sperm characteristics in the evaluation of male infertility. *Fertility and Sterility*, *85*(3), 629–634. https://doi.org/10.1016/j.fertnstert.2005.08.024

Nangia, A. K., Luke, B., Smith, J. F., Mak, W., & Stern, J. E. (2011). National study of factors influencing assisted reproductive technology outcomes with male factor infertility. *Fertility and Sterility*, *96*(3), 609–614. https://doi.org/10.1016/j.fertnstert.2011.06.026

Naz, R. K., & Rajesh, P. B. (2004). Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. *Reproductive Biology and Endocrinology : RB&E*, *2*, 75. https://doi.org/10.1186/1477-7827-2-75

Nicopoullos, J., Vicens-Morton, A., Lewis, S., Lee, K., Larsen, P., Ramsay, J., Yap, T., & Minhas, S. (2019). Novel use of COMET parameters of sperm DNA damage may increase its utility to diagnose male infertility and predict live births following both IVF and ICSI. *Human Reproduction (Oxford, England)*, *34*. https://doi.org/10.1093/humrep/dez151

Nosrati, R., Graham, P. J., Zhang, B., Riordon, J., Lagunov, A., Hannam, T. G., Escobedo, C., Jarvi, K., & Sinton, D. (2017). Microfluidics for sperm analysis and selection. *Nature Reviews Urology*, *14*(12), Article 12. https://doi.org/10.1038/nrurol.2017.175

Nosrati, R., Vollmer, M., Eamer, L., San Gabriel, M. C., Zeidan, K., Zini, A., & Sinton, D. (2014). Rapid selection of sperm with high DNA integrity. *Lab on a Chip*, *14*(6), 1142–1150. https://doi.org/10.1039/c3lc51254a

Nybo Andersen, A.-M., Hansen, K. D., Andersen, P. K., & Davey Smith, G. (2004). Advanced Paternal Age and Risk of Fetal Death: A Cohort Study. *American Journal of Epidemiology*, *160*(12), 1214–1222. https://doi.org/10.1093/aje/kwh332

O'Donnell, L. (2015). Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis*, *4*(2), e979623. https://doi.org/10.4161/21565562.2014.979623

O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., & Stanton, P. G. (2011). Spermiation. *Spermatogenesis*, *1*(1), 14–35. https://doi.org/10.4161/spmg.1.1.14525

O'Flaherty, C., & Matsushita-Fournier, D. (2017). Reactive oxygen species and protein modifications in spermatozoa†. *Biology of Reproduction*, *97*(4), 577–585. https://doi.org/10.1093/biolre/iox104

O'Flynn O'Brien, K. L., Varghese, A. C., & Agarwal, A. (2010). The genetic causes of male factor infertility: A review. *Fertility and Sterility*, *93*(1), 1–12. https://doi.org/10.1016/j.fertnstert.2009.10.045

O'Neill, C. L., Parrella, A., Keating, D., Cheung, S., Rosenwaks, Z., & Palermo, G. D. (2018). A treatment algorithm for couples with unexplained infertility based on sperm chromatin assessment. *Journal of Assisted Reproduction and Genetics*, *35*(10), 1911–1917. https://doi.org/10.1007/s10815-018-1270-x

Orthwein, A., Fradet-Turcotte, A., Noordermeer, S. M., Canny, M. D., Brun, C. M., Strecker, J., Escribano-Diaz, C., & Durocher, D. (2014). Mitosis Inhibits DNA Double-Strand Break Repair to Guard Against Telomere Fusions. *Science*, *344*(6180), 189–193. https://doi.org/10.1126/science.1248024

Palmer, N. O., Bakos, H. W., Fullston, T., & Lane, M. (2012). Impact of obesity on male fertility, sperm function and molecular composition. *Spermatogenesis*, *2*(4), 253–263. https://doi.org/10.4161/spmg.21362

Panner Selvam, M. K., Agarwal, A., Henkel, R., Finelli, R., Robert, K. A., Iovine, C., & Baskaran, S. (2020). The effect of oxidative and reductive stress on semen parameters and functions of physiologically normal human spermatozoa. *Free Radical Biology & Medicine*, *152*, 375–385. https://doi.org/10.1016/j.freeradbiomed.2020.03.008

Panner Selvam, M. K., Ambar, R. F., Agarwal, A., & Henkel, R. (2021). Etiologies of sperm DNA damage and its impact on male infertility. *Andrologia*, *53*(1), e13706. https://doi.org/10.1111/and.13706

Parks, J. E., & Lynch, D. V. (1992). Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology*, *29*(2), 255–266. https://doi.org/10.1016/0011-2240(92)90024-v

Peluso, A., Caruso, T., Landi, A., & Capobianco, A. (2019). The Dynamics of Hole Transfer in DNA. *Molecules*, *24*(22), Article 22. https://doi.org/10.3390/molecules24224044

Petersen, B. M., Boel, M., Montag, M., & Gardner, D. K. (2016). Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Human Reproduction (Oxford, England)*, *31*(10), 2231–2244. https://doi.org/10.1093/humrep/dew188

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. *Indian Journal of Clinical Biochemistry*, *30*(1), 11–26. https://doi.org/10.1007/s12291-014-0446-0

Pinto, S., Carrageta, D. F., Alves, M. G., Rocha, A., Agarwal, A., Barros, A., & Oliveira, P. F. (2021). Sperm selection strategies and their impact on assisted reproductive

technology outcomes. *Andrologia*, *53*(2), e13725. https://doi.org/10.1111/and.13725

Popescu, D. P., Choo-Smith, L.-P., Flueraru, C., Mao, Y., Chang, S., Disano, J., Sherif, S., & Sowa, M. G. (2011). Optical coherence tomography: Fundamental principles, instrumental designs and biomedical applications. *Biophysical Reviews*, *3*(3), 155. https://doi.org/10.1007/s12551-011-0054-7

Preece, D., Chow, K. W., Gomez-Godinez, V., Gustafson, K., Esener, S., Ravida, N., Durrant, B., & Berns, M. W. (2017). Red light improves spermatozoa motility and does not induce oxidative DNA damage. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/srep46480

Punab, M., Poolamets, O., Paju, P., Vihljajev, V., Pomm, K., Ladva, R., Korrovits, P., & Laan, M. (2017). Causes of male infertility: A 9-year prospective monocentre study on 1737 patients with reduced total sperm counts. *Human Reproduction*, *32*(1), 18–31. https://doi.org/10.1093/humrep/dew284

Quinn, M. M., Jalalian, L., Ribeiro, S., Ona, K., Demirci, U., Cedars, M. I., & Rosen, M. P. (2018). Microfluidic sorting selects sperm for clinical use with reduced DNA damage compared to density gradient centrifugation with swim-up in split semen samples. *Human Reproduction (Oxford, England)*, *33*(8), 1388–1393. https://doi.org/10.1093/humrep/dey239

Rael, L. T., Bar-Or, R., Kelly, M. T., Carrick, M. M., & Bar-Or, D. (2015). Assessment of Oxidative Stress in Patients with an Isolated Traumatic Brain Injury Using Disposable Electrochemical Test Strips. *Electroanalysis*, *27*(11), 2567–2573. https://doi.org/10.1002/elan.201500178

Raghunathan, R., Singh, M., Dickinson, M. E., & Larin, K. V. (2016). Optical coherence tomography for embryonic imaging: A review. *Journal of Biomedical Optics*, *21*(5), 050902. https://doi.org/10.1117/1.JBO.21.5.050902

Rashki Ghaleno, L., Alizadeh, A., Drevet, J. R., Shahverdi, A., & Valojerdi, M. R. (2021). Oxidation of Sperm DNA and Male Infertility. *Antioxidants*, *10*(1), Article 1. https://doi.org/10.3390/antiox10010097

Rehman, R., Zahid, N., Amjad, S., Baig, M., & Gazzaz, Z. J. (2019). Relationship Between Smoking Habit and Sperm Parameters Among Patients Attending an Infertility Clinic. *Frontiers in Physiology*, *10*. https://www.frontiersin.org/articles/10.3389/fphys.2019.01356

Repetto, M., Semprine, J., Boveris, A., Repetto, M., Semprine, J., & Boveris, A. (2012). Lipid Peroxidation: Chemical Mechanism, Biological Implications and Analytical Determination. In *Lipid Peroxidation*. IntechOpen. https://doi.org/10.5772/45943

Rex, A. S., Wu, C., Aagaard, J., & Fedder, J. (2019). Implementation of an in-house flow cytometric analysis of DNA fragmentation in spermatozoa. *Asian Journal of Andrology*, *22*(3), 246–251. https://doi.org/10.4103/aja.aja_51_19

Ribas-Maynou, J., & Benet, J. (2019). Single and double strand sperm DNA damage: Different reproductive effects on male fertility. *Genes*, *10*(2). https://doi.org/10.3390/genes10020105

Ribas-Maynou, J., Garcia-Peiro, A., Abad, C., Guedan, M. J., Navarro, J., & Benet, J. (2012). Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups. *Human Reproduction (Oxford, England)*, *27*, 652–658. https://doi.org/10.1093/humrep/der461

Ribas-Maynou, J., García-Peiró, A., Fernandez-Encinas, A., Amengual, M. J., Prada, E., Cortés, P., Navarro, J., & Benet, J. (2012). Double Stranded Sperm DNA Breaks, Measured by Comet Assay, Are Associated with Unexplained Recurrent Miscarriage in Couples without a Female Factor. *PLOS ONE*, *7*(9), e44679. https://doi.org/10.1371/journal.pone.0044679

Ribas-Maynou, J., Novo, S., Torres, M., Salas-Huetos, A., Rovira, S., Antich, M., & Yeste, M. (2022). Sperm DNA integrity does play a crucial role for embryo development after ICSI, notably when good-quality oocytes from young donors are used. *Biological Research*, *55*(1), 41. https://doi.org/10.1186/s40659-022-00409-y

Ribas-Maynou, J., Yeste, M., Becerra-Tomás, N., Aston, K. I., James, E. R., & Salas-Huetos, A. (2021). Clinical implications of sperm DNA damage in IVF and ICSI: Updated systematic review and meta-analysis. *Biological Reviews*, *96*(4), 1284–1300. https://doi.org/10.1111/brv.12700

Ricci, E., Viganò, P., Cipriani, S., Somigliana, E., Chiaffarino, F., Bulfoni, A., & Parazzini, F. (2017). Coffee and caffeine intake and male infertility: A systematic review. *Nutrition Journal*, *16*, 37. https://doi.org/10.1186/s12937-017-0257-2

Riffo, M. S., & Parraga, M. (1996). Study of the acrosome reaction and the fertilizing ability of hamster epididymal cauda spermatozoa treated with antibodies against phospholipase A2 and/or lysophosphatidylcholine. *Journal of Experimental Zoology*, *275*(6), 459–468. https://doi.org/10.1002/(SICI)1097-010X(19960815)275:6<459::AID-JEZ8>3.0.CO;2-N

Roberts, M., & Jarvi, K. (2009). Steps in the investigation and management of low semen volume in the infertile man. *Journal of the Canadian Urological Association*, *3*(6), 479–485. https://doi.org/10.5489/cuaj.1180

Robine, J.-M., Cournil, A., Henon, N., & Allard, M. (2003). Have centenarians had younger parents than the others? *Experimental Gerontology*, *38*(4), 361–365. https://doi.org/10.1016/s0531-5565(02)00245-0

Robinson, L., Gallos, I. D., Conner, S. J., Rajkhowa, M., Miller, D., Lewis, S., Kirkman-Brown, J., & Coomarasamy, A. (2012). The effect of sperm DNA fragmentation on miscarriage rates: A systematic review and meta-analysis. *Human Reproduction*, *27*(10), 2908–2917. https://doi.org/10.1093/humrep/des261

Robles, V., & Martínez-Pastor, F. (2013). Flow cytometric methods for sperm assessment. *Methods in Molecular Biology (Clifton, N.J.)*, *927*, 175–186. https://doi.org/10.1007/978-1-62703-038-0_16

Rooij, D. de. (2001). Proliferation and differentiation of spermatogonial stem cells. *Reproduction*, *121*(3), 347–354. https://doi.org/10.1530/rep.0.1210347

Rubes, J., Selevan, S. G., Evenson, D. P., Zudova, D., Vozdova, M., Zudova, Z., Robbins, W. A., & Perreault, S. D. (2005). Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. *Human Reproduction*, *20*(10), 2776–2783. https://doi.org/10.1093/humrep/dei122

Russell, L. D., Ettlin, R. A., Hikim, A. P. S., & Clegg, E. D. (1993). Histological and Histopathological Evaluation of the Testis. *International Journal of Andrology*, *16*(1), 83–83. https://doi.org/10.1111/j.1365-2605.1993.tb01156.x

Said, T. M., & Land, J. A. (2011). Effects of advanced selection methods on sperm quality and ART outcome: A systematic review. *Human Reproduction Update*, *17*(6), 719–733. https://doi.org/10.1093/humupd/dmr032

Saleh, R. A., Agarwal, A., Sharma, R. K., Nelson, D. R., & Thomas, A. J. (2002). Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: A prospective study. *Fertility and Sterility*, *78*(3), 491–499. https://doi.org/10.1016/s0015-0282(02)03294-6

Saleh, R. A., & Hcld, A. A. (2002a). Oxidative Stress and Male Infertility: From Research Bench to Clinical Practice. *Journal of Andrology*, *23*(6), 737–752. https://doi.org/10.1002/j.1939-4640.2002.tb02324.x

Saleh, R. A., & Hcld, A. A. (2002b). Oxidative Stress and Male Infertility: From Research Bench to Clinical Practice. *Journal of Andrology*, *23*(6), 737–752. https://doi.org/10.1002/j.1939-4640.2002.tb02324.x

Samavat, J., Cantini, G., Lotti, F., Di Franco, A., Tamburrino, L., Degl'Innocenti, S., Maseroli, E., Filimberti, E., Facchiano, E., Lucchese, M., Muratori, M., Forti, G., Baldi, E., Maggi, M., & Luconi, M. (2018). Massive Weight Loss Obtained by Bariatric Surgery Affects Semen Quality in Morbid Male Obesity: A Preliminary Prospective Double-Armed Study. *Obesity Surgery*, *28*(1), 69–76. https://doi.org/10.1007/s11695-017-2802-7

Sampson, D. M., Dubis, A. M., Chen, F. K., Zawadzki, R. J., & Sampson, D. D. (2022). Towards standardizing retinal optical coherence tomography angiography: A review. *Light: Science & Applications*, *11*(1), Article 1. https://doi.org/10.1038/s41377-022-00740-9

Samuel, R., Feng, H., Jafek, A., Despain, D., Jenkins, T., & Gale, B. (2018). Microfluidic—Based sperm sorting & analysis for treatment of male infertility. *Translational Andrology and Urology*, *7*(Suppl 3), S336–S347. https://doi.org/10.21037/tau.2018.05.08

Schlesinger, M. H., Wilets, I. F., & Nagler, H. M. (1994). Treatment outcome after varicocelectomy. A critical analysis. *The Urologic Clinics of North America*, *21*(3), 517–529.

Schuster, T. G., Cho, B., Keller, L. M., Takayama, S., & Smith, G. D. (2003). Isolation of motile spermatozoa from semen samples using microfluidics. *Reproductive BioMedicine Online*, *7*(1), 75–81. https://doi.org/10.1016/S1472-6483(10)61732-4

Seidel, C. A. M., Schulz, A., & Sauer, M. H. M. (1996). Nucleobase-Specific Quenching of Fluorescent Dyes. 1. Nucleobase One-Electron Redox Potentials and Their Correlation with Static and Dynamic Quenching Efficiencies. *The Journal of Physical Chemistry*, *100*(13), 5541–5553. https://doi.org/10.1021/jp951507c

Selevan, S. G., Borkovec, L., Slott, V. L., Zudová, Z., Rubes, J., Evenson, D. P., & Perreault, S. D. (2000). Semen quality and reproductive health of young Czech men exposed to seasonal air pollution. *Environmental Health Perspectives*, *108*(9), 887–894. https://doi.org/10.1289/ehp.00108887

Sepehr, A., Armstrong, W. B., Guo, S., Su, J., Perez, J., Chen, Z., & Wong, B. J. F. (2008). Optical coherence tomography of the larynx in the awake patient. *Otolaryngology--Head and Neck Surgery*, *138*(4), 425–429. https://doi.org/10.1016/j.otohns.2007.12.005

Sergerie, M., Laforest, G., Bujan, L., Bissonnette, F., & Bleau, G. (2005). Sperm DNA fragmentation: Threshold value in male fertility. *Human Reproduction (Oxford, England)*, *20*(12), 3446–3451. https://doi.org/10.1093/humrep/dei231

Shaman, J. A., Yamauchi, Y., & Ward, W. S. (2007). The sperm nuclear matrix is required for paternal DNA replication. *Journal of Cellular Biochemistry*, *102*(3), 680–688. https://doi.org/10.1002/jcb.21321

Shamsi, M. B., Imam, S. N., & Dada, R. (2011). Sperm DNA integrity assays: Diagnostic and prognostic challenges and implications in management of infertility. *Journal of Assisted Reproduction and Genetics*, *28*(11), 1073–1085. https://doi.org/10.1007/s10815-011-9631-8

Sharma, R., & Agarwal, A. (2020). Sperm Processing and Selection. In S. J. Parekattil, S. C. Esteves, & A. Agarwal (Eds.), *Male Infertility* (pp. 647–659). Springer International Publishing. https://doi.org/10.1007/978-3-030-32300-4_52

Sharma, R., Agarwal, A., Rohra, V. K., Assidi, M., Abu-Elmagd, M., & Turki, R. F. (2015). Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reproductive Biology and Endocrinology : RB&E*, *13*, 35. https://doi.org/10.1186/s12958-015-0028-x

Sharma, R., Ahmad, G., Esteves, S. C., & Agarwal, A. (2016). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using bench top flow cytometer for evaluation of sperm DNA fragmentation in fertility laboratories: Protocol, reference values, and quality control. *Journal of Assisted Reproduction and Genetics*, *33*(2), 291–300. https://doi.org/10.1007/s10815-015-0635-7

Sharma, R., Iovine, C., Agarwal, A., & Henkel, R. (2021). TUNEL assay—Standardized method for testing sperm DNA fragmentation. *Andrologia*, *53*(2), e13738. https://doi.org/10.1111/and.13738

Sharma, R. K., Sabanegh, E., Mahfouz, R., Gupta, S., Thiyagarajan, A., & Agarwal, A. (2010). TUNEL as a test for sperm DNA damage in the evaluation of male

infertility. *Urology*, *76*(6), 1380–1386. https://doi.org/10.1016/j.urology.2010.04.036

Sheflin, L. G., Fucile, N. W., & Spaulding, S. W. (1991). HMG 14 and protamine enhance ligation of linear DNA to form linear multimers: Phosphorylation of HMG 14 at Ser 20 specifically inhibits intermolecular DNA ligation. *Biochemical and Biophysical Research Communications*, *174*(2), 660–666. https://doi.org/10.1016/0006-291x(91)91468-r

Shen, H. M., Chia, S. E., & Ong, C. N. (1999). Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *Journal of Andrology*, *20*(6), 718–723.

Shen, H., & Ong, C. (2000). Detection of oxidative DNA damage in human sperm and its association with sperm function and male infertility. *Free Radical Biology & Medicine*, *28*(4), 529–536. https://doi.org/10.1016/s0891-5849(99)00234-8

Shirota, K., Yotsumoto, F., Itoh, H., Obama, H., Hidaka, N., Nakajima, K., & Miyamoto, S. (2016). Separation efficiency of a microfluidic sperm sorter to minimize sperm DNA damage. *Fertility and Sterility*, *105*(2), 315-321.e1. https://doi.org/10.1016/j.fertnstert.2015.10.023

Sies, H. (1993). Strategies of antioxidant defense. *European Journal of Biochemistry*, *215*(2), 213–219. https://doi.org/10.1111/j.1432-1033.1993.tb18025.x

Sikka, S. C., Rajasekaran, M., & Hellstrom, W. J. (1995). Role of oxidative stress and antioxidants in male infertility. *Journal of Andrology*, *16*(6), 464–468.

Simon, L., & Carrell, D. T. (2013). *Sperm DNA Damage Measured by Comet Assay*. *927*, 137–146. https://doi.org/10.1007/978-1-62703-038-0_13

Simon, L., Murphy, K., Shamsi, M. B., Liu, L., Emery, B., Aston, K. I., Hotaling, J., & Carrell, D. T. (2014). Paternal influence of sperm DNA integrity on early embryonic development. *Human Reproduction*, *29*(11), 2402–2412. https://doi.org/10.1093/humrep/deu228

Singer, R., Sagiv, M., Barnet, M., Levinsky, H., Segenreich, E., Fuchs, Y., Mendes, E., & Yehoshua, H. (1991). Low energy narrow band non-coherent infrared illumination of human semen and isolated sperm*. *Andrologia*, *23*(2), 181–184. https://doi.org/10.1111/j.1439-0272.1991.tb02532.x

Slama, R., Bouyer, J., Windham, G., Fenster, L., Werwatz, A., & Swan, S. H. (2005). Influence of Paternal Age on the Risk of Spontaneous Abortion. *American Journal of Epidemiology*, *161*(9), 816–823. https://doi.org/10.1093/aje/kwi097

Smith, R., Kaune, H., Parodi, D., Madariaga, M., Rios, R., Morales, I., & Castro, A. (2006). Increased sperm DNA damage in patients with varicocele: Relationship with seminal oxidative stress. *Human Reproduction*, *21*(4), 986–993. https://doi.org/10.1093/humrep/dei429

Smith, T. B., Dun, M. D., Smith, N. D., Curry, B. J., Connaughton, H. S., & Aitken, R. J. (2013). The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *Journal of Cell Science*, *126*(Pt 6), 1488–1497. https://doi.org/10.1242/jcs.121657

Steiner, A. Z., Hansen, K. R., Barnhart, K. T., Cedars, M. I., Legro, R. S., Diamond, M. P., Krawetz, S. A., Usadi, R., Baker, V. L., Coward, R. M., Huang, H., Wild, R., Masson, P., Smith, J. F., Santoro, N., Eisenberg, E., Zhang, H., & Reproductive Medicine Network. (2020). The effect of antioxidants on male factor infertility: The Males,

Antioxidants, and Infertility (MOXI) randomized clinical trial. *Fertility and Sterility*, *113*(3), 552-560.e3. https://doi.org/10.1016/j.fertnstert.2019.11.008

Storr, A., Venetis, C., Cooke, S., Kilani, S., & Ledger, W. (2018). Time-lapse algorithms and morphological selection of day-5 embryos for transfer: A preclinical validation study. *Fertility and Sterility*, *109*(2), 276-283.e3. https://doi.org/10.1016/j.fertnstert.2017.10.036

Stringer, J. M., Winship, A., Liew, S. H., & Hutt, K. (2018). The capacity of oocytes for DNA repair. *Cellular and Molecular Life Sciences: CMLS*, *75*(15), 2777–2792. https://doi.org/10.1007/s00018-018-2833-9

Suganuma, R., Yanagimachi, R., & Meistrich, M. L. (2005). Decline in fertility of mouse sperm with abnormal chromatin during epididymal passage as revealed by ICSI. *Human Reproduction (Oxford, England)*, *20*(11), 3101–3108. https://doi.org/10.1093/humrep/dei169

Sultan, S., Patel, A. G., El-Hassani, S., Whitelaw, B., Leca, B. M., Vincent, R. P., le Roux, C. W., Rubino, F., Aywlin, S. J. B., & Dimitriadis, G. K. (2020). Male Obesity Associated Gonadal Dysfunction and the Role of Bariatric Surgery. *Frontiers in Endocrinology*, *11*, 408. https://doi.org/10.3389/fendo.2020.00408

Sun, Y., Saha, L. K., Saha, S., Jo, U., & Pommier, Y. (2020). Debulking of topoisomerase DNA-protein crosslinks (TOP-DPC) by the proteasome, non-proteasomal and non-proteolytic pathways. *DNA Repair*, *94*, 102926. https://doi.org/10.1016/j.dnarep.2020.102926

Swain, J. E., Lai, D., Takayama, S., & Smith, G. D. (2013). Thinking big by thinking small: Application of microfluidic technology to improve ART. *Lab on a Chip*, *13*(7), 1213–1224. https://doi.org/10.1039/c3lc41290c

Syed, S. H., Larin, K. V., Dickinson, M. E., & Larina, I. V. (2011). Optical coherence tomography for high-resolution imaging of mouse development in utero. *Journal of Biomedical Optics*, *16*(4), 046004. https://doi.org/10.1117/1.3560300

Takae, S., Tsukada, K., Sato, Y., Okamoto, N., Kawahara, T., & Suzuki, N. (2017). Accuracy and safety verification of ovarian reserve assessment technique for ovarian tissue transplantation using optical coherence tomography in mice ovary. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/srep43550

Takeshima, T., Yumura, Y., Kuroda, S., Kawahara, T., Uemura, H., & Iwasaki, A. (2017). Effect of density gradient centrifugation on reactive oxygen species in human semen. *Systems Biology in Reproductive Medicine*, *63*(3), 192–198. https://doi.org/10.1080/19396368.2017.1294214

Talhout, R., Schulz, T., Florek, E., van Benthem, J., Wester, P., & Opperhuizen, A. (2011). Hazardous Compounds in Tobacco Smoke. *International Journal of Environmental Research and Public Health*, *8*(2), 613–628. https://doi.org/10.3390/ijerph8020613

Tamburrino, L., Marchiani, S., Montoya, M., Elia Marino, F., Natali, I., Cambi, M., Forti, G., Baldi, E., & Muratori, M. (2012). Mechanisms and clinical correlates of sperm DNA damage. *Asian Journal of Andrology*, *14*(1), 24–31. https://doi.org/10.1038/aja.2011.59

Tandara, M., Bajić, A., Tandara, L., Bilić-Zulle, L., Šunj, M., Kozina, V., Goluža, T., & Jukić, M. (2014). Sperm DNA integrity testing: Big halo is a good predictor of embryo quality and pregnancy after conventional IVF. *Andrology*, *2*(5), 678–686. https://doi.org/10.1111/j.2047-2927.2014.00234.x

Tejada, R. I., Mitchell, J. C., Norman, A., Marik, J. J., & Friedman, S. (1984). A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertility and Sterility*, *42*(1), 87–91. https://doi.org/10.1016/s0015-0282(16)47963-x

Tello-Mora, P., Hernández-Cadena, L., Pedraza, J., López-Bayghen, E., & Quintanilla-Vega, B. (2018). Acrosome reaction and chromatin integrity as additional parameters of semen analysis to predict fertilization and blastocyst rates. *Reproductive Biology and Endocrinology*, *16*(1), 102. https://doi.org/10.1186/s12958-018-0408-0

Ten, J., Guerrero, J., Linares, Á., Rodríguez-Arnedo, A., Morales, R., Lledó, B., Llácer, J., & Bernabeu, R. (2022). Sperm DNA fragmentation on the day of fertilisation is not associated with assisted reproductive technique outcome independently of gamete quality. *Human Fertility*, *25*(4), 706–715. https://doi.org/10.1080/14647273.2021.1877364

Torres-Flores, U., & Hernández-Hernández, A. (2020). The Interplay Between Replacement and Retention of Histones in the Sperm Genome. *Frontiers in Genetics*, *11*, 780. https://doi.org/10.3389/fgene.2020.00780

Tough, S. C., Faber, A. J., Svenson, L. W., & Johnston, D. W. (2003). Is Paternal Age Associated with an Increased Risk of Low Birthweight, Preterm Delivery, and Multiple Birth? *Canadian Journal of Public Health = Revue Canadienne de Santé Publique*, *94*(2), 88–92. https://doi.org/10.1007/BF03404578

Tremellen, K. (2008). Oxidative stress and male infertility—A clinical perspective. *Human Reproduction Update*, *14*(3), 243–258. https://doi.org/10.1093/humupd/dmn004

Turner, K. A., Rambhatla, A., Schon, S., Agarwal, A., Krawetz, S. A., Dupree, J. M., & Avidor-Reiss, T. (2020). Male Infertility is a Women's Health Issue—Research and Clinical Evaluation of Male Infertility Is Needed. *Cells*, *9*(4), 990. https://doi.org/10.3390/cells9040990

Tusell, L., Latre, L., Ponsa, I., Miró, R., Egozcue, J., & Genescà, A. (2004). Capping of radiation-induced DNA breaks in mouse early embryos. *Journal of Radiation Research*, *45*(3), 415–422. https://doi.org/10.1269/jrr.45.415

Umezu, K., Xia, T., & Larina, I. V. (2022). Dynamic volumetric imaging and cilia beat mapping in the mouse male reproductive tract with optical coherence tomography. *Biomedical Optics Express*, *13*(6), 3672–3684. https://doi.org/10.1364/BOE.459937

Vaamonde, D., Da Silva-Grigoletto, M. E., García-Manso, J. M., Barrera, N., & Vaamonde-Lemos, R. (2012). Physically active men show better semen parameters and hormone values than sedentary men. *European Journal of Applied Physiology*, *112*(9), 3267–3273. https://doi.org/10.1007/s00421-011-2304-6

Vaamonde, D., Garcia-Manso, J. M., & Hackney, A. C. (2017). Impact of physical activity and exercise on male reproductive potential: A new assessment questionnaire. *Revista Andaluza de Medicina Del Deporte*, *10*(2), 79–93. https://doi.org/10.1016/j.ramd.2016.11.017

Vanegas, J. C., Chavarro, J. E., Williams, P. L., Ford, J. B., Toth, T. L., Hauser, R., & Gaskins, A. J. (2017). Discrete survival model analysis of a couple's smoking pattern and outcomes of assisted reproduction. *Fertility Research and Practice*, *3*, 5. https://doi.org/10.1186/s40738-017-0032-2

Vasan, S. S. (2011). Semen analysis and sperm function tests: How much to test? *Indian Journal of Urology : IJU : Journal of the Urological Society of India*, *27*(1), 41–48. https://doi.org/10.4103/0970-1591.78424

Vasilyeva, T. A., Marakhonov, A. V., Sukhanova, N. V., Kutsev, S. I., & Zinchenko, R. A. (2020). Preferentially Paternal Origin of De Novo 11p13 Chromosome Deletions Revealed in Patients with Congenital Aniridia and WAGR Syndrome. *Genes*, *11*(7), 812. https://doi.org/10.3390/genes11070812

Vessey, W., Perez-Miranda, A., Macfarquhar, R., Agarwal, A., & Homa, S. (2014). Reactive oxygen species in human semen: Validation and qualification of a chemiluminescence assay. *Fertility and Sterility*, *102*(6), 1576-1583.e4. https://doi.org/10.1016/j.fertnstert.2014.09.009

Vilfan, I. D., Conwell, C. C., & Hud, N. V. (2004). Formation of Native-like Mammalian Sperm Cell Chromatin with Folded Bull Protamine*. *Journal of Biological Chemistry*, *279*(19), 20088–20095. https://doi.org/10.1074/jbc.M312777200

Villani, M. T., Morini, D., Spaggiari, G., Falbo, A. I., Melli, B., La Sala, G. B., Romeo, M., Simoni, M., Aguzzoli, L., & Santi, D. (2022). Are sperm parameters able to predict the success of assisted reproductive technology? A retrospective analysis of over 22,000 assisted reproductive technology cycles. *Andrology*, *10*(2), 310–321. https://doi.org/10.1111/andr.13123

Wang, C., & Swerdloff, R. S. (2014). Limitations of semen analysis as a test of male fertility and anticipated needs from newer tests. *Fertility and Sterility*, *102*(6), 1502–1507. https://doi.org/10.1016/j.fertnstert.2014.10.021

Wang, J., Baker, A., Subramanian, M. L., Siegel, N. H., Chen, X., Ness, S., & Yi, J. (2022). Simultaneous visible light optical coherence tomography and near infrared OCT

angiography in retinal pathologies: A case study. *Experimental Biology and Medicine*, *247*(5), 377–384. https://doi.org/10.1177/15353702211063839

Wang, K., Gao, Y., Wang, C., Liang, M., Liao, Y., & Hu, K. (2022). Role of Oxidative Stress in Varicocele. *Frontiers in Genetics*, *13*. https://www.frontiersin.org/articles/10.3389/fgene.2022.850114

Wang, S., & Larin, K. V. (2015). Optical coherence elastography for tissue characterization: A review. *Journal of Biophotonics*, *8*(4), 279–302. https://doi.org/10.1002/jbio.201400108

Ward, W. S. (2010). Function of sperm chromatin structural elements in fertilization and development. *Molecular Human Reproduction*, *16*(1), 30–36. https://doi.org/10.1093/molehr/gap080

Ward, W. S. (2017). Eight tests for sperm DNA fragmentation and their roles in the clinic. *Translational Andrology and Urology*, *6*(Suppl 4), Article Suppl 4. https://doi.org/10.21037/tau.2017.03.78

Ward, W. S., Kimura, Y., & Yanagimachi, R. (1999). An intact sperm nuclear matrix may be necessary for the mouse paternal genome to participate in embryonic development. *Biology of Reproduction*, *60*(3), 702–706. https://doi.org/10.1095/biolreprod60.3.702

World Health Organization. (2010). *WHO Laboratory Manual for the Examination and Processing of Human Semen.* (5th Edition). World Health Organization, WHO Press.

World Health Organization. (2021). *WHO laboratory manual for the examination and processing of human semen*. https://www.who.int/publications-detail-redirect/9789240030787

Wu, F.-J., Lin, T.-Y., Sung, L.-Y., Chang, W.-F., Wu, P.-C., & Luo, C.-W. (2017). BMP8A sustains spermatogenesis by activating both SMAD1/5/8 and SMAD2/3 in spermatogonia. *Science Signaling*, *10*(477), eaal1910. https://doi.org/10.1126/scisignal.aal1910

Wyck, S., Herrera, C., Requena, C. E., Bittner, L., Hajkova, P., Bollwein, H., & Santoro, R. (2018). Oxidative stress in sperm affects the epigenetic reprogramming in early embryonic development. *Epigenetics & Chromatin*, *11*(1), 60. https://doi.org/10.1186/s13072-018-0224-y

Xu, D.-X., Shen, H.-M., Zhu, Q.-X., Chua, L., Wang, Q.-N., Chia, S.-E., & Ong, C.-N. (2003). The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. *Mutation Research*, *534*(1–2), 155–163. https://doi.org/10.1016/s1383-5718(02)00274-7

Yamaguchi, K., Hada, M., Fukuda, Y., Inoue, E., Makino, Y., Katou, Y., Shirahige, K., & Okada, Y. (2018). Re-evaluating the Localization of Sperm-Retained Histones Revealed the Modification-Dependent Accumulation in Specific Genome Regions. *Cell Reports*, *23*(13), 3920–3932. https://doi.org/10.1016/j.celrep.2018.05.094

Yin, Q., Yang, C.-H., Strelkova, O. S., Sun, Y., Gopalan, S., Yang, L., Dekker, J., Fazzio, T. G., Li, X. Z., Gibcus, J., & Rando, O. J. (2022). *Revisiting chromatin packaging in mouse sperm* (p. 2022.12.26.521943). bioRxiv. https://doi.org/10.1101/2022.12.26.521943

Yoshioka, K., Suzuki, C., & Onishi, A. (2008). Defined system for in vitro production of porcine embryos using a single basic medium. *The Journal of Reproduction and Development*, *54*(3), 208–213. https://doi.org/10.1262/jrd.20001

Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., de Mouzon, J., Sokol, R., Rienzi, L., Sunde, A., Schmidt, L., Cooke, I. D., Simpson, J. L., & van der Poel, S. (2017). The International Glossary on Infertility and Fertility Care, 2017. *Fertility and Sterility*, *108*(3), 393–406. https://doi.org/10.1016/j.fertnstert.2017.06.005

Zhao, J., Zhang, Q., Wang, Y., & Li, Y. (2014). Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: A systematic review and meta-analysis. *Fertility and Sterility*, *102*(4), 998-1005.e8. https://doi.org/10.1016/j.fertnstert.2014.06.033

Zheng, W.-W., Song, G., Wang, Q.-L., Liu, S.-W., Zhu, X.-L., Deng, S.-M., Zhong, A., Tan, Y.-M., & Tan, Y. (2018). Sperm DNA damage has a negative effect on early embryonic development following in vitro fertilization. *Asian Journal of Andrology*, *20*(1), 75–79. https://doi.org/10.4103/aja.aja_19_17

Zhou, Q., & Griswold, M. D. (2008). Regulation of spermatogonia. In *StemBook [Internet]*. Harvard Stem Cell Institute. https://doi.org/10.3824/stembook.1.7.1

Zhu, C., Chen, F., Zhang, S., She, H., Ju, Y., Wen, X., Yang, C., Sun, Y., Dong, N., Xue, T., Liu, K., Li, F., & Cui, H. (2022). Influence of sperm DNA fragmentation on the clinical outcome of in vitro fertilization-embryo transfer (IVF-ET). *Frontiers in Endocrinology*, *13*, 945242. https://doi.org/10.3389/fendo.2022.945242

Zhu, X. B., Chen, Q., Fan, W.-M., Niu, Z.-H., Xu, B.-F., & Zhang, A.-J. (2019). Sperm DNA fragmentation in Chinese couples with unexplained recurrent pregnancy loss. *Asian Journal of Andrology*, *22*. https://doi.org/10.4103/aja.aja_60_19

Zini, A., Boman, J. M., Belzile, E., & Ciampi, A. (2008). Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: Systematic review and meta-analysis. *Human Reproduction*, *23*(12), 2663–2668. https://doi.org/10.1093/humrep/den321

Zini, A., Finelli, A., Phang, D., & Jarvi, K. (2000). Influence of semen processing technique on human sperm DNA integrity. *Urology*, *56*(6), 1081–1084. https://doi.org/10.1016/s0090-4295(00)00770-6

Zini, A., & Libman, J. (2006). Sperm DNA damage: Clinical significance in the era of assisted reproduction. *CMAJ : Canadian Medical Association Journal*, *175*(5), 495–500. https://doi.org/10.1503/cmaj.060218

Zini, A., & Sigman, M. (2009). Are tests of sperm DNA damage clinically useful? Pros and cons. *Journal of Andrology*, *30*(3), 219–229. https://doi.org/10.2164/jandrol.108.006908

ZyMot Fertility. (2019). *ZyMot Fertility*. DxNow, Inc. https://genetikaneked.hu/wp-content/uploads/2022/05/zymot-Handout-ZyMot_Fertility-Introduction_and_Data_Spotlights_FERTILE.pdf

10. Appendix Section

10.1. Survey Preview

Beyond semen analysis: which sperm characteristics are useful in diagnosing and managing male infertility?

Page 1

Thank you for participating in this survey! We are a team of andrologists and reproductive geneticists from the University of Kent, working on improving diagnostic testing for male infertility.

This survey aims to assess how urologists use standard semen analysis data to diagnose and manage infertility in their male patients, to discover which additional sperm tests are useful (and why), and identify potential new avenues for test development that may be of use. The survey will take approximately 15 minutes to complete and your responses will be collected anonymously.

Page 2: Section A: Use of semen analysis in your practice

1. What country do you practice in?

- [ ] Afghanistan
- [ ] Akrotiri
- [ ] Albania
- [ ] Algeria
- [ ] American Samoa
- [ ] Andorra
- [ ] Angola
- [ ] Anguilla
- [ ] Antarctica
- [ ] Antigua and Barbuda
- [ ] Argentina
- [ ] Armenia
- [ ] Aruba
- [ ] Ashmore and Cartier Islands
- [ ] Australia
- [ ] Austria
- [ ] Azerbaijan
- [ ] Bahamas, The
- [ ] Bahrain
- [ ] Bangladesh
- [ ] Barbados
- [ ] Bassas da India
- [ ] Belarus
- [ ] Belgium
- [ ] Belize
- [ ] Benin
- [ ] Bermuda
- [ ] Bhutan

- [ ] Bolivia
- [ ] Bosnia and Herzegovina
- [ ] Botswana
- [ ] Bouvet Island
- [ ] Brazil
- [ ] British Indian Ocean Territory
- [ ] British Virgin Islands
- [ ] Brunei
- [ ] Bulgaria
- [ ] Burkina Faso
- [ ] Burma
- [ ] Burundi
- [ ] Cambodia
- [ ] Cameroon
- [ ] Canada
- [ ] Cape Verde
- [ ] Cayman Islands
- [ ] Central African Republic
- [ ] Chad
- [ ] Chile
- [ ] China
- [ ] Christmas Island
- [ ] Clipperton Island
- [ ] Cocos (Keeling) Islands
- [ ] Colombia
- [ ] Comoros
- [ ] Congo, Democratic Republic of the
- [ ] Congo, Republic of the
- [ ] Cook Islands
- [ ] Coral Sea Islands
- [ ] Costa Rica

- [ ] Cote d'Ivoire
- [ ] Croatia
- [ ] Cuba
- [ ] Cyprus
- [ ] Czech Republic
- [ ] Denmark
- [ ] Dhekelia
- [ ] Djibouti
- [ ] Dominica
- [ ] Dominican Republic
- [ ] Ecuador
- [ ] Egypt
- [ ] El Salvador
- [ ] Equatorial Guinea
- [ ] Eritrea
- [ ] Estonia
- [ ] Ethiopia
- [ ] Europa Island
- [ ] Falkland Islands (Islas Malvinas)
- [ ] Faroe Islands
- [ ] Fiji
- [ ] Finland
- [ ] France
- [ ] French Guiana
- [ ] French Polynesia
- [ ] French Southern and Antarctic Lands
- [ ] Gabon
- [ ] Gambia, The
- [ ] Gaza Strip
- [ ] Georgia
- [ ] Germany

- [ ] Ghana
- [ ] Gibraltar
- [ ] Glorioso Islands
- [ ] Greece
- [ ] Greenland
- [ ] Grenada
- [ ] Guadeloupe
- [ ] Guam
- [ ] Guatemala
- [ ] Guernsey
- [ ] Guinea
- [ ] Guinea-Bissau
- [ ] Guyana
- [ ] Haiti
- [ ] Heard Island and McDonald Islands
- [ ] Holy See (Vatican City)
- [ ] Honduras
- [ ] Hong Kong
- [ ] Hungary
- [ ] Iceland
- [ ] India
- [ ] Indonesia
- [ ] Iran
- [ ] Iraq
- [ ] Ireland
- [ ] Isle of Man
- [ ] Israel
- [ ] Italy
- [ ] Jamaica
- [ ] Jan Mayen
- [ ] Japan

- [ ] Jersey
- [ ] Jordan
- [ ] Juan de Nova Island
- [ ] Kazakhstan
- [ ] Kenya
- [ ] Kiribati
- [ ] Korea, North
- [ ] Korea, South
- [ ] Kuwait
- [ ] Kyrgyzstan
- [ ] Laos
- [ ] Latvia
- [ ] Lebanon
- [ ] Lesotho
- [ ] Liberia
- [ ] Libya
- [ ] Liechtenstein
- [ ] Lithuania
- [ ] Luxembourg
- [ ] Macau
- [ ] Macedonia
- [ ] Madagascar
- [ ] Malawi
- [ ] Malaysia
- [ ] Maldives
- [ ] Mali
- [ ] Malta
- [ ] Marshall Islands
- [ ] Martinique
- [ ] Mauritania
- [ ] Mauritius

- [ ] Mayotte
- [ ] Mexico
- [ ] Micronesia, Federated States of
- [ ] Moldova
- [ ] Monaco
- [ ] Mongolia
- [ ] Montenegro
- [ ] Montserrat
- [ ] Morocco
- [ ] Mozambique
- [ ] Namibia
- [ ] Nauru
- [ ] Navassa Island
- [ ] Nepal
- [ ] Netherlands
- [ ] Netherlands Antilles
- [ ] New Caledonia
- [ ] New Zealand
- [ ] Nicaragua
- [ ] Niger
- [ ] Nigeria
- [ ] Niue
- [ ] Norfolk Island
- [ ] Northern Mariana Islands
- [ ] Norway
- [ ] Oman
- [ ] Pakistan
- [ ] Palau
- [ ] Panama
- [ ] Papua New Guinea
- [ ] Paracel Islands

- [ ] Paraguay
- [ ] Peru
- [ ] Philippines
- [ ] Pitcairn Islands
- [ ] Poland
- [ ] Portugal
- [ ] Puerto Rico
- [ ] Qatar
- [ ] Reunion
- [ ] Romania
- [ ] Russia
- [ ] Rwanda
- [ ] Saint Helena
- [ ] Saint Kitts and Nevis
- [ ] Saint Lucia
- [ ] Saint Pierre and Miquelon
- [ ] Saint Vincent and the Grenadines
- [ ] Samoa
- [ ] San Marino
- [ ] Sao Tome and Principe
- [ ] Saudi Arabia
- [ ] Senegal
- [ ] Serbia
- [ ] Seychelles
- [ ] Sierra Leone
- [ ] Singapore
- [ ] Slovakia
- [ ] Slovenia
- [ ] Solomon Islands
- [ ] Somalia
- [ ] South Africa

- [ ] South Georgia and the South Sandwich Islands
- [ ] Spain
- [ ] Spratly Islands
- [ ] Sri Lanka
- [ ] Sudan
- [ ] Suriname
- [ ] Svalbard
- [ ] Swaziland
- [ ] Sweden
- [ ] Switzerland
- [ ] Syria
- [ ] Taiwan
- [ ] Tajikistan
- [ ] Tanzania
- [ ] Thailand
- [ ] Timor-Leste
- [ ] Togo
- [ ] Tokelau
- [ ] Tonga
- [ ] Trinidad and Tobago
- [ ] Tromelin Island
- [ ] Tunisia
- [ ] Turkey
- [ ] Turkmenistan
- [ ] Turks and Caicos Islands
- [ ] Tuvalu
- [ ] Uganda
- [ ] Ukraine
- [ ] United Arab Emirates
- [ ] United Kingdom
- [ ] United States

- [ ] Uruguay
- [ ] Uzbekistan
- [ ] Vanuatu
- [ ] Venezuela
- [ ] Vietnam
- [ ] Virgin Islands
- [ ] Wake Island
- [ ] Wallis and Futuna
- [ ] West Bank
- [ ] Western Sahara
- [ ] Yemen
- [ ] Zambia
- [ ] Zimbabwe

2. Describe your practice (tick any that apply)

- [ ] Government-run/public health urology clinic
- [ ] Private urology clinic
- [ ] Government-run/public health andrology clinic
- [ ] Private andrology clinic
- [ ] Other

2.a. If you selected Other, please specify:

3. Why is a semen analysis important? (tick any that apply)

- ☐ Indicator of obstruction of the male reproductive tract
- ☐ Indicator of male accessory gland dysfunction
- ☐ Indicator of testicular dysfunction
- ☐ Indicator of infection/inflammation
- ☐ Indicator of immunological infertility
- ☐ Indicator of toxin exposure
- ☐ Indicator of genetic infertility
- ☐ Indicator of other pathology (other, please specify)
- ☐ Other

3.a. If you selected Other, please specify:

4. How relevant are the parameters presented below in a semen analysis?

	not at all relevant	slightly relevant	moderately relevant	very relevant
Motility	○	○	○	○
Morphology	○	○	○	○
Appearance	○	○	○	○
pH	○	○	○	○
Volume	○	○	○	○
Antibodies	○	○	○	○
Leukocytes	○	○	○	○
Round cells	○	○	○	○
Vitality	○	○	○	○

4.a. Please share any comments on the utility of different semen parameters in diagnosis

Page 3: SECTION B: Which types of follow-up tests do you use?

Have you referred patients for any of the following types of test?

5. Sperm aneuploidy testing

- ○ Yes
- ○ No

5.a. Approximately how many referrals do you make per year for this type of test?

- ○ 1-5
- ○ 6-10
- ○ 11-25
- ○ 26+

5.a.i. Please indicate the main reasons for referral

5.b. Please indicate one or more reasons why this test is not useful for your patients

- ☐ I am unfamiliar with this test
- ☐ I am unaware that this test is available
- ☐ This type of test is never useful / is not medically informative
- ☐ I would use this test but it is not cost effective
- ☐ I would use this test if it were more readily available
- ☐ It is not appropriate for my patients

- [ ] Other

5.b.i. If you selected Other, please specify:

6. Sperm DNA fragmentation testing

- ○ Yes
- ○ No

6.a. Approximately how many referrals do you make per year for this type of test?

- ○ 1-5
- ○ 6-10
- ○ 11-25
- ○ 26+

6.a.i. Please indicate the main reasons for referral

6.b. Please indicate one or more reasons why this test is not useful for your patients

- [ ] I am unfamiliar with this test
- [ ] I am unaware that this test is available

- [ ] This type of test is never useful / is not medically informative
- [ ] I would use this test but it is not cost effective
- [ ] I would use this test if it were more readily available
- [ ] It is not appropriate for my patients
- [ ] Other

6.b.i. If you selected Other, please specify:

7. Sperm oxidative damage testing (ROS test)

- ○ Yes
- ○ No

7.a. Approximately how many referrals do you make per year for this type of test?

- ○ 1-5
- ○ 6-10
- ○ 11-25
- ○ 26+

7.a.i. Please indicate the main reasons for referral

7.b. Please indicate one or more reasons why this test is not useful for your patients

- [ ] I am unfamiliar with this test
- [ ] I am unaware that this test is available
- [ ] This type of test is never useful / is not medically informative
- [ ] I would use this test but it is not cost effective
- [ ] I would use this test if it were more readily available
- [ ] It is not appropriate for my patients
- [ ] Other

7.b.i. If you selected Other, please specify:

8. Any other followup test of sperm characteristics

- ○ Yes
- ○ No

8.a. Yes (please specify which test, approximate number per year and reasons for referral)

Page 4: SECTION C: Which sperm tests are most useful to you and why?

9. Which types of DNA damage are most important to measure?

	Not at all relevant	Slightly relevant	Moderately relevant	Very relevant	Don't know
Double strand breaks (DSBs)	○	○	○	○	○
Single strand breaks (SSBs)	○	○	○	○	○
Oxidised bases	○	○	○	○	○
Adducts	○	○	○	○	○
Abasic sites	○	○	○	○	○

9.a. Please share any comments on the use of sperm DNA damage measurement in diagnosis and management of male infertility

10. Which of the following sperm DNA damage assays have you used and why? (tick all that apply)

- ☐ Sperm Chromatin Structure Assay (SCSA)
- ☐ Acridine Orange test
- ☐ Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)
- ☐ Sperm Chromatin Dispersion assay (SCD / Halosperm)
- ☐ SpermComet
- ☐ None of the above

- [ ] Other

10.a. If you selected Other, please specify:

11. Would it be useful for andrology service providers to to provide a “suite” of different DNA fragmentation tests to facilitate a holistic overview of sperm DNA damage?

- ○ Yes
- ○ No

11.a. If yes, which tests are most important to include and why?

12. Are there any existing sperm tests (named above or otherwise) that you would like your patients to have better access to?

13. Are there any other critical aspects of sperm health that are not adequately tested at

present?

Page 5: Thank you

Thank you once again for your time. Your answers will be used to understand how semen analysis and other sperm tests are used by urology professionals to diagnose and manage infertility, and to identify unmet needs in the community.