The Effect of Polyunsaturated Fatty Acids on Bovine Sperm, *in vitro*

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Submitted in accordance with the academic requirements for the Degree of Master of Science to the Department of Life Sciences, Faculty of Science and Engineering, University of Limerick

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Declaration

I hereby declare that I am the sole author of this thesis and that it has not been submitted for any other University or higher institution, or for any other academic award in this University. References and acknowledgements have been made, where necessary, to the work of others.

Signature: _________________________ Date: _________________________

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Dedicated to my parents
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ABP</td>
<td>Androgen Binding Protein</td>
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<td>AI</td>
<td>Artificial Insemination</td>
</tr>
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<td>ALA</td>
<td>Alpha-Linolenic acid</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BSP</td>
<td>Bovine Seminal Plasma</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood Testis Barrier</td>
</tr>
<tr>
<td>BWW</td>
<td>Biggers Whitten and Whittingham</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium ions</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CH₃</td>
<td>Methyl group</td>
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<tr>
<td>CM-H₂DCFDA</td>
<td>Chloromethyl-2,7-dichlorofluorescein diacetate</td>
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<tr>
<td>COOH</td>
<td>Carboxyl group</td>
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<td>CoQ</td>
<td>Co-Enzyme Q</td>
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<tr>
<td>DF</td>
<td>Dilution Factor</td>
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<td>DGLA</td>
<td>Dihomo-gamma-Linolenic acid</td>
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<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<tr>
<td>diH₂O</td>
<td>Distilled Water</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DPA</td>
<td>Docosapentaenoic acid</td>
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<tr>
<td>ES</td>
<td>Ectoplasmic Specialisation</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>Fe^{2+}/Fe^{3+}</td>
<td>Free Iron Radicals</td>
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<td>FMP</td>
<td>Forward Motility Protein</td>
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<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<td>GnRH</td>
<td>Gonadatrophin Releasing Hormone</td>
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<td>GPX</td>
<td>Glutathione Peroxidise</td>
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<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>HOCl</td>
<td>Hypochlorous acid</td>
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<tr>
<td>IGC</td>
<td>Immature Germ Cells</td>
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<td>Lutenising Hormone</td>
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<td>Lipid Peroxidation</td>
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<td>Myristic Acid</td>
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<td>MMP</td>
<td>Mitochondrial Membrane Potential</td>
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<td>MUFA</td>
<td>Monounsaturated Fatty Acids</td>
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<tr>
<td>n-3</td>
<td>Omega 3 Fatty acid</td>
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</tr>
<tr>
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</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NO-</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>Nitric Dioxide</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH Oxidase</td>
</tr>
<tr>
<td>O₂</td>
<td>Molecular oxygen</td>
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<td>O₂⁻</td>
<td>Superoxide</td>
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<td>Propidium Iodide</td>
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<tr>
<td>PSA</td>
<td>Prostate-Specific Antigen</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated Fatty acid</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>RNS</td>
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Preface

Artificial Insemination (AI) is the single most important tool in facilitating the genetic improvement of dairy cows. Both frozen and liquid semen are used in AI, however the majority (~95%) of insemination doses worldwide (and in Ireland) employ use of frozen semen (Vishwanath & Shannon, 2000). Only 5% of insemination doses used in Ireland are liquid semen, however, during peak breeding season of April and May, this number could rise to 25%. Use of frozen semen in AI has the benefit of long term storage as it causes the biochemical and developmental changes in sperm to stop, however, after the freeze thaw process, more than 50% of sperm cells are damaged (Vishwanath & Shannon, 2000), meaning a higher concentration of sperm cells per dose is required and therefore, less insemination doses per ejaculate are achieved. The use of stored fresh semen would remove this problem of damaged sperm cells after a freeze thaw process. Liquid semen would also allow a lower concentration of sperm cells per insemination dose, thereby, maximising the use of semen from high genetic merit bulls. It has been reported that although liquid bull semen maintains motility for up to 4 weeks post collection, \textit{in vivo} fertility declines significantly after 4-5 days post collection (Vishwanath & Shannon, 1997). The reason for this sudden drop in fertility remains to be elucidated; however, it could be attributed to a number of factors, such as changes in the lipid profile and a subsequent loss in cell membrane fluidity, perhaps due to attack from reactive oxygen species (ROS). The addition of long chain fatty acids to bull sperm \textit{in vitro} may have a stabilising effect on the sperm membrane, through fatty acids constituents being incorporated into the sperm cell membrane, as reported in a study by Neill & Masters (1971) or by inhibiting or reducing the lipid peroxidation (LPO) of the sperm membrane, which is high in polyunsaturated fatty acids.
Chapter 1 – Literature Review
Male Reproductive Physiology

1.1 Male Reproductive System

The male reproductive system consists of internal (series of ducts and accessory glands) and external (penis, testis and epididymis – within scrotum) organs (Figure 1.1). Sperm cells, produced in the testis, travel through the series of internal ducts (efferent ducts, rete testes, vas deferens and urethra) and are expelled through the urethra located in the penis. As sperm travel through the male reproductive system, the accessory glands, seminal vesicles, prostate and bulbourethral glands add secretions to the cells, forming seminal plasma, the liquid portion of semen. A mixture of sperm cells, the seminal plasma and other components such as leukocytes, proteins and lipids form semen.

Figure 1.1: Reproductive Tract of the Bull, displaying the external organs (penis and testis within the scrotum) and internal organs (ducts and accessory glands) (Ashwood, 2009).
1.1.1 Testes

The testes, which are responsible for the production of sperm cells and male steroid hormones (testosterone and dihydrotestosterone; Kenagy & Trombulak, 1986), are held within a skin-like sac called the scrotum (Figure 1.1) and are surrounded by two layers of tissue. The inner most of these layers is known as the tunica albuginea (Polguj et al., 2011), while, the outer layer is known as the tunica vaginalis (Garriga et al., 2009; Figure 1.2). Tunica albuginea is a fibrous capsule, composed of collagen fibres and densely populated with blood vessels (Polguj et al., 2011), whereas, the tunica vaginalis is a mesothelium lined sac, that covers the majority of the testis, except the posterior border (Garriga et al., 2009). This abundance in blood vessels, both arterial and venous, allows for the important heat transfer, that occurs as blood from the body enters the testes through the testicular artery, and is cooled by the blood in the veins returning to the heart, which is at a lower temperature (Brito et al., 2004).

Figure 1.2: Cross section of a testis, also displaying epididymis. Adapted from Wollaston (2011).
Before puberty, at approximately 25 weeks of age, the testes of the bull undergo a rapid phase of growth until they enter puberty, at 37-50 weeks of age. This rapid growth period involves a significant increase in the length and diameter of the seminiferous tubules, proliferation of germ cells into mature germ cells, and an increase in the number of Leydig and Sertoli cells (Rawlings et al., 2008). An average scrotal circumference of 28 cm is often used as the defining point of puberty in the bull, however, this is interchangeable between breeds (Lunstra et al., 1978; Rawlings et al., 2008). In addition, a bull is reported to have reached puberty when its ejaculate contains greater than 50 million total sperm cells, and 10% of the cells are motile (Wolf et al., 1965).

The testicular size often differs between adults of the species, is inversely proportional to body mass and proportional to sperm production (Kenagy & Trombulak, 1986; Latif et al., 2010). As animal size increases, the share of body mass allocated to the testes decreases; studies conducted in the rat and human demonstrated that the testis-body ratio was 0.8% and 0.08%, respectively, however, the much larger bull had a testis-body ratio of 0.1% (Kenagy & Trombulak, 1986), which does not differ significantly from the human. This deviation from the trend seen in the case of the bull, where a higher than expected body mass was dedicated to the testes, could be due to the need for higher sperm production in the bull in comparison to the human or the rat (Kenagy & Trombulak, 1986). This suggests that the larger the testes, the greater potential for sperm production. It has been reported that bulls with a higher scrotal circumference, and therefore a larger testicular size, display greater semen volume, sperm concentration and total number of sperm per ejaculate (Latif et al., 2010). It was mentioned previously that adults of a species differ in testicular size, a trait which is seen in bulls. Larger bulls like Angus and Charolais showed larger scrotal circumferences, and therefore, testicular size compared to Hereford and Polled Hereford bulls
In addition to this, testes were found to be larger in animals who copulate more frequently than in those who do not copulate as often (Harcourt et al., 1981), thereby, proposing that mating system has an effect on testicular size. The size of the testes varies throughout the year in seasonal breeding animals, for example, rams and stallions. This increase in the size of the testes suggests a greater potential for producing sperm at the necessary times (Kenagy & Trombulak, 1986). In the same way, by removing one testicle, the other will increase in size, again allowing greater sperm producing potential (Kenagy & Trombulak, 1986).

As the increase in testicular size can be linked to a greater potential for sperm production at the time of greater requirement (Kenagy & Trombulak, 1986), it is clear that the increase in testicular size in seasonal breeders correlates with an increase in sperm production (Aguirre et al., 2007). In addition to this, the increase in the size of the testes during breeding season is linked to an increase in testosterone levels (Johnson & Thompson Jr, 1983; Aguirre et al., 2007) and lutenising hormone levels (Clay et al., 1987). In stallions, the increased sperm production is reported to stem from an increased population of undifferentiated spermatogonia (Johnson & Tatum, 1989), while numbers of Sertoli and Leydig cells also are greater during breeding season (Johnson & Nguyen, 1986; Johnson & Thompson Jr, 1986; Johnson & Thompson Jr, 1987), leading to an increase in testicular size. Disruption to sperm production is caused when testes are damaged or their descent is obstructed in some way.

Failure of the testes to descend into the scrotal sac is referred to as cryptorchidism (Hutson et al., 1997). Cryptorchidism results in a disruption of spermatogenesis and sterility in bilateral cryptorchidism (failure of two testes to descend) cases (Scott, 1961; Kellaway et al., 1971) and in untreated and post-pubertal cases of unilateral cryptorchidism (failure of one testes to
descend) (Scott, 1961). In addition to this, it can also cause the degeneration of the testes due to higher than normal temperatures, reviewed by Hutson et al. (1997) and reduced production of testosterone when testis remains undescended (Gendrel et al., 1980).

1.1.2 Scrotum and Temperature Regulation in the Testes

The testes are held outside the body, in a sac known as the scrotum, which is composed of four layers, namely, skin, tunica dartos, scrotal fascia and parietal vaginal tunic (Morresey, 2007; Figures 1.1 & 1.3). The tunica dartos layer consists of smooth muscle and fibroelastic tissue, while the scrotal fascia is a loose connective tissue that allows the inner most layer, the parietal vaginal tunic, to move freely (Morresey, 2007).

Figure 1.3: Cross section of testis and relevant layers, including four layers of the scrotum. (Adapted from Steinbachs, 2011).
The presence of an external testes is a species dependent feature. Werdelin & Nilsonne (1999) reported that mammals could be scrotal or ascrotal while their testes are descended or non-descended. Since the 1920’s, it was suggested that thermoregulation was the primary reason for an external testes (Moore, 1926). While this is its primary function, it is now known that mammals with undescended and descended but ascrotal testes carry out normal spermatogenesis, reviewed by Werdelin & Nilsonne (1999). Other secondary reasons for the presence of a scrotum include its involvement in drawing attention from other members of the species (Portman, 1952; Werdelin & Nilsonne, 1999); while the apparently hostile environment of the scrotum can be perceived as a training ground of sorts for spermatozoa in order to prepare the sperm cells for the difficult journey through the female reproductive tract (Werdelin & Nilsonne, 1999).

Optimum bovine testicular temperature is maintained between 2 and 6 °C below normal body temperature to allow normal spermatogenesis to occur (Kastelic et al., 1996) and for maintenance of viable sperm (Appell et al., 1977). Temperature regulation of the testes is achieved through a number of different methods in the bull. The scrotum has a higher than normal number of sebaceous and sweat glands in the skin (Morresey, 2007; Figure 1.3), which are essential to allow the testes to exist at a lower temperature than normal body temperature. The higher number of large sweat glands (Amakiri, 1974; Morresey, 2007) in the scrotal skin also allows for greater moisture evaporation from the scrotum, per unit area of skin, than any other part of the body (Robertshaw & Vercoe, 1980). In addition to this, the muscle in the tunica dartos (Figure 1.3), can alter its shape and change the location of the scrotum in relation to the body by increasing or decreasing the thickness of the muscular wall between the testes and the body when temperatures rise and fall (Waites & Moule, 1961). Finally, the skin of the scrotum is thin while the testes are surrounded by a plentiful supply of
blood vessels, to allow for heat radiation out of the scrotum (Gallup Jr et al., 2009; Kastelic et al., 1996).

1.1.3 Organisational Structure of the Testes

The testis is composed of four different compartments, interstitial, vascular (or tubular), basal and adluminal (Parks et al., 2003; Figure 1.4). The interstitial and tubular compartments are separated from the basal and adluminal compartments by a membrane, known as the basement membrane, which is composed of peritubular (myoid) cells and lamina propria (fibrous tissue) (Parks et al., 2003). Interstitial and vascular compartments are composed of Leydig cells (otherwise known as Interstitial cells of Leydig), lymphocytes, lymphatic vessels and blood vessels (Parks et al., 2003; Fijak & Meinhardt, 2006; Figure 1.4).

Inside the parenchyma (working tissue) of the testes, sperm cells are produced in the walls of the highly convoluted seminiferous tubules, in a process known as spermatogenesis (Hess & Franca, 2009; Moura et al., 2011). Each seminiferous tubule is surrounded by myoid peritubular cells, which provides the tubule with structure and support and allows the tube to contract in order for the spermatozoa to move through the tubule (Fijak & Meinhardt, 2006). Basal and adluminal compartments are contained within the seminiferous tubules. The basal compartment is composed mainly of the basement membrane, Sertoli cells and contains spermatagonia, the germ cells which undergo spermatogenesis (Parks et al., 2003; Figure 1.4). The adluminal compartment contains the developing spermatocytes, more developed sperm cells and Sertoli cells. The adluminal compartment is separated from the basal compartment by the presence of Sertoli cell junctions, creating two compartments with separate environments for cells at different stages of spermatogenesis. Immature sperm cells exist below the blood-testis barrier (BTB) in the basal compartment which move towards the
adluminal compartment as they engage in spermatogenesis (Dym & Fawcett, 1970; Onoda et al., 1990).

Figure 1.4: Compartments of adult bull testes, cross section of one seminiferous tubule, both basal and adluminal compartments are within the seminiferous tubule (Amann, 1983; Parks et al., 2003)
Sertoli cells, also known as sustentacular cells, are the only somatic cells located within the seminiferous tubules. The main function of Sertoli cells is the “nursing” of developing germ cells (Amann & Schanbacher, 1983) by providing the appropriate environment for sperm cells at different stages of spermatogenesis, physical support and junctional complexes which allow selective movements (Griswold, 1998).

The main junctional complex or barrier that the Sertoli cells form is the BTB. It is composed of tight Sertoli cell – Sertoli cell junctions (Morrow et al., 2009), which separates the basal compartment from the adluminal compartment (Mruk & Cheng, 2004; Figure 1.4). The BTB is one of the tightest barriers in the mammalian body (Mruk & Cheng, 2004; Abbott et al., 2010); however, it must open periodically, to allow for the passage of germ cells during spermatogenesis, meaning that the Sertoli-Sertoli cell junctions and Sertoli-germ cell junctions must be disassembled and reassembled in order to do so (Mruk & Cheng, 2004). The mechanism which causes this opening of the BTB, and thus the movement of sperm cells, remains to be elucidated. The main purpose of the BTB (likewise the Blood Brain Barrier - BBB) is to regulate movement of ions, blocking the movement of molecules into the adluminal compartment, thereby, protecting the maturing sperm cells (Mruk & Cheng, 2004; Morrow et al., 2009; Abbott et al., 2010), while also protecting the post-meiotic spermatids from attack from the immune system (Fijak & Meinhardt, 2006). In order for the Sertoli cells to successfully provide nourishment and support for the sperm cells and to for the BTB, there must be a large number of Sertoli cells in the seminiferous tubule, which are obtained up to puberty.
In prenatal life, the seminiferous tubule contains Sertoli cells in the basal compartment and some sparsely distributed germ cells, or gonocytes, which continue to form after birth and all throughout their adult life. Sertoli cell numbers increase rapidly between birth and puberty; the time of initiation and duration of this rapid rise in Sertoli cell number is species dependent (Parks et al., 2003) In the bull, Sertoli cells begin to proliferate at 4 weeks of age and the adult population of Sertoli cells is usually present at 30-40 weeks of age (Rawlings et al., 2008). Between birth and puberty, there is a five-fold increase in the number of Sertoli cells in the bull, however post puberty; there is no increase in the number of Sertoli cells in adult males (Hochereau-de Reviers et al., 1987).

1.1.5 Sperm Production

Spermatogenesis, which begins at puberty, involves the proliferation and transformation of epithelial germ cells into finalised male gametes (Parks et al., 2003). It begins at the wall of the seminiferous tubule, where spermatogonia located along the basement membrane (Brinster, 2002) progress inwards towards the lumen of the seminiferous tubule, taking approximately 61 days in the bull (Johnson et al., 2000; Figures 1.4 and 1.5). Before cells begin to proliferate into more recognisable sperm cells, gonocytes differentiate into testicular stem cells, otherwise known as Type A spermatogonia (Brinster, 2002); these have no chromatin in the nucleus (de Rooij & Russell, 2000). Spermatogenesis can be segregated into three main stages, as described by Parks et al. (2003), spermatocytogenesis, meiosis and spermiogenesis and is initiated when the Type A spermatogonia begin to differentiate and multiply (Parks et al., 2003; Figure 1.5).

The first stage of spermatogenesis is known as spermatocytogenesis, which involves the mitotic division of Type A spermatogonia, to form more differentiated and mature Type A
spermatogonia, followed by Intermediate (In) spermatogonia (i.e. containing an intermediate amount of chromatin) and finally Type B spermatogonia (i.e. contains chromatin in the nucleus) (Amann & Schanbacher, 1983; de Rooij & Russell, 2000). Following this, Type B spermatogonia divide by mitosis to from two primary spermatocytes which enter meiosis (Amann & Schanbacher, 1983; Figure 1.5). The entire process of spermatocytogenesis takes 21 days in the bull (Johnson et al., 2000).

Figure 1.5: Spermatogenesis begins at the basement membrane and ends with the release of spermatids into the lumen of the seminiferous tubule, (adapted from Embryology (2011a)).
The second stage of spermatogenesis is meiosis, where the two primary spermatocytes undergo meiosis, forming secondary spermatocytes, as developing sperm cells move towards the adluminal compartment, through the BTB (Russell, 1977; Johnson et al., 2000; Figure 1.5). Meiosis allows the number of chromosomes in the sperm cell to be halved in the primary spermatocytes (4n) forming secondary spermatocytes (2n); following which the secondary spermatocytes undergo meiosis again, forming haploid (n), round spermatids (Parks et al., 2003; Figure 1.5). The process of meiosis takes approximately 23 days in the bull (Johnson et al., 2000).

The final stage of spermatogenesis is known as spermiogenesis, where immature, round, haploid spermatids with spherical nuclei undergo differentiation into the recognisable sperm cell of the species (Johnson et al., 2000; Parks et al., 2003) and takes approximately 17 days to complete in the bull (Figure 1.5). This process of differentiation is widely studied and occurs in four distinct stages known as the golgi stage, capping stage, acrosomal stage and maturation stage (Figure 1.6). In the golgi phase of spermiogenesis, the golgi apparatus in round spermatids consists of a dense granule surrounded by a vesicle (Susí et al., 1971; Figure 1.6). Synthesis of the acrosome occurs when the golgi apparatus begins to release pro-acrosomal granules which then fuse together, forming a structure called the acrosomal vesicle (Moreno et al., 2000). This vesicle then attaches to the nucleus and continues to grow as more pro-acrosomal granules are released from the golgi apparatus and fuse to the forming acrosomal vesicle (Susí et al., 1971; Figure 1.6). In the capping phase, the acrosomal vesicle formed during the golgi phase, flattens over the nuclear surface, forming a small cap (Escalier et al., 1991; Figure 1.6). This is followed by the acrosomal stage, which involves the acrosomal vesicle covering the majority of the nucleus, acrosomal proteins condensing into a
structure called the acrosomal granule and chromatin condensing (Escalier et al., 1991; Moreno et al., 2000; Burgos & Fawcett, 1955; Figure 1.6).

**Figure 1.6:** Phases of Spermiogenesis, including golgi phase, cap phase, acrosome phase and maturation phase (Steinbachs, 2011).

While these major changes occur within the sperm head, the midpeice and tail of the sperm cell are also developing rapidly. The sperm cell begins to lengthen after the capping phase, triggered by a continuous expulsion of excess cytoplasm (Burgos & Fawcett, 1955) which contains unused mitochondria, ribosomes and lipids, along with the now unnecessary golgi apparatus (Moreno et al., 2000). The elongation of the sperm cell also causes the mitochondria to move away from the nucleus, towards what will become the midpiece of the cell (Figure 1.6). The midpiece is located between the sperm head and tail and contains the mitochondrial sheath, consisting of mitochondria joined end to end in the bull, and in total occupies approximately 80% of the midpiece (Bahr & Engler, 1970). The end step of
spermiogenesis, referred to as the maturation phase, involves the lengthening of the sperm cell and allows the developing flagellum, seen in the acrosomal phase, to transform into the sperm tail (Johnson et al., 2000; Figure 1.5). The chromatin of the nucleus is also fully condensed, and the acrosome, complete with digestive enzymes, covers nearly the entire nucleus, except the section connecting to the midpiece (Johnson et al., 2000).

After the cytoplasm is expelled from the sperm cell during spermiogenesis, a structure known as a residual body, which is the excess cytoplasm, can remain attached to the midpiece-principal piece of the tail junction (Cooper & Yeung, 2003; Figure 1.6). These residual bodies are normally lost as the sperm cells travel towards the seminiferous tubule lumen, or are phagocytised by the Sertoli cells (Breucker et al., 1985). If the residual body remains attached to a sperm cell after it is released into the seminiferous tubule lumen, it is then known as a cytoplasmic droplet. These are normally lost during transit through the epididymis (Cooper & Yeung, 2003).

Spermatogenesis ends with spermiation, the release of sperm cells from the seminiferous epithelium into the lumen of the seminiferous tubule (Johnson et al., 2000). The bull produces approximately 35 million sperm cells per day (Hess & Franca, 2009). Spermatogenesis is regulated and driven indirectly by hormones such as Follicle Stimulating Hormone (FSH), Lutenising Hormone (LH) and testosterone; the action of FSH and LH is carried out on Sertoli and Leydig cells, respectively (Eddy, 2002).

1.1.6 Regulation of Hormones, Leydig and Sertoli Cells

FSH, a gonodatrophin, is released by the anterior pituitary to stimulate the Sertoli cells to secrete fluid, androgen binding protein (ABP), activin and inhibin (Amann & Schanbacher,
Androgens of the male reproductive system, testosterone and dihydrotestosterone, are secreted by Leydig cells, which are located in-between the seminiferous tubules (Amann & Schanbacher, 1983; Figure 1.7). LH, secreted by the anterior pituitary, binds to its receptor on the Leydig cells and causes an increase in intracellular cyclic adenosine monophosphate (cAMP) levels (Dufau, 1988). This facilitates the transport of cholesterol to the inner mitochondrial membrane, where cholesterol begins the initial stage of steroidogenesis to testosterone (Jefcoate et al., 1992). These androgens (testosterone and dihydrotestosterone) are produced by the Leydig cells in response to LH, and to some extent, FSH (Amann, 1983; Figure 1.8), levels of which are controlled by gonadotrophin releasing hormone (GnRH), which is released by the hypothalamus (Amann, 1983). GnRH pulses induce an increase in LH pulse activity by the anterior pituitary, therefore stimulating the Leydig cells to secrete higher levels of testosterone (Amann, 1983; Figure 1.8). In contrast, increasing levels of testosterone decrease the release of GnRH, resulting in reduced LH pulse activity from the anterior pituitary.

The importance of testosterone is evident by its role in the male. Testosterone is responsible for (i) production and maturation of sperm, (ii) secondary male characteristics, such as bone growth and deepening of the voice and hair grown pattern and (iii) libido (de Souza & Hallak, 2011). The release of ABP, activin and inhibin from the Sertoli cells is controlled in
similar fashion to the control of testosterone release from the Leydig cells, involving a series of both positive and negative feedback loops.

Figure 1.7: Leydig cells, or Interstitial Cells of Leydig of the interstitial and vascular/tubular compartments, (adapted from Amann (1983); Parks et al. (2003)).

Figure 1.8: Regulation of hormones by the Leydig and Sertoli cells in the male reproductive tract.
Both FSH and testosterone work in association with each other to maximise spermatogenesis (Shupe et al., 2011). Both support germ cell development and suppress apoptosis, while testosterone alone has been reported to allow spermiation to occur by improving adhesion of spermatids to Sertoli cells, as reviewed by McLachlan et al. (1996). A testis specific cell-cell, actin based adherence junction type, known as ectoplasmic specialisation (ES) allows developing spermatids to adhere to the Sertoli cells of the adluminal compartment, as reviewed by Cheng & Mruk (2002), thereby, facilitating the movement of developing sperm cells through the seminiferous epithelium towards the lumen of the seminiferous tubule (Wong et al., 2005). The same study reported that endogenous testosterone suppression in an in vivo model induces germ cell loss from the seminiferous epithelium due to a disruption of ES adhesion function. The movement of spermatids through the seminiferous epithelium towards the lumen of the seminiferous tubule is regulated by the effect that endogenous testosterone has on ES in the adluminal compartment (Cheng & Mruk, 2002; Wong et al., 2005). After cells are released into the lumen of the seminiferous tubule, they pass through a structure known as the rete testis, which is a network of intertwining slit-like channels which are lined by a flat epithelium (Fijak & Meinhardt, 2006), before entering the epididymis.

### 1.1.7 Epididymis

Sperm cells travel from the rete testis to the epididymis through the efferent ducts (Figure 1.2). The epididymis (Figure 1.2 and 1.9) is a highly convoluted tubule, consisting of three separate regions known as the caput (head), corpus (body) and cauda (tail) epididymis (Caballero et al., 2010; Figures 1.2 and 1.9). The caput epididymis, along with the efferent ducts, re-absorbs rete testis fluid (Gatti et al., 2004). In addition to this function, both the caput and corpus epididymis are responsible for early and late stage maturation of sperm cells (Cornwall, 2009). The cauda epididymis is the main site of storage for mature sperm cells.
Acott & Hoskins (1978) reported that it can take up to 11 days for sperm cells to pass through the epididymis in the bull, however, this is in contrast to Amann & Schanbacher (1983), who reviewed transit time through the epididymis, concluding that it is variable between breeds of bulls, averaging a journey of 5.6 days through the epididymis in Hereford bulls and 8.3 days for Holstein bulls.

**Figure 1.9:** The testis and highly convoluted epididymis (caput, corpus and cauda epididymis). The ductus deferens is also labelled, (Adapted from About-Cancer (2011)).

The organisational structure of an epididymis (Figure 1.10) consists of an outer myoid layer and an inner epithelium layer composed of principal, basal, narrow, apical and halo cells, and a hollow centre, known as the lumen. The principal cells, which compose 80% of the inner epididymal layer, are responsible for the majority of proteins that are secreted by the epididymis into the lumen while unnecessary proteins are eradicated by the clear cells (Cornwall, 2009). Narrow, apical and clear cells are responsible for secreting H\(^+\) ions,
therefore, forming an acidic environment inside the lumen (Pietrement et al., 2006; Kujala et al., 2007). Changes in epididymal lumen pH can affect sperm maturation (Acott & Carr, 1984; Carr et al., 1985; Yeung et al., 2004). In addition to pH, it is vital for conditions inside the lumen to support the maturation of sperm cells. Optimum conditions for the epididymal lumen include a plentiful supply of inorganic ions and organic molecules for sperm cells, optimum bovine testicular temperature and low oxidative stress (Cornwall, 2009).

**Figure 1.10:** Cross section diagram of an epididymis, displaying the different cells of the epididymis and their organisation (Cornwall, 2009).

**1.1.8 Sperm Maturation and Storage**

**1.1.8.1 Changes in the Sperm Membrane Lipid Profile**

During transit through the epididymis, sperm undergo a complex remodelling of the plasma membrane. This involves the uptake of secreted epididymal glycoproteins, the removal or utilisation of specific phospholipids from the membrane bilayer, the processing of glycoproteins that exist in the cell already or have been acquired by endoproteolysis and the
re-positioning of protein and lipid constituents to different membrane domains (Jones, 1998). Lipids of the sperm cell membrane include phospholipids, free fatty acids, sterols, di- and triglycerides (one glycerol molecule with two or three fatty acid chains attached, respectively). Phospholipids constitute the main part of the sperm cell membrane, due to their ability to form lipid bilayers. They consist of a phosphate group, made up of phosphorus, oxygen and hydrogen, two fatty acids and a simple polar molecule attached to a glycerol molecule (Figure 1.11). Phospholipids are named for the polar inorganic group that is attached to the glycerol molecule, which include ethanolamine, choline and insitosol. For this literature review, changes in the lipid profile only, in particular phospholipids will be discussed in this section; the biochemistry of fatty acids will be dealt with in Section 1.4.

Figure 1.11: Structural and space filling model of Phospholipid with Choline polar organic group. The phospholipid is phosphocholine (Campbell & Reece, 2005)
The remodelling of the sperm cell membrane has been investigated in a number of models. Boar sperm showed a distinct change in lipids of the sperm plasma membrane as they travelled through the epididymis, where a decrease was seen in phospholipids such as phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, in addition to free fatty acids, cholesterol and the major glycolipids in boar sperm while an increase was seen in other phospholipids, such as phosphatidylcholine, sphingomyelin and polyphosphoinositides, demosterol, cholesterol sulphate and diacylglycerol (Nikolopoulou et al., 1985). The same study also reported that the same phospholipid-protein and cholesterol-phospholipid ratio was seen in boar sperm cells from the three regions of the epididymis.

Poulos et al. (1973) evaluated testicular, cauda and ejaculated bull sperm phospholipids, reporting that testicular sperm contained double the amount of phospholipids than cauda and ejaculated sperm and that levels of all the major phospholipids (phosphatidylcholine, phosphatidylethanolamine, cardiolipin, phosphatidylserine and sphingomyelin) decreased significantly during epididymal maturation, however, the main phospholipid component, choline plasmalogen, only changed slightly. The amount of major phospholipid bound fatty acid also decreased during epididymal transit, with reductions in absolute concentration in Palmitic acid (PA; C16:0, Saturated fatty acid), Stearic acid (SA; C18.0, SFA), Oleic acid (OA; C18:1, n-9) and Arachidonic acid (AA; C20:4, n-6), with the biggest reduction observed in PA (Poulos et al., 1973). In addition to this, the same study also reported that there was only minor differences in the phospholipid profile of cauda sperm compared to the profile of ejaculated sperm (Poulos et al., 1973), indicating that sperm stored in the cauda are matured. In cauda epididymal sperm, it has been suggested that sperm tails have a higher polyunsaturated fatty acid (PUFA) content than the sperm head, where the main omega 3 fatty acids include docosapentaenoic acid (DPA; 22:5, n-3) and alpha-linolenic acid (ALA;
18:3, n-3) while the main omega 6 fatty acids are linoleic acid (LA; 18:2, n-6) and adrenic acid (22:4, n-6) (Ahluwalia & Holman, 1969). Interestingly, the same study found that docosahexaenoic acid (DHA; 22:6, n-3) was present only in traces in bull sperm tails, and in low amounts in both sperm heads and seminal plasma, however, Poulos et al. (1973) reported that DHA is one of the main fatty acids of caudal and ejaculated whole bull sperm.

It is the remodelling of the sperm cell membrane as it passes through the epididymis that provides them with the ability to bind to and fertilise the female gamete (Jones, 1998). The ability of human sperm cells to bind to hamster oocytes was significantly greater in sperm collected from the caput epididymis in comparison to sperm collected from the corpus epididymis (Hinrichsen & Blaquier, 1980). In addition to this, it is reported that sperm cells acquire the ability to capacitate and undergo acrosome reaction during their journey through the epididymis (Cummins, 1976; Hinrichsen & Blaquier, 1980).

The lipid profile of the sperm plasma membrane also has a role in sperm survival during the cryopreservation process, as reviewed by Bailey et al. (2000). Lipids of sperm cells, which otherwise appear undamaged and unaffected after cryopreservation, display a compromised ability to diffuse within the membrane (James et al., 1999), thereby, reducing the ability to carry out important biochemical reactions relating to fertilisation, including the acrosome reaction and fusion with the oocyte. The success of cryopreservation of semen varies with species, and is not reliable in species such as stallions and rams, due to the number of non-viable cells after the freeze thaw process, reviewed by Bailey et al. (2000). A similar pattern is observed in boar sperm; although the remodelling of the lipid profile of the boar sperm cell membrane increases fluidity of the membrane, it reduces the cell’s resistance to cold shock (Nikolopoulou et al., 1985) thereby causing the reduced number of viable cells after
cryopreservation. In addition to this, the lipid profile remodelling has been suggested as a possible reason for decreased hypotonic resistance (Druart et al., 2009), a characteristic seen cauda epididymal sperm but not in pre-epididymal sperm. The ratio of unsaturated to saturated fatty acids in the sperm plasma membrane has been linked to a species sperm cell susceptibility to cold shock (White, 1993). Species which have a greater number of unsaturated fatty acids in their sperm plasma membranes, for example boar, bull and ram sperm, show high susceptibility to damage from cryopreservation, while those with reduced unsaturated fatty acids, for example humans, display a greater resilience to withstand cold shock, reviewed by Royere et al. (1996); Bailey et al. (2000). This contrasts to results from Giraud et al. (2000), who reported higher amounts of PUFAs in sperm indicates an increased adaptability of sperm to freeze thaw. As bull semen contains high amounts of PUFAs (Ahluwalia & Holman, 1969) in comparison to boar semen, it suggests a greater resilience to damage from cryopreservation. In addition, results from Nikolopoulou et al. (1985) suggest that a better response of sperm cells to cryopreservation is observed in cells with a higher membrane fluidity, therefore, a higher ratio of unsaturated fatty acids.

1.1.8.2 Progressive Motility

Sperm cells are immotile until they reach the corpus epididymis (Yeung et al., 1997; Gatti et al., 2004), due to the low levels of intracellular cAMP inhibiting movement of the sperm tail in the caput and corpus epididymis. Until this point, sperm cells have travelled by peristaltic contractions of the smooth muscle surrounding the epididymal ducts (Amann & Schanbacher, 1983). Levels of cAMP increase in the corpus epididymis in response to external signals from the sperm plasma membrane, allowing the sperm to become motile (Dacheux & Paquignon, 1980; Pariset et al., 1985). There is evidence to suggest that bovine sperm motility is attained through a glycoprotein known as the bovine sperm forward motility protein (FMP) which
works in association with cAMP (Acott & Hoskins, 1978; Acott et al., 1983). This FMP is produced in the epididymis, perhaps by the epithelial cells of the proximal corpus (Acott et al., 1983) and is bound in an inactive state to the sperm cells during transit, which is activated as the surface of the sperm cell membrane is remodelled (Brandt et al., 1978; Acott & Hoskins, 1978). In vitro addition of FMP to bovine caput epididymal sperm cells did not affect total or curvilinear distance travelled but it did significantly increase linear progression and the percentage of progressively motile sperm (Acott et al., 1983). An increase in cAMP is reported to develop an uncoordinated, thrashing movement of the sperm tail, while the FMP converts this movement to strong progressive motion (Acott & Hoskins, 1981). The positive effect of the FMP on caput epididymal sperm cells was also seen in humans, dogs, boars and rabbits, in vitro (Acott et al., 1979).

1.1.8.3 Storage of Sperm

The cauda epididymis, together with the initial segment of the vas deferens, is responsible for storage of mature sperm cells (Amann & Schanbacher, 1983). A study by Amann & Almquist (1962) reported that an average of 54% of sperm cells are stored in the cauda epididymis in sexually rested bulls. The human epididymis is not as pronounced as in other species, thus, making it a poor reservoir for sperm cells (Bedford, 1994), suggesting that prolonged storage may not be necessary in the human. The cauda epididymis secretes proteins, adhesion molecules and ubiquitin, which is involved in the elimination of defective sperm. The cauda epididymis also secretes a variety of proteins, including (i) enzymes which modify proteins of lipids already on the sperm cell membrane surface, (ii) glycosylation enzymes which cause carbohydrates to attach to a hydroxyl or other functional groups, (iii) proteases, enzymes that catalyse the breakdown of proteins, (iv) proteins involved with sperm maturation, motility, future sperm-egg interactions and sperm protection (i.e. Glutathione S-transferase or
peroxiredoxin isoforms). All components secreted by the cauda epididymis help maintain viability and fertilising potential of mature, stored sperm cells (Girouard et al., 2011).

Following storage in the epididymis, sperm cells enter the vas deferens (Steers, 1994), otherwise known as the ductus deferens, which is an uncoiled and unbranched structure, which stores and protects mature sperm (Snyder et al., 2010; Figures 1.2 and 1.9). The vas deferens, along with the testicular artery, testicular vein, and lymphatic vessels form the spermatic cord, which, along with the testes, is surrounded by the cremaster muscle (Polgúj et al., 2011; Libman et al., 2010; Kayalioglu et al., 2008; Figure 1.12).

During ejaculation, the epididymis and vas deferens contract to promote sperm movement towards the urethra (Coolen et al., 2004; Vignozzi et al., 2008), located in the penis. The rhythmic contraction of the perineum muscles, including the bulbospongiosus (surrounds the urethral bulb) and ischiocavernosus muscles, anal and urethral sphincters and levator ani muscles (Figure 1.13) cause male ejaculation of semen into the urethra (Coolen et al., 2004).

1.1.9 Accessory Glands of the Male Reproductive Tract

There are three accessory glands of the male reproductive tract; these are the bulbourethral glands, the prostate gland and the seminal vesicles which contribute 25%, 5% and 50% of components to total seminal plasma in the bull, respectively (Barszcz et al., 2011). The secretions from the accessory glands contribute to the fluid part of semen, known as the seminal plasma, which is fundamental to the survival of sperm cells in vivo in the female reproductive tract.
The bulbourethral glands (also known as the Cowpers glands) (Figure 1.1) secretions consist of mucin, sialic acid, galactose and salts in a slight viscous, clear, aqueous fluid (Rodríguez-Martínez et al., 2011), which, during ejaculation, neutralises any traces of acidic urine remaining in the urethra (Chughtai et al., 2005). In some species, the boar and stallion for example, the bulbourethral glands produce a gel like secretion which can coagulate the entire ejaculate; this is usually removed during semen collection and storage (Rodríguez-Martínez et al., 2011). Mucin is a protein coagulant responsible for the formation of gels, while sialic acid is reported to influence the structural integrity of the cell membrane and the acrosome (Levinsky et al., 1983). Galactose is a sugar which is present as an energy source for sperm motility and metabolism (Hiipakka & Hammerstedt, 1978).
Figure 1.13: Bull perineum muscles, including muscles associated with erection and ejaculation, namely the bulbospongiosus and ischiocavernosus muscles. Urethra and anal sphincters not displayed as urinary tract not included.

The second accessory gland to contribute to semen is the prostate gland, which secretes a slightly acidic fluid, rich in citrate and zinc, and contains peptides and proteins (phosphatase and prostate-specific antigen – PSA) (Rodríguez-Martínez et al., 2011; Figure 1.1). Zinc can play a role in maintaining normal sperm cell physiology (Gavella & Lipovac, 1998) while citrate acts as a major zinc ligand (Arver, 1982). PSA is an anti-coagulant, serine protease, which degrades the gel formed by mucin (Suarez & Pacey, 2006) and is aided by the enzyme phosphatase (Tanaka et al., 2004).
The final accessory gland to contribute to semen is the seminal vesicles, which secrete an alkaline fluid which is rich in fructose, peptides and prostaglandins (Rodríguez-Martínez et al., 2011; Figure 1.1). Fructose, similar to galactose, is necessary as an energy source for motility and metabolism (Schoenfeld et al., 1979; Lewis-Jones et al., 1996). Bull semen contains Prostaglandin E1 (PGE₁) and E2 (PGE₂) in equal amounts, where a high sperm concentration is associated with decreased levels of prostaglandins (Mai & Kinsella, 1980). PGE₁ and PGE₂ are known to play a role in uterine contractility in pigs (Mueller et al., 2006), aiding sperm transport through the female reproductive tract and is likely to have a similar role could be observed in cattle.

1.1.10 Seminal Plasma

Seminal plasma is a combination of fluids secreted by the cauda epididymis and the accessory glands of the male reproductive tract, which, along with sperm cells and round cells (cells of the ducts, epididymis or accessory glands and leukocytes) and cell vesicles (epididymidosomes and prostaticsomes) forms semen (Rodríguez-Martínez et al., 2011). Seminal plasma differs between species, and even within individuals of a species, in respect to volume and composition (Rodríguez-Martínez et al., 2011). Seminal plasma is normally viewed as a transport mechanism for sperm cells, and therefore is removed from some species and replaced with extenders when storing or freezing semen for artificial insemination (AI) (Rodríguez-Martínez et al., 2011). In the bull, however, seminal plasma is not removed but diluted with a suitable extender for the purpose intended; therefore, diluting the positive role that seminal plasma might have, in vivo.

Previous evidence in boars, suggests that the removal of seminal plasma from sperm cells caused changes in motility patterns, including an increase in hyperactivity (Cremades et al.,
In addition to this, seminal plasma was shown to provide boar sperm cells with additional protection against cold shock (Pursel *et al.*, 1973) while both human and boar seminal plasma provided their sperm cells with protection against oxidative stress, due to the presence of antioxidants (Alvarez *et al.*, 1987; Roca *et al.*, 2005). Furthermore, seminal plasma of rodents, boars and humans contain signalling agents (transforming growth factor β, cytokines and prostaglandins) that disrupt the female’s destructive immune system, and allow sperm cells into the female reproductive tract unharmed, thus, improving conception rates (Letterio & Roberts, 1998; Weiner, 2001; Robertson, 2007).

Bovine seminal plasma contains an array of lipids and proteins. In particular, bovine seminal plasma (BSP) contains phospholipid binding proteins known as BSP-A1/A2, BSP-A3 and BSP-30-kDa (Desnoyers & Manjunath, 1992) which are secreted by the seminal vesicles. The BSP proteins bind to choline phospholipids, which are high density lipoproteins, during ejaculation and in doing so, cause an efflux of phospholipids and cholesterol from mature sperm, thereby modifying the cell membrane and triggering capacitation (Thérien *et al.*, 1999). Bull caudal epididymal sperm, when incubated with accessory gland fluid from the same bull, also resulted in decreased sperm motility, decreased sperm viability and an increased incidence of acrosome reacted sperm (Way *et al.*, 2000). Therefore, while these BSP proteins are vital to trigger capacitation in the female reproductive tract, they are thought to be detrimental to sperm *in vitro*. It is for this reason that it is important to dilute semen as quickly as possible after collection for storage and use in AI. When egg yolk based extender was used to dilute bovine semen, the phospholipid and cholesterol efflux from sperm was completely reversed, there was a cholesterol and phospholipid gain, indicating a decreased binding of BSP proteins to the sperm cell. Dilution of bovine semen with a non-egg yolk based diluent, i.e. Tris-glycerol extender, did not have the same effect in reversing
phospholipid and cholesterol efflux (Bergeron et al., 2004). It has been proposed that there are two methods by which egg yolk can help protect sperm cells and extend their life in vitro. The first method suggested by Bergeron et al. (2004) is that the association of low density lipoproteins in egg yolk can prevent the BSP proteins binding to sperm cells, thereby, preventing the phospholid and cholesterol efflux from sperm. Secondly, the low density lipoprotein lipid, or the whole low density lipoprotein, could in fact be incorporated into the sperm cell plasma membrane, and therefore, preserve the integrity of the plasma membrane (Bergeron et al., 2004).

Lipids constitute 1.35% of BSP, which similar to sperm cells themselves, are reported to be composed mainly of phospholipids (~70%) and cholesterol (~20%) (Komarek et al., 1964). This is in contrast to a study by Pursel et al. (1973) who found that of the total lipids found in BSP, ~30% were phospholipids and ~20% were cholesterol. Phospholipids in BSP are composed mainly of choline and ethanolamine phosphatides (~54% and ~27%, respectively) and sphingomyelin (~14%), which are also seen in bovine sperm cells, however, two extra lipids are found in BSP, not seen in their sperm, namely, polyglycerol phosphatide (~5%) and cerebroside (trace) (Pursel et al., 1973). Within the phospholipids of BSP, OA was found to be the most abundant MUFA in choline phosphatides, while in ethanolamine phosphatides LA was the most available PUFA (Pursel et al., 1973).

1.2 Matured Bovine Sperm Structure

Sperm cell structure is a species dependent characteristic (Johnson et al., 2000), however, in mammals, it is widely accepted that it generally consists of a sperm head, midpiece and tail, with an acrosome covering two-thirds of the sperm head (Figures 1.14 and 1.15). The head of the sperm cell consists of a homogenous, flattened, highly condensed nucleus, containing
The DNA content and organisation in the nucleus usually determines the shape of the sperm head (Sailer et al., 1996). The nucleus thickens to the posterior of the sperm head, in most species, in order to allow the connection between head and tail (Saacke & Almquist, 1964a). The equatorial segment of the sperm head forms when the acrosome swells and then deteriorates, leaving a posterior portion intact, which appears as a band about the centre of the nucleus (Saacke & Almquist, 1964a; Figure 1.15).

**Figure 1.14:** The mature sperm structure, where A = sperm head, B = neck, C = midpiece, D = principal piece and E = end piece. Finer structures include 1 = plasma membrane, 2 = outer acrosomal membrane, 3 = acrosome, 4 = inner acrosomal membrane, 5 = nucleus, 6 = proximal centriole, 7 = distal centriole, 8 = course outer fibres, 9 = mitochondria, 10 = axoneme, 11 = annulus and 12 = fibres, (Adapted from Embryology (2011b)).

The acrosome, a cap like structure, contains enzymes, such as acrosin and hyaluronidase (Lenzi et al., 1996) which are necessary to penetrate the layers of cumulus cells around the matured oocyte (Johnson et al., 2000; Figure 1.15). The acrosome is thicker to the front of the
head, thus, forming a ridge to one side of the nucleus and consists of three layers, namely a
dense inner and outer membrane with an electron rich middle layer (Saacke & Almquist,
1964a).

Figure 1.15: Sperm cell head structure, plasma membrane is located outside the acrosome,
the acrosome covers the majority of the nucleus (Senger, 2003)

The midpiece, connected to the head by the neck, consists of mitochondria arranged in a
helix, which, in the bull is composed of three main spirals, with mitochondria joined end to
dead (Saacke & Almquist, 1964b; Bahr & Engler, 1970; Figure 1.14 and 1.16), where it is
suggested that the strands or spirals of mitochondria can begin or end at any location along
the midpiece (Saacke & Almquist, 1964b). The bovine sperm tail consists of fibres arranged
in a $9 + 9 + 2$ pattern (Saacke & Almquist, 1964b), which represents nine outer, coarse fibres,
nine microtubule doublets and one microtubule doublet in the centre, respectively (Figures
1.16). The nine outer, coarse fibres run from the neck throughout most of the tail (Figure 1.16
lower panel), becoming narrower as they progress down the tail, ending with the principal piece, at different intervals (Saacke & Almquist, 1964b). Inside these coarse outer fibres, are nine microtubule doublets with one microtubule doublet in the centre (Figures 1.16), which run through the midpiece and the tail (Saacke & Almquist, 1964b; Porter & Sale, 2000). The sperm tail is connected to the midpiece by the annulus (Kwitny et al., 2010), which is also surrounded by the nine course, outer fibre structure. The end piece of the tail consists of only the central axoneme covered by plasma membrane (Figure 1.14). Sperm are surrounded by a plasma membrane which is composed of lipids (Ahluwalia & Holman, 1969; Lenzi et al., 2000) and proteins (Flesch & Gadella, 2000).

**Figure 1.16:** Upper panel: General structure of an axoneme, 9 outer microtubule doublets with an inner doublet. Microtubules are connected to each other and to the inner doublet by
dynein arms, which are motor proteins, responsible for the bending out the sperm tail. Adapted from Afzelius (1998). Lower panel: Cross section of a normal human sperm axoneme from principal piece of cell, where the fibrous sheath is seen, indicated by the asterix, the 9 + 2 structure of the axoneme inside the sheath. Adapted from Chemes & Alvarez Sedo (2012).

**Lipids and Fatty Acids**

1.3 Introduction to Lipids

Lipids are a wide and diverse group of molecules that play a vital role in mammalian cell structure and function. They are used as storage compounds, an energy source, as signalling molecules and are involved in membrane trafficking, regulating membrane proteins and creating sub-compartments in membranes (Shevchenko & Simons, 2010). In mammalian cells, the main lipids found are phospholipids, sterols and glycerides (mono, di and tri), phospholipids being the most representative lipid fraction in sperm cell membranes (Table 1.1).

Lipids are a prominent part of the cellular plasma membrane, where amphipathic phospholipids form an asymmetric bilayer, with phosphatidylcholines and sphingolipids in the outer layer and phosphatidylethanolamines, phosphatidylserines and phosphoinositides in the inner layer, which are in contact with the cytosol (Shevchenko & Simons, 2010; Table 1.1). Phospholipids contain a hydrophilic head and a hydrophobic tail, a property which aids in the formation of bilayers by allowing the self-association of the hydrophobic tail, driven by water, while the hydrophilic heads interact with each other and with the aqueous environment surrounding them (Van Meer *et al.*, 2008). Cholesterol, a non-polar molecule, is the main
sterol of mammalian cells and is present in both leaflets of the lipid bilayer (Van Meer et al., 2008; Shevchenko & Simons, 2010).

Fatty acids are vital components of phospholipids and di- and triglycerides, where they are attached to a glycerol molecule in both, with an additional polar, organic molecule adjoined to the glycerol molecule of the phospholipid. Phospholipids and diglycerides contain two fatty acid chains while triglycerides contain three. When these fatty acids are not part of a larger structure, they are known as free fatty acids.

1.4 Saturated and Unsaturated Fatty Acids

1.4.1 Saturated, Monounsaturated and Polyunsaturated Fatty Acids

Fatty acids are located in membrane lipids and storage oils as phospholipids, esters, ethers, glycerides, glycolipids, phosphonolipids, glycosphingolipids, sulpholipids and lipoproteins, as reviewed by (Gill & Valivety, 1997). The fatty acids of cell membranes differ in composition through their carbon chain length, the number of double bonds and the position of the first double bond from the methyl end in the fatty acid (Stubbs et al., 1981). Saturated fatty acids (SFAs) have no double bonds in their hydrocarbon chain (Figure 1.17), while unsaturated fatty acids contain a double bond at various positions along the chain (Figure 1.18-1.21). Fatty acids consist of a hydrocarbon chain with a methyl group (CH$_3$) at one end with a carboxyl group (COOH) at the other end (Gill & Valivety, 1997).
Table 1.1: Structure of common lipids, including phospholipids, diglycerides, triglycerides, sterols and free fatty acids, with common examples (ALevelNotes, 2011)

<table>
<thead>
<tr>
<th>Type of Lipid</th>
<th>Lipid Structure</th>
<th>Examples in Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td><img src="image" alt="Phospholipid Diagram" /></td>
<td>Phosphatidylcholine, Phosphatidylethanolamine, Phosphatidylserine, Phosphoinositides</td>
</tr>
<tr>
<td>Diglyceride</td>
<td><img src="image" alt="Diglyceride Structure" /></td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td><img src="image" alt="Triglyceride Structure" /></td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Sterol</td>
<td><img src="image" alt="Sterol Structure" /></td>
<td></td>
</tr>
<tr>
<td>Free Fatty Acid</td>
<td><img src="image" alt="Free Fatty Acid Structure" /></td>
<td>Palmitic acid, Linoleic acid, Alpha-linoleic acid, Oleic acid</td>
</tr>
</tbody>
</table>
The most common SFAs in human sperm cells are PA (16:0) and stearic acid (SA; 18:0) (Aitken et al., 2006; Koppers et al., 2010), both of which contain no double bonds in the hydrocarbon chain (Figure 1.17). Due to the absence of double and triple bonds, saturated fatty acids are packed a lot tighter in cell membranes and therefore, reduce membrane fluidity. Membrane fluidity increases as levels of membrane unsaturation increases (Lenzi et al., 1996).

![Structure of saturated fatty acids (SFAs), palmitic acid (PA; 16:0) (Upper Panel) and stearic acid (SA; 18:0) (Lower Panel), no double bonds, 16 and 18 carbons in the hydrocarbon chains, respectively. Both have COOH groups to the end with methyl (CH₃) end to the left, C and H atoms not marked.](image)

**Figure 1.17:** Structure of saturated fatty acids (SFAs), palmitic acid (PA; 16:0) (Upper Panel) and stearic acid (SA; 18:0) (Lower Panel), no double bonds, 16 and 18 carbons in the hydrocarbon chains, respectively. Both have COOH groups to the end with methyl (CH₃) end to the left, C and H atoms not marked.

Unsaturated fatty acids can be described as monounsaturated or polyunsaturated, where monounsaturated fatty acid (MUFA) components contain only one double bond and PUFAs contain more than one. MUFAs and PUFAs are then again classified into three families, omega 3, 6 and 9 unsaturated fatty acids according to the distance of the first double bond from the methyl terminal (Lenzi et al., 1996).
1.4.2 Omega 3, 6 and 9 Fatty Acids

Omega 3 fatty acids are fatty acids in which the first double bond is located on the third carbon-carbon double bond from the methyl end of the hydrocarbon chain of the fatty acid. The first double carbon-carbon bond of omega 6 fatty acids is located on the sixth carbon from the methyl end while omega 9 fatty acids have the first carbon-carbon double bond on the ninth carbon from the methyl end group. Omega 3, 6 and 9 fatty acids can also be denoted as n-3, n-6 and n-9 or ω-3, ω-6 and ω-9 fatty acids, respectively. Common n-3 fatty acids in many species of sperm include ALA (18:3), DHA (22:6), DPA (22:5) and eicosapentaenoic acid (EPA; 20:5) (Figure 1.18). Arachidonic acid (AA; 20:4) and LA (18:2) are two of the most well-known n-6 fatty acids in sperm cells (Figure 1.19) while oleic acid (OA; 18:1) is the most prominent n-9 fatty acid family (Figure 1.20).

![Figure 1.18: Structure of n-3 polyunsaturated fatty acids (PUFAs), alpha-linolenic acid (ALA; 18:3), docosapentaenoic acid (DPA; 22:5); docosahexaenoic acid (DHA 22:6) and...](image)
eicosapentaenoic acid (EPA; 20:5) (top, second, third and final panel, respectively), with 18, 22, 22 and 20 carbons and 3, 5, 6 and 5 double bonds on the carbon chain in ALA, DPA, DHA and EPA, respectively. The first double bond in all four is on third carbon atom from methyl end. Adapted from Gill & Valivety (1997).

**Figure 1.19:** Structure of n-6 polyunsaturated fatty acids (PUFAs), arachidonic acid (AA; 20:4) and linoleic acid (LA; 18:2) (top and bottom panel, respectively), with 20 and 18 carbons and 4 and 2 double bonds on the carbon chain in AA and LA, respectively. First double bond in both is on sixth carbon atom from methyl end (Adapted from Gill & Valivety (1997)).

**Figure 1.20:** Structure of n-9 monounsaturated fatty acids (MUFA), oleic acid (OA; 18:1) with 18 carbons in the carbon chain, 1 double bond on the carbon chain; located on the ninth carbon atom from the methyl end of the hydrocarbon chain.
Higher animals, like the bull, boar and human, are unable to manufacture fatty acids with carbon chains longer than 18 carbons, due to a deficiency in the appropriate desaturase enzymes. These animals have $\Delta^4$-desaturase, $\Delta^5$-desaturase, $\Delta^6$-desaturase and $\Delta^9$-desturase, the number indicating the location that the desaturase enzyme places the double bond in the carbon chain (Gill & Valivety, 1997). They do not contain $\Delta^1$-desaturase, $\Delta^2$-desaturase and $\Delta^3$-desaturase enyzmes which could form ALA, LA and OA, otherwise known as the parent fatty acids of n-3, n-6 and n-9 fatty acids, respectively. These fatty acids are usually found in the diet and longer chain fatty acids are synthised by the metabolism of the fatty acids by elongation and desaturation reactions (Lenzi et al., 1996; Figure 1.21). This process is referred to as de novo synthesis of fatty acids (Gill & Valivety, 1997).

**Figure 1.21:** Metabolism of parent fatty acids ALA (n-3) and LA (n-6) into longer carbon chain fatty acids with relevant enzymatic reactions to form the fatty acids (Lenzi et al., 1996).
1.5 Polyunsaturated Fatty Acids and Fertility

Unsaturated fatty acids have been widely reported, in both human and animal models, to have beneficial effects on the cardiovascular (Bønaa et al., 1990; Harper & Jacobson, 2001) and nervous (Alessandri et al., 2004; Brenna & Diau, 2007) systems. This focus has extended to the role on PUFA’s on female fertility in more recent years (Childs et al., 2008; Hammiche et al., 2010) and to a lesser extent, male fertility, as reviewed by Wathes et al. (2007).

The main focus of work on the effect of PUFAs on male fertility has centred around the inclusion of fatty acids in the diet (Comhaire & Mahmoud, 2003) or on the comparison of the lipid profile of sperm between fertile and infertile males (Aksoy et al., 2006; Safarinejad et al., 2010). Supplementation of dietary fish oils, rich in long chain fatty acids, has been demonstrated to successfully modify the fatty acid profile of sperm across a range of species, such as cockerel, human, boars and bulls, respectively (Kelso et al., 1997b; Comhaire et al., 2000; Rooke et al., 2001; Castellano et al., 2010; Gholami et al., 2010), however, the effects of this are still unclear. An increase in the proportion of n-3 fatty acids in ejaculated boar sperm led to the number of progressively motile cells being increased after being fed with tuna oil (Rooke et al., 2001), however, others found no beneficial effects in terms of semen production (Castellano et al., 2010) or the lifespan of boar sperm stored at 17 °C or following cryopreservation (Castellano et al., 2010; de Graaf et al., 2007). Furthermore, supplementation of DHA in the diet was found to improve motility, speed and viability of fresh Holstein bull sperm, with no effect on frozen sperm (Gholami et al., 2010).

Fatty acids, as a component of phospholipids and di- and triglycerides, and as free fatty acids, are an integral part of sperm cell membranes, with both saturated and unsaturated fatty acids identified in the plasma membrane surrounding both the sperm head and tail of many
different species (Ahluwalia & Holman, 1969; Poulos et al., 1973; Zalata et al., 1998). It has been reported that the fatty acid composition of sperm cells help determine physiological characteristics. For instance, n-3 fatty acids are important for improvements in human sperm development, motility and cryogenic tolerance (Safarinejad et al., 2010), while also being necessary in maintaining lipid bilayer properties (Farooqui et al., 2000).

Cell membrane fluidity, which is dependent on the sperm lipid and fatty acid constitution, is vital for normal cell function (Lenzi et al., 1996). In addition to sperm membrane fluidity increasing as levels of unsaturation increase (Hall et al., 1991; Lenzi et al., 1996), important biochemical reactions such as acrosome reaction, sperm-oocyte fusion and fertilisation are dependent on high levels of membrane fluidity (Safarinejad et al., 2010). An increase in the saturated fatty acid content would be expected to disrupt cell function as it limits cell membrane fluidity (Koppers et al., 2010), due to the tight packing and lack of double bonds in SFAs. The same study reported that PA was the single most prominent fatty acid of human sperm, followed by DHA and then SA, while the other fatty acids contribute to less than 6% of total fatty acids.

1.6 Fatty Acid Profile of Mature Sperm
As sperm travel through the epididymis (Sections 1.1.7 & 1.1.8), they undergo a process of maturation, where their membrane proteins and lipids are restructured into the species dependent membrane profiles (Jones, 1998), while attaining the ability to gain progressive motility and the ability to fertilise an oocyte (Hinrichsen & Blaquier, 1980; Gatti et al., 2004). Bull sperm, in particular, lose half their phospholipid content, with a reduction in all major phospholipid fractions (Poulos et al., 1973). Fatty acids, as a major component of phospholipids, also undergo a major reduction during epididymal transit (Ahluwalia &
Holman, 1969; Poulos et al., 1973), while retention of fatty acids (SFA, MUFAs and PUFAs) is indicative of immature and defective sperm cells (Koppers et al., 2010).

To the best of the authors knowledge, there is no published work on the fatty acid profile of sperm cell membranes or epididymal and testicular fluid, where fatty acid concentration is given in local concentrations. The majority of work, which is mentioned below, gives fatty acid concentration as a percentage of total fatty acids. Ahluwalia & Holman (1969) fractionated bull semen into sperm heads and tails and reported that mature sperm tails contained more n-3 PUFAs than the sperm head membrane, while n-6 PUFAs were higher in sperm heads than the tails. Similar n-3 and n-6 patterns by the same study were found in human sperm. A higher percentage of n-6 fatty acids (28%) were found in total bull sperm than n-3 fatty acids (23%), where the main n-6 fatty acids identified were Adrenic acid (22:4), LA, DGLA and AA (13.5, 5.2, 3.6 and 3.6%, respectively). The main n-3 fatty acid identified was DPA (20.8%), while DHA was found in trace amounts only, in both bull and human sperm, while, OA and its metabolites (n-9 fatty acids) were not present in recordable amounts in either bull or human semen (sperm heads and tails and seminal plasma). The results from the study by Ahluwalia & Holman (1969) contrasted to both Poulos et al. (1973) and Koppers et al. (2010), who reported that DHA was one of the main fatty acids of caudal and ejaculated bull sperm and ejaculated human sperm, respectively. More recently a study by Lenzi et al. (2000) has suggested that up to 60% of PUFA in normal human sperm consists of DHA, however Zalata et al. (1998) reported that DHA accounted for 34% of sperm head PUFAs and 14% of sperm tail PUFAs. Both these studies suggest that DHA is the most prominent PUFA of human sperm cell membranes. In contrast to the earlier work by Ahluwalia & Holman (1969), Zalata et al. (1998) found that human sperm heads contained a higher level of PUFAs than sperm tails. PA and SA have been identified as the most
prominent saturated fatty acids of whole human sperm (Lenzi et al., 2000; Koppers et al., 2010).

Additional evidence demonstrated that human sperm fatty acid composition from asthenozoospermic (low motility and viability) males differed to that from normospermic (normal) males, where, the former showed lower levels of DHA but higher OA (Zalata et al., 1998; Conquer et al., 1999) while unsaturated fatty acids, as a whole, were reduced in the asthenozoospermic males compared to normospermic males (Calamera et al., 2003). In addition to this, infertile human males were found to have higher levels of n-6 PUFAs, which was linked to a decreased sperm concentration, decreased motility and higher abnormal cell count (Safarinejad et al., 2010). High levels of n-3 PUFAs (ALA, DHA, DPA and EPA) were associated with sperm development, improved motility and morphology and cryogenic resistance (Safarinejad et al., 2010).

In contrast to the common theory that fatty acids are lost as they mature, Lenzi et al. (2000) reported that immature germ cells (IGC – defined as spermatogonia, spermatocytes and spermatids), isolated from mature sperm cells using a Percoll gradient, contained lower levels of long chain PUFAs but SFAs and parent n-3 and n-6 PUFAs (namely; ALA and LA) levels were elevated. The percentage of SFAs in matured sperm cells were similar to those in IGCs, however levels of long chain PUFAs were increased in IGCs. This increase in long chain PUFAs coincided with a reduction in parent n-3 and n-6 fatty acids (ALA and LA), thereby, demonstrating that sperm have an active metabolism as they mature, which causes an elongation and desaturation of essential fatty acids (Lenzi et al., 2000). The superabundance of unsaturated fatty acids, often seen in IGCs, leave sperm cells extremely susceptible to
Reactive Oxygen Species

1.8 Reactive Oxygen Species Formation

The majority of reactive oxygen species (ROS) are free radicals, a group of highly reactive and short lived chemical species, with one or more unpaired electrons (Agarwal et al., 2006), which includes the superoxide anion (O$_{2^-}$), the hydroxyl radical (OH$^-$) and the hydroperoxyl radical (HO$_{2^-}$). Hydrogen peroxide (H$_2$O$_2$) is a ROS that is not part of the free radical group as it does not have any unpaired electrons, leaving it quite stable in comparison to other ROS (Sikka, 2001). A smaller group of free radicals, known as reactive nitrogen species (RNS), sometimes described as a subset of ROS (Darley-Usmar et al., 1995; Davidson et al., 1997), are also detrimental to sperm cells. These include nitric oxide (NO$^-$), nitric dioxide (NO$_2^-$) and peroxynitrite (NO$_3^-$$\cdot$).

A fine balance exists between the positive and negative role that ROS can play in cells, in particular sperm cells. ROS can be of benefit to the sperm cell as a signalling molecule, reviewed by Sanocka & Kurpisz (2004); Kothari et al. (2010), and are involved in vital cell processes such as capacitation, hyperactivation (Griveau et al., 1994) and the acrosome reaction (Aitken et al., 1995; Section 1.9). However, as sperm cells contain a high level of fatty acids, in particular long chain PUFAs, they are extremely susceptible to ROS attack, due to the presence of the double bonds.
\[ O_2 + e^- \xrightarrow{\text{oxidase}} O_2^- \]

**Figure 1.22:** The 1 electron reduction of diatomic oxygen, where \( e^- \) indicates an electron. The reaction can occur with or without the presence of oxidase enzymes. Adapted from Sikka (2001).

Superoxide is formed through the reduction of molecular oxygen (\( O_2 \)), leaving an end product of \( O_2^- \), the superoxide anion (Figure 1.22). This can then be used to form other ROS through a series of reactions (Wolin, 2000; Figure 1.23-1.25). \( O_2^- \) dismutates (a process of simultaneous oxidation and reduction), in the presence of protons and superoxide dismutase (an anti-oxidant) to form hydrogen peroxide (\( H_2O_2 \)), also producing the short lived, hydroperoxyl (\( HO_2^- \)), an intermediary free radical (Sikka, 2001; Figure 1.24). This finding has also been reported by Alvarez *et al.* (1987), who found that \( H_2O_2 \) was generated by spontaneous protonation (addition of a proton) of the superoxide anion or in the presence of superoxide dismutase (Figure 1.23). \( H_2O_2 \), as outlined previously, is relatively stable in comparison to the other ROS, as it is not a free radical, however, this does not subtract from the damaging effects it can have on cells. It has been reported that \( H_2O_2 \) has a greater biological permeability than other ROS, allowing it to pass through cell and nuclear membranes relatively easily, making it one of the most detrimental ROS to sperm cells (Baumber *et al.*, 2000).

\[
2(O_2^-) + 2H^+ \xrightarrow{\text{SOD}} 2HO_2^- \rightarrow H_2O_2 + O_2
\]

**Figure 1.23:** Generation of hydrogen peroxide (\( H_2O_2 \)) by superoxide (\( O_2^- \)), where SOD indicates superoxide dismutase. An intermediate hydroperoxyl (\( HO_2^- \)) species is formed before \( H_2O_2 \), which in itself is a type of ROS. Molecular oxygen (\( O_2 \)) is also released from
the reaction, leaving it possible for superoxide to be formed again. Adapted from Sikka (2001).

The presence of excess $O_2^-$ and $H_2O_2$ causes the equilibrium to shift in favour of the Haber Weiss reaction occurring (Aitken et al., 1989; Figure 1.24). This reaction allows the formation of hydroxyl radicals (OH·), which are extremely reactive, short lived and considered to be the most toxic of ROS (Sikka, 2001). Due to their reactivity, OH· initiate LPO (Aitken et al., 1989; Kothari et al., 2010), which can be described as the loss of fatty acids from the cell membrane due to ROS attack (Kothari et al., 2010) greatly reducing membrane fluidity and integrity (Aitken et al., 1989), as well as being highly detrimental to DNA (Wellejus et al., 2000).

$$H_2O_2 + O_2^- \rightarrow OH^\cdot + OH^- + O_2$$

**Figure 1.24:** The Haber-Weiss reaction occurs in the presence of excess hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^-$), resulting in formation of hydroxyl radicals (OH·) and again molecular oxygen. Adapted from Aitken et al. (1989).

In addition to the Haber Weiss reaction, hydroxyl radicals (OH·) are formed through the Fenton reaction, in which $H_2O_2$ reacts with free iron ions ($Fe^{2+}$) (Sikka, 2001; Figure 1.25). Iron ions ($Fe^{2+}/Fe^{3+}$) are ubiquitous (Wellejus et al., 2000) and an iron binding glycoprotein has been found in human seminal plasma (Quinlivan, 1968). Therefore, as iron is available in human seminal plasma, the Fenton reaction is free to occur when $H_2O_2$ becomes available.
\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO} + \text{HO}. \]

**Figure 1.25**: The Fenton Reaction. H\(_2\)O\(_2\) can react with free iron ions (Fe\(^{2+}\)) to produce OH· radicals (Adapted from Sikka (2001)).

### 1.9 Physiological Role of ROS in Sperm Cells

The role that ROS fulfil in semen, either beneficial or detrimental, is somewhat dependent on certain factors, mainly, the type of ROS involved, the time and location they are produced at, their duration of contact and in what amount they are produced (Griveau & Lannou, 1997), as well as external factors like temperature, availability of oxygen and the levels of antioxidants (Agarwal & Saleh, 2002). While sperm cells travel through the male reproductive tract they gain the ability to move progressively however it is during their journey through the female reproductive tract, they undergo a series of biochemical reactions in order to gain the ability to fertilise the oocyte (Choudhry et al., 1995). It is thought that ROS play a vital role in capacitation, hyperactivation and the acrosome reaction, although the mechanism of their action is not yet fully understood.

Capacitation is a process which takes place in the female reproductive tract, involving the removal of the sperm acrosomes glycoprotein coat and seminal plasma proteins, in order to allow the acrosome reaction to occur. Hyperactivation is usually linked to capacitation, as it has been observed in sperm cells while they are undergoing capacitation however, it has been reported that one can occur without the other, reviewed by Marquez & Suarez (2004). Hyperactivation is a form of sperm motility that involves rapid, asymmetrical movement of the sperm tail and allows sperm to detach from the wall of the oviduct, to find the oocyte in the oviduct, to penetrate mucus in the female oviduct and finally to penetrate cumulus cells and the zona pellucida of the oocyte, as reviewed by Suarez & Ho (2003). When NO and O\(_2\)-
were generated artificially in semen, capacitation was triggered in human cells (Zini et al., 1995; de Lamirande & Gagnon, 1993b), while O$_2^-$, stimulated sperm cells to exhibit hyperactivated motility (de Lamirande & Gagnon, 1993b). Hydrogen peroxide (H$_2$O$_2$) has been demonstrated to have a significant effect at promoting hyperactivation and subsequent capacitation in human sperm cells, *in vitro*. Levels of both processes decreased with the introduction of catalase, an anti-oxidant specific to H$_2$O$_2$ (Griveau et al., 1994). In other models, capacitation in stallion sperm was promoted by the addition of H$_2$O$_2$ and O$_2^-$ (Baumber *et al.*, 2003) while hyperactivation levels in hamster sperm were maintained at a reduced level by NO during its transit through the epididymis (Suarez, 2008). Although it has been reported by many studies that ROS play a role in capacitation and hyperactivation, it remains to be demonstrated their mechanisms of action.

During capacitation, changes occur in the sperm cells in order for the acrosome reaction to occur, another biochemical process that sperm cells undergo in the female reproductive tract. The acrosome reaction involves the expulsion of acrosomal contents, which mainly contains enzymes, including acrosin and hyaluronidase (Lenzi et al., 1996). In a study by Aitken *et al.* (1995), the acrosome reaction was induced artificially in human sperm cells by the addition of the divalent cation ionophore, A23187. Treatments which had catalase added in addition to the ionophore prevented the acrosome reaction, indicating that H$_2$O$_2$ plays a vital role in this biochemical reaction. The acrosome reaction, in addition to capacitation and hyperactivation are referred to as “redox regulated” events or “oxidative” events (de Lamirande *et al.*, 1997). Although all studies mentioned on the redox regulated events were carried out *in vitro*; all studies have suggested that similar effects may occur *in vivo*, inside the model. It has been suggested that the generation of ROS enhances tyrosine phosphorylation (the addition of a phosphate group (PO$_4^{3-}$) to a protein, carried out on the tyrosine residue), and it is this process
that triggers a cascade of intracellular events that leads to the acrosome reaction occurring in human sperm cells which aids in the sperm cell fusing with the oocyte (Aitken et al., 1995). The mechanism by which ROS (mainly $\text{H}_2\text{O}_2$) causes tyrosine phosphorylation remains to be elucidated, however, Aitken et al. (1995) suggested that it may be a combination of stimulation of tyrosine kinase activity or inhibition of tyrosine phosphatise activity, collectively or on their own.

Finally, low levels of LPO, caused by ROS, have been reported to enhance the ability of human sperm to bind to the zona pellucida, as suggested by Aitken et al. (1989) while a similar result has also been found in mouse spermatozoa where ROS generation was stimulated with iron and ascorbic acid (Kodama et al., 1996). This effect could be attributed to changes in order of the sperm cell membrane, caused by LPO, as suggested by Aitken et al. (1989). Excessive generation of ROS produced by sperm cells themselves and other endogenous sources cause OS and LPO.

### 1.10 Source and Generation of ROS in Semen

Sperm cells, both normal and immature, contribute to ROS in semen; however immature sperm are deemed to be a more prominent source of ROS, reviewed by Griveau & Lannou, (1997); Kothari et al. (2010). It has been reported that immature and defective sperm cells cause higher generation of ROS than normal sperm cells in an ejaculate (Aitken & Clarkson, 1987; Koppers et al., 2008). Possible mechanisms for production of ROS in both normal and mature sperm cells and defective and immature sperm cells are outlined.

Adenosine triphosphate (ATP) is a molecule that facilitates the transfer of energy within cells and is vital for motility in sperm. ATP is produced by sperm cells during anaerobic and
aerobic respiration. ATP production from glycolysis (anaerobic respiration) is limited but rapid, and therefore, can be considered a main source of ATP in sperm cells; however it does not involve the mitochondria (Nascimento et al., 2008). In addition to this, glycolysis, unlike aerobic respiration, is not restricted to the mitochondria (Stendardi et al., 2011), it occurs in the cytosol of the cell, and therefore, ATP would be distributed more evenly in the sperm cell.

The cause of excessive free radical generation has not yet been established (Koppers et al., 2008) for most mammalian species, therefore, it is inaccurate to claim that the ETC is responsible for the majority of ROS generation from sperm cells, however, ROS generation by human spermatozoa has been linked to mitochondria (Koppers et al., 2008). Aerobic respiration, or oxidative phosphorylation, is a far more energy efficient method of respiration for sperm cells, where fifteen times more ATP molecules are generated by the mitochondria, than during glycolysis (Alberts et al., 2002). The process of aerobic respiration requires oxygen and occurs in the mitochondria of the cell, located in the sperm midpiece. Aerobic respiration is carried out through the electron transport chain (ETC), which is an extremely effective energy production method, however, one of the by-products of the ETC is the generation of ROS, as electrons leak from the ETC during respiration to go on to form superoxide (Turrens, 2003).

The mitochondrial ETC consists of four multi-protein complexes (Complex I - IV) and a series of electron carriers, like flavoproteins, iron-sulfur proteins, ubiquinone and cytochromes (Liu et al., 2002). Electrons are input into the ETC through complex I and complex II, where molecules nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) donate electrons to complex I and succinate
(C₄H₆O₄) donates electrons to complex II (Liu et al., 2002; Figure 1.26). Succinate is known as a flavin adenine dinucleotide (FAD) linked substrate, where FAD is a coenzyme that is associated with redox reactions in the body. Electrons then are free to travel down the ETC by an electron carrier, known as coenzyme Q (CoQ) or ubiquinone towards complex III, followed by transport to complex IV by cytochrome C, (Liu et al., 2002; Figure 1.26). At various points along the ETC, electrons leak out and form superoxide, which in turn can be converted to other types of ROS, such as hydroperoxyl, hydrogen peroxide and hydroxide radicals (Figure 1.26). Superoxide is formed at complex I, just before electrons enter complex III (Figure 1.26) while O₂ is fully reduced to water (H₂O) at the end of the ETC (Liu et al., 2002).

Figure 1.26: Mitochondrial Electron Transport System (ETC), depicting complex I, II, III and IV. Complex I and II accept electrons from NADH/NADPH and succinate (C₄H₆O₄), respectively. Adapted from Liu et al. (2002).
1.10.1 Nicotinamide adenine dinucleotide/Nicotinamide adenine dinucleotide phosphate as Proton Donors for ROS Generation using NADPH Oxidases

Nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are reducing agents which provide protons ($H^+$) within a cell (Figure 1.27). NADPH or NADH can cause increased ROS generation through the provision of electrons for the formation of free radicals, by the reduction of oxygen to superoxide (Aitken et al., 1994).

It had been suggested that excess NADPH located in the residual cytoplasmic droplet could trigger a NADPH oxidase (NOX) system in the human sperm plasma membrane (Aitken et al., 1997; Armstrong et al., 2002). The same studies suggest that human sperm generate ROS using the NOX5 enzyme. The NOX5 system is triggered as levels of calcium ions ($Ca^{2+}$) in the cell cytosol rise, producing superoxide (Bánfi et al., 2001). NADPH oxidases are plasma enzymes (Babior, 1999) that catalyse the generation of ROS by allows electrons to flow from NADPH across the cellular membrane to molecular oxygen, in order to form superoxide (Fulton, 2009) by reduction (Figure 1.27).

$$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+$$

**Figure 1.27:** Transfer of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) through the NADPH oxidase system, creating superoxide, NADP$^+$ and a proton, as suggested by Schirmer et al. (1983).

The addition of exogenous NADPH to viable, motile human sperm induced an increase in the rate of superoxide generation (Aitken et al., 1997). The same study also revealed that in order for the addition of NADPH to be effective at stimulating ROS, it was necessary for it to
penetrate the sperm cell membrane; this was supported by the results that sperm with purposely damaged cell membranes showed a higher tendency to absorb NADPH and as a consequence, form ROS. This suggests that intracellular NADPH may be the cause of ROS generation, or provide electrons for the production of ROS (Figures 1.26 and 1.27). The excess cytoplasm associated with IGCs suggests excess NADPH, providing the electrons for an increased level of ROS associated with immature sperm cells. This production of NADPH in the cytoplasm, in a system known as the monophosphate shunt (Said et al., 2005), is controlled by the enzyme glucose-6-phosphate dehydrogenase (Huszar et al., 1997), thereby, indicating that activity of glucose-6-phosphate dehydrogenase is correlated with ROS generation (Aitken et al., 1997). This enzyme also has a role in controlling the rate of glucose flux (Said et al., 2005) and the presence of the enzyme itself is a marker for immature human sperm cells (Aitken et al., 1994).

1.10.2 Oxidative Deamination of Aromatic Amino Acids in Bull Semen as a Mechanism for ROS Generation

The oxidative deamination (removal of an amine group) or dehydrogenation (removal of a hydrogen) of aromatic amino acids, phenylalanine, tyrosine and tryptophan can produce H$_2$O$_2$ and ammonia in bull semen (Tosic & Walton, 1950; MacMillan et al., 1972). These processes of deamination and dehydrogenation are carried out in the presence of D-amino acid oxidase, which is bound in an inactive form to motile sperm, but is released and activated after their death, producing H$_2$O$_2$ which is detrimental to the remaining, living sperm (Shannon & Curson, 1972). An increase in H$_2$O$_2$ production has been found when these amino acids, phenylalanine, tyrosine and tryptophan, were added to semen (MacMillan et al., 1972). Low levels of these amino acids were found in ejaculated bull semen (Sexton et al., 1971)
indicating that the aromatic amino acids must be formed during storage (MacMillan et al., 1972).

1.10.3 Leukocytes

Leukocytes are another important source of ROS in semen, as they use ROS production as a defence mechanism against pathogens (Tremellen, 2008). It has been reported that low levels of ROS generated, when seminal plasma is present, are not detrimental to spermatozoa, however, ROS generated by leukocytes when there is an infection in the genitourinary (reproduction and urinary) tract can damage sperm and impair sperm function (Ochsendorf, 1998). In addition to this, leukocytes use the NOX system to produce excess ROS, as reviewed by Griveau & Lannou (1997). Kessopoulou et al. (1992) has reported that leukocytes are the dominant source of ROS in semen rather than sperm cells themselves.

1.11 Oxidative Stress and Lipid Peroxidation in Sperm Cells

Oxidative stress (OS) occurs when there is an imbalance between the biochemical processes which produce ROS and those processes which remove ROS (Safarinejad et al., 2010). High levels of ROS or RNS are detrimental to cells, by way of oxidation of lipids, amino acids and carbohydrates, as reviewed by Sanocka & Kurpisz (2004), while also causing mutations in DNA. The oxidation of lipids and proteins is damaging to the mitochondrial membrane and the plasma membrane due to its high concentration of PUFAs (Gharagozloo & Aitken, 2011). In addition to this, damage to DNA from ROS can also occur either in the nuclear genetic material or in the mitochondrial genetic material (Gharagozloo & Aitken, 2011). It has been reported that the mechanism by which ROS damage sperm cell DNA involves the ROS attacking the DNA bases, in particular guanine, and their phosphodiester bonds (bonds involved in DNA, between a phosphate group and two five carbon sugars, over ester bonds),
thereby, destabilising the DNA structure and causing DNA fragmentation (Kemal Duru et al.,
2000; Gharagozloo & Aitken, 2011).

As previously mentioned, ROS are particularly detrimental to lipids of the plasma and
mitochondrial membrane due to their oxidising effect they have. The attack of ROS on
PUFAs of the sperm cell membrane triggers the process of lipid peroxidation (Kothari et al.,
2010), which involves a series of chain reactions taking place, causing degradation of the
sperm cell membrane and the mitochondrial membrane (Sikka et al., 1995; Gharagozloo &
Aitken, 2011). Immature sperm cells contain a superabundance of PUFAs (Ollero et al.,
2001), which are a prime target for attack by free radicals due to the presence of double or
triple bonds (Sanocka & Kurpisz, 2004; Kothari et al., 2010), which allow the ROS to begin
degradation of the PUFA carbon chain (Griveau & Lannou, 1997; Figure 1.28).

![Figure 1.28](image)

**Figure 1.28:** The process of lipid peroxidation (LPO) which occurs when lipids are exposed
to reactive oxygen species (ROS).
Lipid peroxidation occurs in three stages, known as Initiation, Propogation and Termination (Figure 1.28). Initiation is triggered by either an OH\textsuperscript{-} radical or \( \text{HO}_2 \) due to their high energy status (Blake \textit{et al.}, 1987), causing lipids to lose an electron (oxidised), in the form of a hydrogen atom (Sanocka & Kurpisz, 2004), forming lipid radicals (Kothari \textit{et al.}, 2010; Reaction 1, Figure 1.29). These lipid radicals then react with molecular oxygen, forming peroxyl radicals (Griveau & Lannou, 1997; Figure 1.28 and Reaction 2, Figure 1.29).

\[
\begin{align*}
LH + R^\circ & \rightarrow L^\circ + RH \\
L^\circ + \text{O}_2 & \rightarrow \text{LOO}^\circ
\end{align*}
\]

**Figure 1.29:** Reactions of the Initiation Stage, where \( L \) = lipid and \( R \) = alkyl radical. The free radical attacks the lipid to stabilise itself, leaving a lipid radical, with a stabilised radical. This lipid radical then reacts with molecular oxygen, forming a peroxyl radical. Adapted from Griveau & Lannou (1997).

The lipid radical and peroxyl radical are then free to attack other PUFAs within the membrane, causing further loss of electrons, leaving the once tight and organised sperm plasma membrane broken down, as reviewed by Griveau & Lannou, 1997; Reaction 1 and 2, Figure 1.30). The peroxyl radical can continue to oxidise PUFAs, forming further lipid radicals and lipid hydroperoxide while doing so (Sanocka & Kurpisz, 2004; Reaction 2, Figure 1.30). Due to the presence of ubiquitous iron ions, reactions like the Fenton reaction (Reaction 3 in Figure 1.30) can occur, forming more hydroxyl radicals, which in turn can initiate further LPO to occur (Griveau & Lannou, 1997).
Due to the nature of the LPO reactions, and as peroxyl and alkyl radicals are regenerated, propagation could potentially continue indefinitely, or until all the PUFAs have been attacked (Sanocka & Kurpisz, 2004), however, termination is triggered when radical species react with each other or are targeted by scavenging antioxidants (Griveau & Lannou, 1997; Figure 1.31).

1.11.1 Effects of Lipid Peroxidation on Sperm Cell Functionality
The main damaging effect that ROS exert over sperm cells is LPO of the sperm cell plasma membrane, however, excessive levels of ROS, and its subsequent LPO can also cause disruptions in sperm cell functionality, such as motility and DNA mutations (Aitken et al.,
This could be because the sperm cell plasma membrane not only covers the sperm head, but the midpiece and the majority of the sperm tail also.

Xanthine-xanthine oxidase is a reaction system involving an enzyme (xanthine oxidase) which artificially generates ROS by the univalent and divalent reduction of molecular oxygen, generating $O_2^-\cdot$ and $H_2O_2$ (Sikka, 1995). The addition of xanthine-xanthine oxidase to human sperm caused reduced numbers of sperm cells fusing with the oocyte (Aitken et al., 1993). ROS, in particular $H_2O_2$, can cause damaging effects to the fluidity of sperm cell membranes (Agarwal et al., 2003), contributing to the loss in functionality. This is further strengthened by (Lenzi et al., 2000; Aksoy et al., 2006), who reported that a low concentration of unsaturated fatty acids in the sperm cell membrane reduces cell fluidity. Aitken et al. (1993) has suggested that the changes in the sperm cell membrane from LPO could have a direct effect on its ability to initiate the membrane fusion events, like the acrosome reaction and fusion with the oocyte. The same study found that of the ROS produced by the xanthine oxidase system ($H_2O_2$ and $O_2^-\cdot$), $H_2O_2$ was the most detrimental to the sperm, as addition of superoxide dismutase (SOD – scavenger for $O_2^-\cdot$) had no significant effect at alleviating damage caused by the xanthine generated ROS, but addition of catalase did.

In addition to damage to the sperm cell membrane and its fluidity, sperm cell motility was decreased when xanthine-xanthine oxidase was added to human spermatozoa, due to the increased generation of ROS (Aitken et al., 1993). NO has also been found to reduce sperm cell motility in human sperm (Weinberg et al., 1995), while addition of $H_2O_2$ also decreased motility in stallion sperm (Baumber et al., 2000). The reported loss in sperm cell motility
could be attributed to a drop in ATP generation, which occurs when ROS target the PUFAs of the mitochondrial membrane (Sikka, 2001), allowing ATP molecules to leak out.

ROS, like the lipid peroxyl or alkoxyl radical, can oxidise bases of DNA (guanine) or can cause covalent bonding within DNA which results in strands breaking or cross linking, reviewed by Sikka (2001). It has been reported that infertile human males actually display an increased amount of DNA fragmentation and increased ROS generation (Irvine et al., 2000), linking high levels of ROS to breakages in strands of DNA, both in the nuclear and mitochondrial genomes (Aitken & Krausz, 2001). In addition to this, (Irvine et al., 2000) found that semen quality decreased, particularly concentration and motility, as DNA fragmentation increased in sperm cells.

An investigation by Wang et al. (2003) revealed that an increase in ROS production is associated with a reduction in mitochondrial membrane potential (MMP). The MMP is an electrochemical gradient which pumps protons from inside the mitochondria to the outside of the mitochondria (Evenson et al., 1982; Wang et al., 2003). It has been suggested that the MMP level provides an estimate of the metabolic function of a cell and could be an indicator of functional integrity of spermatozoa (Wang et al., 2003), as there is a positive correlation MMP and sperm motility and viability (Evenson et al., 1982; Auger et al., 1993).

When cells undergo oxidative stress, and are damaged by ROS, apoptosis, or programmed cell death is triggered (Sakkas et al., 1999). It is thought that ROS stimulates mitochondria to release cytochrome c, which is a signalling molecule that activates a cascade of events that cause cell apoptosis (Kothari et al., 2010). Cell apoptosis is also initiated by the inflammatory production of the ROS, hypochlorous acid (HOCl), by leukocytes. This HOCl can oxidise
cell components (Kothari et al., 2010) like the lipid membrane, DNA and other proteins or trigger other signalling cascades (Said et al., 2004) like the cytochrome c cascade.

1.12 Role of Antioxidants

Antioxidants, which are present in the body as enzymatic or non-enzymatic, can be taken in by the diet (Sanocka & Kurpisz, 2004), have been shown to be present in seminal plasma (Zini et al., 2002) and have scavenging properties (Baker & Aitken, 2004), which dispose of and suppress the formation of ROS (Sikka, 2001). Enzymatic antioxidants cause ROS to break down while non-enzymatic antioxidants neutralise ROS attack chemically (Tremellen, 2008). It is widely reported that spermatozoa are vulnerable to OS due not only to their high PUFA content but also because of their deficiency in intracellular antioxidant systems as they have a limited amount of cytoplasm in the sperm head (Gharagozloo & Aitken, 2011) and a lack of recovery system (Aitken & Clarkson, 1987; de Lamirande & Gagnon, 1993a). Infertile males are reported to show a reduced amount of antioxidant activity than fertile patients (Fujii et al., 2003; Safarinejad et al., 2010), mainly SOD and catalase, suggesting that oral antioxidant treatment for infertility may be a justified action (Gharagozloo & Aitken, 2011).

1.12.1 Enzymatic Antioxidants

Enzymatic antioxidants include superoxide dismutase (SOD), catalase and glutathione peroxidise (GPX) (Sanocka & Kurpisz, 2004) and can be found in the male reproductive tract (testis, prostate, seminal vesicles, vas deferens and epididymis), the seminal plasma and sperm cells themselves, as reviewed by Vernet et al. (2004).
SOD can be found in both sperm cells and in seminal plasma (Zini et al., 1993) and works to spontaneously break down O$_2^-$ into O$_2$ and H$_2$O (Sikka, 2001). SOD has been reported to be vital in protecting spermatozoa against LPO and maintain sperm motility (Alvarez et al., 1987; Kobayashi et al., 1991). Similarly, when SOD was added to bull semen milk based extender, sperm cell motility was improved (Foote et al., 2002). A study by de Lamirande & Gagnon (1993a) suggested that the scavenging ability of SOD was 40% lower in men with sperm which underwent spontaneous hyperactivation compared to men with normal sperm.

While some investigations have found that there is a link between human male infertility and a reduced level of SOD in seminal plasma (Alkan et al., 1997; Sanocka et al., 1997), other studies have not found this association (Miesel et al., 1997; Zini et al., 2000; Hsieh et al., 2002). The majority of studies, however, report a definite association between decreased levels of catalase and male infertility (Miesel et al., 1997; Sanocka et al., 1997; Zini et al., 2000). Hydrogen peroxide (H$_2$O$_2$) has been suggested to be one of the most detrimental ROS to human sperm (Aitken & Clarkson, 1987; Baumber et al., 2000) and catalase works specifically to decompose this ROS into H$_2$O and O$_2$. A definitive study by Shannon et al. (1984) demonstrated that the inclusion of catalase (4.5 µg) to 5 and 20% egg yolk Caprogen, significantly increased the non-return rate in bulls. However, when catalase was added directly to stored sperm cells however, there was no significant effect on sperm cell motility, viability and fertilising ability, after inclusion for 72 h (Vandemark et al., 1950).

Glutathione peroxidase (GPX) is a selenium containing enzyme antioxidant (Flohe et al., 1973) that allows the glutathione factor of enzymes to reduce hydroperoxides (Tremellen, 2008) and remove the peroxyl radical (ROO-) from semen, including the peroxide from
When GPX is inhibited in vitro, the level of LPO increases dramatically, thus, demonstrating its significance as an antioxidant in semen (Twigg et al., 1998), whilst males with leukospermia caused by OS displayed reduced GPX activity within spermatozoa (Therond et al., 1996). The scavenging ability of GPX depends on the glutathione being regenerated by glutathione reductase (GPR), by allowing the glutathione of GPX to become oxidised and thus makes it available to use again (Tremellen, 2008).

1.12.2 Non-Enzymatic Antioxidants

Non-enzymatic antioxidants include α-tocopherol (Vitamin E), ascorbate (or Vitamin C), glutathione, amino acids (taurine and hypotaurine), albumin, protosomes and β-carotene which are all present within semen (Sanocka & Kurpisz, 2004; Tremellen, 2008). These non-enzymatic antioxidants, otherwise known as low molecular mass antioxidants (Gharagozloo & Aitken, 2011) neutralise ROS attack chemically (Tremellen, 2008), however, albumin works by allowing itself to become oxidised, instead of the sperm cell (Twigg et al., 1998) and protostomes, secreted by the prostate can fuse with leukocytes in semen, thereby, reducing their generation of ROS (Saez et al., 1998). As was found with all three enzyme based antioxidants mentioned above, there was a significantly lower amount of non-enzymatic antioxidant activity in infertile males than in normal males (Gürbüz et al., 2003; Koca et al., 2003; Moustafa et al., 2004; Song et al., 2006). A wide variety of studies where non-enzymatic antioxidants have been added to the diet of males have been carried out, as reviewed by Gharagozloo & Aitken (2011) and results are still inconclusive.

Vitamin C first came to light as a potential antioxidant through a study by Fraga et al. (1996), on smokers. Work by Dawson et al. (1987) and Dawson et al. (1992) has demonstrated that supplementation of ascorbic acid (a form of vitamin C) to the male diet, results in elevated
levels of ascorbic acid in seminal plasma after 3 weeks inclusion in the diet. The addition of 200 mg of ascorbic acid had no effect on motility, but it significantly increased sperm cell viability, while addition of 1000 mg of ascorbic acid had no effect on viability, but it greatly increased sperm cell motility (Dawson et al., 1987). Akmal et al. (2006) reported that a twice daily oral supplement of 1000 mg given to infertile men for a period of two months resulted in an increase in sperm concentration, sperm motility and reduced sperm cell abnormality.

Vitamin E has been reported by a number of studies to be effective at reducing amounts of ROS in semen, *in vitro*, thereby, improving motility and viability and reducing LPO (Bansal & Bilaspuri, 2009). The same study has suggested that the addition of Vitamin E to sperm could improve semen quality for use in AI. In contrast to this study, others have found that Vitamin E has no beneficial or detrimental effects on any sperm parameters (Giovenco et al., 1987; Moilanen & Hovatta, 1995).

1.13 Artificial Insemination with Fresh and Frozen-Thawed Semen

AI is the single most important technique devised to facilitate the genetic improvement in animals, a fact which is highly evident from the genetic progress achieved in the dairy industry since the 1970’s. The two main types of semen used in AI are frozen and liquid. Approximately 95% of all insemination doses used worldwide, and in Ireland, are frozen semen (Vishwanath & Shannon, 2000). Currently in Ireland, liquid semen accounts for only approximately 5% of all insemination doses used nationally (Total insemination doses used in 2011 was 856,660; Source ICBF) however, this may rise to 25% during the peak dairy breeding season of April and May.
The cryopreservation of semen causes the biochemical and developmental changes in sperm to stop, allowing the long term storage of sperm cells. However, the cryopreservation and subsequent thawing process results in a significant proportion (>50%) of damaged sperm (Vishwanath & Shannon, 2000). This is mainly due to the induction of a series of modifications to the sperm membrane such as the premature induction of capacitation (‘cryocapacitation’) and the acrosome reaction, a reduction in their ability to penetrate artificial mucus in vitro (Al Naïb et al., 2011a) as well as a reduction in the in vivo and in vitro fertilising ability (Ward et al., 2001). Post thawing, although lipids of the sperm cell membrane are apparently undamaged, they display a compromised ability to diffuse within the cell membrane (James et al., 1999), thereby, reducing the fertilising capacity of the sperm. In addition to this, a significant drop in sperm motility and viability is usually associated with cryopreserved bull sperm, as reviewed by Watson (1995). Finally, sperm head morphology was reduced in cryopreserved sperm in comparison to non-cryopreserved sperm (Gravance et al., 1998), perhaps indicating a loss in plasma membrane components, acrosomal exocytosis or nuclear overcondensation (Gravance et al, 1998). To combat this reduction in fertilising ability a relatively high sperm number of 20 million sperm per AI dose is the norm.

The use of liquid sperm is a viable alternative to frozen-thawed sperm and its main advantage is that a lower concentration of sperm per dose can be used (norm in Ireland is 5 million sperm per dose), due to the absence of any freeze-thaw injury to sperm. This enables AI companies to obtain a higher number of doses per ejaculate which maximises the use of elite bulls (Vishwanath & Shannon, 2000). However, the main disadvantage of liquid semen is its finite lifespan and is not used in Ireland after 60 h post collection due to concerns over lower fertility of aged sperm.
Liquid semen can be stored at ambient temperature or at 5 °C. Storage at 5 °C reduces the metabolic activity of sperm and thus is likely to result in less reactive oxygen species production, however, storage at lower temperature has been shown to disrupt the work of the sodium-potassium pump, resulting in an increase of intracellular sodium, which is detrimental to sperm (Makler et al., 1981). Bovine sperm stored at ambient temperature, is normally stored in Caprogen media (Ireland and New Zealand). Caprogen is an egg yolk based diluent which is purged in N gas so as to generate anaerobic conditions and limit sperm cell metabolism. Work by Vishwanath & Shannon (1997) showed a very slow decline in sperm motility stored in Caprogen over a 4 week period, however, the majority of doses were found to be incapable of fertilising an oocyte on the tenth day after collection. Similarly, bovine sperm stored at 5 °C, in a bicarbonate based media, gassed with CO₂ showed similar motility results, with 56% sperm motile after 40 days of storage, however, by Day 3 of storage, fertility was reduced by up to 15% (Bartlett Jr & Van Demark, 1962). This rapid drop in fertility could be attributed to extracellular oxidative stress, effect of removal/dilution of seminal plasma and endogenous ROS production, as reviewed by Vishwanath & Shannon (2000). The decline in fertility could also be linked to a change in the fluidity of the membrane, which is partly governed by its lipid and fatty acid composition (Lenzi et al., 1996; Alvarez & Storey, 1982).

1.14 Irish Dairy Industry

The agri-food sector is Ireland’s largest indigenous manufacturing industry with a gross annual output of over €22 billion and employs some 135,000 people. The dairy industry makes up 28% of Ireland’s gross agriculture output and is by far the most profitable of the farming enterprises. With the abolition of milk quotas within the European Union now imminent (March 31st 2015), the Irish dairy industry has set a target of a 50% increase in
dairy output by the year 2020 (Department of Agriculture, 2010). The underlying basis for Ireland’s profitable dairy sector is due to its seasonal grass-based milk production system (which is similar to New Zealand and parts of Australia) which possesses a significant cost advantage in the form of low-cost milk production. This system is highly dependent on excellent reproductive performance with compact calving to coincide with the start of the grass growing season (Dillon et al., 1995). This contrasts greatly to many other countries (for example North America) where cows are kept and fed indoors, milked all year round, administered hormone therapy (bovine somatotropin: BST) and therefore, compact calving is not of high priority.

While milk production per cow has increased since the 1970’s (Foote, 1996), reproductive performance has dramatically declined (Walsh et al., 2011) and in order to combat this decline in fertility, Ireland, through the Irish Cattle Breeding Federation (ICBF), has implemented an Economic Breeding Index (EBI; Berry et al., 2005). This is a profit index that enables farmers to select the most elite and profitable bulls to breed replacement heifers which will have an increased ability to produce more milk solids per cow per year as well as having an increased ability to become pregnant. Therefore, the intensive use of a relatively small number of high EBI bulls is the best way to reverse the fertility problems in the national dairy herd while maintaining increases in milk production.

In 2009, Ireland began to select dairy bulls for use in AI programmes using genomic selection with potential AI bulls now being identified within weeks of birth. These bulls are entering AI centres at approximately 10 months of age and although they have undergone puberty they yield a low volume of semen and demand for their semen far exceeds supply. Therefore, it is imperative that the industry can maximise the number of insemination doses from these bulls.
In parallel to this, and given that over 60% of dairy inseminations occur in a 6-week period in April/May in Ireland, the extensive use of fresh semen is a viable option, whereby, the number of insemination doses per ejaculate could be increased by up to 4-5 fold compared to frozen semen.

1.15 The Role of Fatty Acids in Improving the Lifespan of Fresh Semen

Work carried out in vitro using various human and animal models (Comhaire et al., 2000; Safarinejad et al., 2010; Rooke et al., 2001; Gholami et al., 2010), suggests that the addition of fatty acids to the diet can lead to beneficial effects for sperm cells. Fatty acid degradation in the sperm cell membrane by ROS significantly reduces membrane fluidity, motility, DNA structure and in vivo fertility (Gharagozloo & Aitken, 2011; Aitken et al., 1993; Aitken & Krausz, 2001; Yumura et al., 2009); although there is limited work done in liquid semen, in vitro. The addition of exogenous fatty acids to sperm cells, in vitro, may combat this problem by stabilising the sperm cell membrane and therefore, extend the fertile lifespan of the sperm cell. Neill & Masters (1971) reported that fatty acids such as myristic (MA; 14:0), PA, SA, OA and LA, were all incorporated into the lipids of the sperm cell membrane within two hours of addition to the cell cultures; myristic acid incorporated to the greatest extent (Neill & Masters, 1971). In general, saturated fatty acids were incorporated more actively than unsaturated fatty acids into lipids (diglycerides), however, SA was not incorporated effectively into any phospholipid, except phosphatidylinositol (Neill & Masters, 1971). Short chain fatty acids, when added to bovine sperm, in vitro, have been demonstrated to maintain viability of sperm at both 5 °C and 37 °C (Shannon, 1962), while unsaturated fatty acids (or long chain fatty acids), when added in vitro to boar sperm, improved motility, viability and acrosome reaction levels (oleic and linoleic, combination of oleic and arachidonic increased acrosome reaction) (Hossain et al., 2007). In contrast, the in vitro addition of unsaturated
fatty acids (arachidonic, linoleic, docosahexaenoic, palmitoleic and oleic) to human sperm has been shown to dramatically increase the number of sperm testing positive for ROS, however, the addition of saturated fatty acids (palmitic and stearic) did not (Aitken et al., 2006; Koppers et al., 2010). Therefore, the effect of long chain unsaturated fatty acids on the functional capacity of liquid bull semen remain to be elucidated and is the focus of this thesis.
Objectives

The objectives of this thesis were to investigate the effect of:

1. The \textit{in vitro} addition of exogenous fatty acids on the ability of stored bovine sperm to penetrate artificial mucus for up to 4 days post collection, stored at 5 °C.

2. The \textit{in vitro} addition of exogenous fatty acids on the generation of Reactive Oxygen Species (H\textsubscript{2}O\textsubscript{2}, OH\textsuperscript{-}, peroxyl radical (ROO\textsuperscript{-}) and peroxynitrite anion (ONOO\textsuperscript{-} )) in bovine sperm, up to 7 days post collection, stored at 5 °C.

3. The \textit{in vitro} addition of exogenous fatty acids on the generation of Superoxide (O2\textsuperscript{-}) in bovine sperm, up to 7 days post collection, stored at 5 °C.

4. The addition of catalase to an egg-yolk based diluent on the generation of Reactive Oxygen Species in bovine sperm, up to 10 days post collection, stored at 5 °C.
Chapter 2 – Experimental Chapter – The Effect of Polyunsaturated Fatty Acids on Bovine Sperm, \textit{in vitro}
Abstract

PUFAs are an integral part of the sperm cell membrane and while they are essential for membrane fluidity, they are a prime target for attack by ROS. This study aimed to investigate the effects of long chain fatty acids (ALA, DHA, EPA, LA, OA and PA) at concentrations of 10-100 µM, on bull sperm stored in vitro, for up to 7 Days. In addition, the effect of the addition of catalase to egg yolk based diluent on the generation of ROS from stored bull sperm, stored in vitro, for up to 10 days, was assessed. Progressive linear motion (PLM) and viability (Experiments 1-3), ability to penetrate artificial mucus (Experiment 1), ROS generation (Experiment 2) and superoxide production (Experiment 3) were assessed. Sperm maintained the ability to penetrate artificial mucus up to and including Day 4, irrespective of treatment (Exp 1). In Experiments 2a and 3, DHA and EPA had detrimental effects on PLM and viability, with cytotoxic effects displayed by 100 µM concentrations of both. OA preserved PLM and viability at levels greater than the control, while delaying the peak of ROS production (Exp 2). PA maintained PLM and viability at levels higher than the control (P < 0.05), while sustaining ROS levels to a minimum, particularly on Days 1 and 3 (P < 0.01) when ROS generation peaked in other treatments. In contrast, superoxide production peaked on Day 0 (Exp 3), and declined thereafter with no significant effect of fatty acid. Thus, findings of this study support the notion that superoxide dominates on Day 0, while its breakdown products from its reactions, hydrogen peroxide and the hydroxyl radical as assessed by CM-H$_2$DCFDA, contribute to ROS generation on subsequent days. The addition of catalase to an egg-yolk based diluent (Exp 2b) showed no significant effect on sperm motility, viability and generation of ROS in comparison to the control.
2.1 Introduction

PUFAs have been widely reported, in both human and animal models, to have beneficial effects on the cardiovascular (Bønaa et al., 1990; Zampolli et al., 2007) and nervous (Alessandri et al., 2004; Brenna & Diau, 2007) systems. In more recent years, there has been an increased focus on the role of PUFA’s on female fertility (Childs et al., 2008; Hammiche et al., 2010) and to a lesser extent, male fertility (Wathes et al., 2007). In the case of male fertility, the main focus of this work has either been on the inclusion of specific fatty acids in the diet as reviewed by Comhaire & Mahmoud (2003) or on the comparison of the lipid profile of sperm between fertile and infertile males (Aksoy et al., 2006; Safarinejad et al., 2010).

Supplementation with dietary fish oils, rich in long chain fatty acids, has been demonstrated to successfully modify the fatty acid profile of the plasma and sperm across a range of species (Kelso et al., 1997a; Comhaire et al., 2000; Rooke et al., 2001; Castellano et al., 2010; Gholami et al., 2010) however, the effects of this are still unclear. The increase in the proportion of n-3 fatty acids led to the number of progressively motile cells in boars being increased (Rooke et al., 2001), however, others found no beneficial effects in terms of semen production (Castellano et al., 2010), the lifespan of sperm stored at 17 °C or following cryopreservation (Castellano et al., 2010; de Graaf et al., 2007). Furthermore, supplementation of n-3, DHA in the diet was found to improve motility, speed and viability of fresh Holstein bull sperm, while no effect on frozen sperm was observed (Gholami et al., 2010).

The fatty acid profile of normal and abnormal human sperm has recently been investigated. Koppers et al. (2010) reported that defective human spermatozoa contain more total fatty
acids (SFAs, MUFAs and PUFAs) than functional sperm and those of the fatty acid pool of defective sperm contain a high proportion of PUFA’s such as DHA. They hypothesise that this is due to a failure in the functional remodelling of the sperm plasma membrane during epididymal transit where fatty acids are normally lost. An abnormally low concentration of unsaturated fatty acids would be expected to reduce the fluidity of the membrane (Lenzi et al., 2000; Aksoy et al., 2006) while superabundance of unsaturated fatty acids would expose sperm to attack by reactive oxygen species (ROS) generation by the sperm mitochondria (Aitken et al., 2006).

_In vitro_ models provide an opportunity to study the effect of exogenous fatty acids on viability, motility, lipid peroxidation and ROS. It has been reported that fatty acids were incorporated into the lipids of sperm cell membranes within two hours of the addition to cells (Neill & Masters, 1971). In human sperm, the _in vitro_ addition of unsaturated fatty acids (arachidonic, linoleic, docosahexaenoic, palmitoleic and oleic) has been shown to dramatically increase the number of sperm testing positive for ROS, however, the addition of saturated fatty acids (palmitic and stearic) did not (Aitken et al., 2006; Koppers et al., 2010). In contrast, motility, viability and acrosome reaction levels were all improved after the _in vitro_ addition of exogenous unsaturated fatty acids (oleic and linoleic, combination of oleic and arachidonic increased acrosome reaction) to boar sperm (Hossain et al., 2007) while the viability of bull sperm was maintained for longer after the addition of short chain fatty acids (Shannon, 1962).

OS occurs in semen when there is an imbalance between the biochemical processes which produce ROS and those processes which remove ROS (Safarinejad et al., 2010). Sperm are reported to be susceptible to ROS attack, not only due to their high PUFA content, but also
due to their lack of internal antioxidant systems as they have a limited amount of cytoplasm in the sperm head (Gharagozloo & Aitken, 2011). For this reason, antioxidants must be taken in by the diet. Oral intake of antioxidants has proven to reap beneficial effects in a number of studies in the human male (Dawson et al., 1987; Comhaire et al., 2000; Bansal & Bilaspuri, 2009). When antioxidants were added to the diluent of sperm cells, the pregnancy rate was increased in cows in comparison to those inseminated with semen without catalase.

There is limited published work on the effect of unsaturated fatty acids on the functional capacity of stored bull sperm. The aim of this study was to investigate the effect of the in vitro addition of a range of exogenous fatty acids to bull sperm stored for up to 7 days on motility, viability, ability to penetrate artificial mucus and the generation of ROS. In addition to this, this study also investigated the effect of the addition of catalase to an egg yolk based diluent on bull sperm stored for up to 10 days on motility, viability and the generation of ROS.

2.2 Materials and Methods

2.2.1 Animal and Semen Processing

Semen was collected in a commercial artificial insemination (AI) centre from three bulls of proven fertility. A fraction of each ejaculate (0.1 - 0.2 mL) was pooled and diluted in 2 - 5 mL of appropriate semen diluent. Following transport at 35 °C to the laboratory within 1 h of collection, the sperm concentration was assessed using a haemocytometer and subsequently diluted. Diluted semen (1 mL per treatment) was stored in an Eppendorf at 5 °C in the presence of the relevant fatty acids for up to 4 days (Experiment 1) or 7 days (Experiments 2a and 3). Sperm were stored in the presence or absence of catalase for up to 10 days (Experiment 2b).
2.2.2 Experimental Design

2.2.2.1 Experiment 1

The aim of this experiment was to examine the effect of exogenous fatty acids on the subsequent ability of stored bull sperm to penetrate artificial mucus in vitro. The sperm mucus penetration test was used to assess the functional ability of sperm. Semen (0.2 mL) from each ejaculate was pooled in 5 mL of an egg-yolk diluent {Sodium Citrate (63 mM); Glycine (124 mM); Glucose (1.5 M); Glycerol solution (1.14% v/v); Penicillin g sodium (1163 U); Catalase solution (17.14 U); Citric acid (0.7 mM), 5% v/v egg yolk, pH 7.4}. On returning to the laboratory, the semen was diluted in the same diluent to \(4 \times 10^6\) sperm/mL. Fatty acids were dissolved in ethanol, and added to diluted sperm samples to bring to the working concentrations. Diluted semen (1 mL per treatment) was then stored in the presence of EPA (C20:5, n-3), ALA (C18:3, n-3) or PA (C16:0, Saturated fat) at concentrations of 0, 10, 50 and 100 µM at 5 °C for up to 4 days. A 0.096 % ethanol control was included (vehicle control) as this was the highest concentration of ethanol used as a solvent for the fatty acids. As a quality control and to account for day to day variation a pool of frozen-thawed sperm from 3 bulls of proven field fertility were included on each day of assessment (results not shown). All treatments were assessed on Days 0, 1, 2, 3 and 4 for PLM, viability and ability to penetrate artificial mucus. Four replicates were completed.

2.2.2.2 Experiment 2

2.2.2.2.1 Experiment 2a

The aim of this experiment was to examine the effect of the addition of fatty acids to bull sperm stored for up to 7 days on the generation of ROS. Following collection at the AI centre an aliquot of each ejaculate (0.1 mL) from 3 bulls was pooled in 2 mL of a citrate based diluent {Sodium Citrate (75.5 mM); Glycine (130.6 mM); Glucose (16.3 mM); Glycerol
solution (1.23% v/v); Penicillin g sodium (1225 U); Catalase solution (17.14 U); Citric acid (0.7 mM); pH 7.4}. Egg yolk was not included in the media for Experiment 2a due to its potential conflicting effects from its fatty acid content. On returning to the laboratory, the semen was diluted to $40 \times 10^6$ sperm/mL in the same diluent at room temperature. Fatty acids were dissolved in ethanol, and added to diluted sperm samples to bring to the working concentrations. Diluted semen (1 mL per treatment) was then stored in the presence of EPA, ALA, PA, LA (C18:2, n-6), OA (C18:1, n-9) or DHA (C22:6, n-3) at concentrations of 0, 10 and 100 µM at 5 °C. Controls as per Experiment 1 were included and all treatments were assessed on Days 0, 1, 3 and 7 for PLM, viability and generation of ROS using the fluorescent stain Chloromethyl-2,7-dichlorofluorescein diacetate (CM-H$_2$DCFDA). CM-H$_2$DCFDA is a general intra-cellular stain which fluoresces green if ROS such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH·), peroxyl radical (ROO·) and the peroxynitrite anion (ONOO‘) are present. Four replicates were completed.

2.2.2.2.1.1 Positive Control for CM-H$_2$DCFDA and the Generation of ROS
H$_2$O$_2$ of increasing concentrations was added to frozen thawed bull sperm to examine the effect on their generation of ROS (H$_2$O$_2$, OH·, ROO· and ONOO‘), forming a positive control for the use of CM-H$_2$DCFDA. A pool of frozen thawed sperm was used from three different bulls. Three replicates were completed.

2.2.2.2.2 Experiment 2b
The aim of this experiment was to examine the generation of reactive oxygen species in bull semen diluted in the presence or absence of catalase. Following collection in the AI centre, an aliquot of each ejaculate (0.1 mL) from 3 bulls was pooled in 2 mL of an egg yolk based diluent with catalase {Sodium Citrate (63 mM); Glycine (124 mM); Glucose (15 mM);
Glycerol solution (1.14% v/v); Penicillin g sodium (1163 U); Catalase solution (17.14 U); Citric acid (0.7 mM); 5% v/v egg yolk; pH 7.4} and without catalase {Sodium Citrate (63 mM); Glycine (124 mM); Glucose (15 mM); Glycerol solution (1.14% v/v); Penicillin g sodium (1163 U); Catalase solution; Citric acid (0.7 mM); 5% v/v egg yolk; pH 7.4}. On returning to the laboratory, the semen was diluted in the relevant diluent to 40 × 10^6 sperm/mL at room temperature. Sperm were assessed on Days 0, 1, 3, 7 and 10 for PLM, viability and generation of ROS using the fluorescent stain CM-H_2DCFDA. Three replicates were completed.

2.2.2.3 Experiment 3

The aim of this experiment was to examine the effect of the addition of fatty acids to stored bull sperm on mitochondrial generation of the superoxide anion. Again, egg yolk was not included in the media as per Experiment 2a due to its fatty acid content. The same treatments were examined as in Experiment 2a and the treatments were assessed on Day 0, 1, 3 and 7 for PLM, viability and generation of superoxide using the fluorescent stain MitoSOX Red. MitoSOX Red is an intra-cellular stain which fluoresces red if the superoxide anion (O_2^{·-}) is present. Four replicates were completed.

2.2.2.3.1 Positive Control for MitoSOX Red and Generation of ROS

H_2O_2 of increasing concentrations was added to fresh bull sperm to examine the effect on their generation of Superoxide (O_2^{·-}), forming a positive control for the use of MitoSOX Red. Semen was collected, diluted and processed as per Experiment 3, without the addition of fatty acids. Samples were assessed on Days 0, 1, 3, 7 and 10. Three replicates were completed.
2.2.3 In vitro Assessment of Sperm Function

2.2.3.1 Progressive Linear Motion

PLM was assessed for each treatment by placing a 5 µL sample on a pre-warmed slide and assessing 100 live cells, on a scale from 0 to 100 (0 indicating no progressive linear motion observed among the live sperm, 100 indicating 100% of live sperm were moving in a progressive linear motion).

2.2.3.2 Viability

Sperm viability was examined using a nigrosin-eosin stain (0.068 M water soluble nigrosin; 0.014 M water soluble eosin; 0.116 M sodium citrate). A sample of sperm from each treatment was added to nigrosin-eosin stain, in a 1:1 ratio (50 µL). This was incubated at 37 °C for 30 sec following which a smear was prepared on a glass slide and allowed to dry on a heated stand at 37 °C. The slide was viewed under a phase contrast microscope (40X) and an average of three counts was taken, where 100 cells were assessed in each count. The nigrosin component forms the dark background, allowing cells to be identified easily while Eosin Y acts as a marker for dead sperm, staining them a pink colour (Figure 2.1).
2.2.3.3 Mucus Penetration Test

The mucus penetration test was performed as per Al Naib et al. (2011b) with minor adaptations. Artificial mucus, which is sodium hyaluronate based, was prepared as per Al Naib et al. (2011b) using a solution known as Map5 (Labstock MicroServices, Ireland). Map5 (3 mL) was diluted in PBS (2 mL), leaving a 60% sodium hyaluronate solution. Flattened capillary tubes, marked at 10, 30 and 70 mm intervals, were loaded with artificial mucus and sealed with crystaseal. One tube was placed vertically in a 1.5 mL Eppendorf, each representing a different fatty acid treatment at different concentrations. Each eppendorf contained 250 µL of sperm diluted to $20 \times 10^6$ sperm/mL in Biggers, Whitten and Whittingham (BWW) media (Fatty Acid Free BSA used, pH 7.4) and stained with Hoechst 33342. Samples were incubated for 10 min in a dry oven at 37 °C, following which; the capillary tubes were placed on a hot plate at 50°C for 30 sec followed by 3 min at 4 °C in
order to immobilise the sperm cells. Spermatozoa, at each mark along the tube (10, 30, 70 mm), were counted using a fluorescent microscope (Olympus BX 60, 40X) in one field of view wide, wall to wall across the tube. For final analysis, sperm number at 10, 30 and 70 mm intervals were pooled and total sperm number at these intervals were recorded and is shown in the results.

Figure 2.2: Staining of sperm cells with Hoechst 33342 (400X), used in the Mucus Penetration Test, stains cell nucleus

2.2.3.4 Measurement of ROS (CM-H$_2$DCFDA)

A representative sample from each treatment (100 µL) was added to 10 mL of filtered PBS followed by centrifugation at 800 g for 10 min at 32 °C. Following the supernatant being removed, the concentration of the pellet was assessed using a haemocytometer, and the sample was diluted to $1.2 \times 10^6$ sperm/mL in pre-warmed PBS (367 µL). CM-H$_2$DCFDA (50
µL) was added to give a final working concentration of 100 µM and samples were incubated for 30 min in a dry oven at 37 °C. Propidium Iodide (PI; 83 µL) was added to each of the samples to give a final working concentration of 50 µM and samples were incubated for a further 15 min. Samples were subsequently washed in 5 mL PBS by centrifugation at 800 g for 10 min at 32 °C following which the sample was analysed on a BD-LSRI. The 488 Ar-Ion laser was used to excite both stains, with the FL1 detector reading CM-H₂DCFDA (Ex/Em 502/523 nm) and the FL2 detector reading PI (Ex/Em 535/617 nm; Figures 2.3 and 2.4). A suitable protocol were established by running relevant controls to set gates, detectors and voltages, while all data were read on the log scale. A total of 10,000 events for each sample were analysed on Cell Quest 3.7, with a flow rate of approx 150 events per second. Confirmation of both stains was obtained by staining cells and viewing under the fluorescent microscope (Olympus BX 60, 40X; Figures 2.3 and 2.4).

2.2.3.5 Use of Hydrogen Peroxide as a Positive Control for CM-H₂DCFDA

H₂O₂ was added to a sample of frozen thawed sperm cells to give a final concentration of 1.05 × 10⁶ sp/mL in 350 µL and final concentrations of 0, 50, 100, 500, 1000 and 2000 µM H₂O₂. PBS was added to bring the volume to 400 µL. Following a 30 min incubation at 37 °C, CM-H₂DCFDA (50 µL) was added to the sample, giving a final concentration of 100 µM CM-H₂DCFDA, followed by a further incubation for 30 mins at 37 °C. Controls with no cells, with only H₂O₂ and CM-H₂DCFDA were included to account for auto-fluorescence. Samples were subsequently washed in 5 mL PBS by centrifugation at 800 g for 10 min at 32 °C following which the sample was analysed on a BD-LSRI as per Section 2.2.3.4 Measurement of ROS (CM-H₂DCFDA). Again confirmation of both stains was obtained by staining cells and viewing under the fluorescent microscope (Olympus BX 60, 40X; Figures 2.3 and 2.4).
Figure 2.3: Staining of sperm cells with CM-H2DCFDA (400X), a general indicator for ROS.

Figure 2.4: Staining of sperm with Propidium Iodide (400X), a viability stain which stains dead cells.
Figure 2.5 Dot plot of FL1 vs FL2. CM-H$_2$DCFDA stains for the generation of ROS, it is excited at 492-495 nm and emits at 517 – 527 nm, and therefore it is picked up by the FL1 (green) detector. PI stains non-viable cells, it excites at 535 nm and emits at 617 nm, and therefore, it is picked up by the FL2 (orange-red) detector.

2.2.3.6 Measurement of Superoxide (MitoSOX Red)

A sample (100 µL) of each treatment media was added to 10 mL of BWW (pH 7.4) and centrifuged at 800 g for 10 min at 32 °C. Following removal of the supernatant, the sperm were diluted to 1.2 × 10$^6$ sperm/mL in a 472.5 µL of BWW. MitoSOX Red stock (25 µL) was added to each sample to give a final working concentration of 2.5 µM and samples were incubated for 15 min in a dry oven at 37 °C. At the end of the incubation period SYTOX Green (2.5 µL) was added to give a final working concentration of 0.25 µM following which samples were incubated for a further 15 min. Samples were then washed in 5 mL BWW, centrifuged at 800 g for 10 min at 32 °C and analysed on a BD-LSRI as per Section 2.2.3.4. STYOX Green was used as a viability stain in the measurement of superoxide instead of PI as two red stains (MitoSOX Red and PI) would
not be suitable for use in the same flow cytometric analysis on the BD-LSRI. The FL1 detector reads SYTOX Green (Ex/Em 504/523 nm) and the FL2 detector reads MitoSOX Red (Ex/Em 510/580 nm) (Figures 2.6 and 2.7). A flow rate of approximately 200 events per second was used to evaluate samples using Cell Quest 3.7. Again confirmation of both stains was obtained by staining cells and viewing under the fluorescent microscope (Olympus BX 60, 40X; Figures 2.6 and 2.7).

**Figure 2.6:** Staining of sperm with SYTOX Green (400X), viability stain that marks dead cells
2.2.3.7 Use of Hydrogen Peroxide as a Positive Control for MitoSOX Red

H₂O₂ was added to a sample of fresh sperm cells on the day of collection, to give a final concentration of 1.22 × 10⁶ sp/mL in 372.5 µL and final concentrations of 0, 5, 50, 100, 1000, 3000 and 5000 µM H₂O₂. Following a 30 min incubation at 37 °C, MitoSOX Red (25 µL) was added to the sample, giving a final concentration of 2.5 µM MitoSOX Red, followed by a further incubation of 15 mins at 37 °C. Samples were subsequently washed in 5 mL BWW by centrifugation at 800 g for 10 min at 32 °C following which the sample was analysed on a BD-LSRI as per Section 2.2.3.6. Again confirmation of both stains was obtained by staining cells and viewing under the fluorescent microscope (Olympus BX 60, 40X; Figure 2.6).
Samples were subsequently washed in 5 mL BWW by centrifugation at 800 g for 10 min at 32 °C following which the sample was analysed on a BD-LSRI. Again, the 488 Ar-Ion laser was used to excite both stains, with the FL1 detector reading SYTOX Green (Ex/Em 504/523 nm) and the FL2 detector reading MitoSOX Red (Ex/Em 510/580 nm). A total of 10,000 events were analysed on Cell Quest 3.7, with flow rate of approx 200 events per second.

2.2.4 Statistical Analysis

As part of the experimental design process the following power test was conducted with beta=0.80 to determine the number of experimental units per treatment:

\[ N = 15.7 \times (\text{CV}\% / \text{d}\%) \], where \text{CV}\% the proportion of the mean that is the standard deviation, and \text{d}\% is the expected difference that you wish to detect as significant. All analyses were carried out using the SAS v9.1.3 software package (SAS Institute, Cary, NC, USA) (SAS/STAT. 2000). Diagnostic tests were used to determine if data had a normal distribution and data that did not approach a normal distribution were transformed using a box-cox transformation (Box & Cox, 1964) in order to meet the assumptions of analysis of variance.

Data presented in this paper shows the non-transformed values of the data, however, all P-values were calculated using the transformed data where required. Data were analysed using a repeated measures model for day that included the fixed effects of day, fatty acid, concentration of fatty acid, all two way interactions, and the three-way interactions of the fixed effects where appropriate. Effects with a P-value <0.25 were retained in the model. Repeated measures for day were fitted using the appropriate covariance function as determined by the Bayesian Information Criterion, and the Tukey adjustment was used for multiple comparisons. Statistical differences were reported when P-values were < 0.05. Results are reported as least square means ± s.e.m unless otherwise stated.
2.3 Results

2.3.1 Experiment 1

2.3.1.1 Motility and Viability

PLM and % live declined in all treatments over the five days (P < 0.01; Figures 2.8 and 2.9). There was an effect of fatty acid on PLM (P < 0.05), with EPA (100 µM) showing the lowest PLM score on Day 4 compared to Day 0 (66.4 ± 3.53% and 90.0 ± 3.10%, respectively; (P < 0.001). Irrespective of concentration, all PA treatments had numerically higher motility values on Day 4 compared to the control, (87.8 ± 3.10%, 84.4 ± 3.53%, 75.4 ± 3.53% for the 10, 50 and 100 µM PA treatments vs 72.4 ± 3.53% for the control) but only 50 µM PA reached significance (P < 0.05; Figure 2.8). Only 50 µM ALA and 50 µM PA treatments showed significantly higher numbers of live sperm compared to the control on Day 4 (ALA 75.5 ± 5.70%; PA 78.1 ± 5.88% and Control 68.8 ± 5.88%; P < 0.05; Figure 2.9).

2.3.1.2 Ability to penetrate artificial mucus

There was no effect of fatty acid by concentration by day interaction on sperm cell ability to penetrate artificial mucus, nor was there any effect of fatty acid, concentration or day. Irrespective of treatment, sperm retained the ability to penetrate mucus up to and including Day 4 (Figure 2.10). No treatment showed a significantly greater or reduced ability to penetrate the artificial mucus in comparison to the control on a respective day. On Day 2, sperm cells stored with 50 µM ALA (384.0 ± 61.55%) showed an elevated numerical value of sperm cells in the tube compared to the control on the same day (210.3 ± 61.55%), a similar pattern was observed on Day 3 (255.8 ± 61.55% and 216.0 ± 61.55%). On Day 4, no treatment showed higher numerical values in comparison to the control.
Figure 2.8: Motility of sperm stored with ALA, EPA (both n-3) or PA (SFA) at concentrations of 10, 50 and 100 µM assessed on Days 0, 1, 2, 3 and 4. A vehicle control was included. Vertical bars represent s.e.m (n = 4 replicates)
Figure 2.9: Viability of sperm stored with ALA, EPA (both n-3) or PA (SFA) at concentrations of 10, 50 and 100 µM assessed on Days 0, 1, 2, 3 and 4. A vehicle control was included. Vertical bars represent s.e.m (n = 4 replicates)
Figure 2.10: Ability of sperm stored with ALA, EPA (both n-3) or PA (SFA) at concentrations of 10, 50 and 100 µM to penetrate artificial mucus, assessed on Days 0, 1, 2, 3 and 4. A vehicle control was included. Vertical bars represent s.e.m (n = 4 replicates)
2.3.2 Experiment 2

2.3.2.1 Experiment 2a

Motility and Viability

PLM decreased over time in all treatments (P < 0.01), but the most dramatic decline in motility was in the EPA and DHA treatments with PLM declining more rapidly in the 100 compared to the 10 µM treatments in both cases up to Day 3 (P < 0.05; Figure 2.11). PLM in the 100 µM DHA treatment on Days 0 and 1 were 69.8 ± 0.31% and 22.5 ± 0.31%, respectively. PA (100 µM) and OA (10 and 100 µM) maintained the highest PLM up to and including Day 7 (45.0 ± 0.35%; 27.0 ± 0.35% and 24.0 ± 0.35%, respectively) in comparison to the control, which contained no progressively motile sperm on Day 7 (P < 0.01).

Similarly to PLM, viability declined over time (P < 0.05; except 10 µM OA), with the most rapid decline in both the 10 and 100 µM EPA and DHA treatments. In the 100 µM EPA treatment viability decreased from 36.7 ± 6.05% on Day 0 to 26.7 ± 6.05% on Day 1 (P < 0.01) while in the 100 µM DHA treatment on Day 0 and 1 viability decreased from 49.0 ± 6.05% to 28.0 ± 6.05%, respectively (P < 0.05; Figure 2.12). Interestingly, even though 100 µM EPA and DHA treatments had the lowest number of viable sperm on Days 1, 3 and 7 (100 µM EPA: 27.6 ± 6.05%; 14.0 ± 6.05% and 4.6 ± 6.97%, 100 µM DHA: 28.0 ± 6.05%; 15.0 ± 6.05% and 4.1 ± 6.97%, respectively), both fatty acids at 10 µM maintained a higher sperm cell viability compared to their 100 µM counterparts on all Days (DHA; P < 0.01, EPA; P < 0.05). Consistent with the PLM test, OA (10 and 100 µM), ALA (10 µM) and PA had the greatest ability to maintain viability, with a score for each on Day 7 of 45.6 ± 6.97%, 39.1 ± 6.97%, 44.6 ± 6.97% and 45.6 ± 6.97%, respectively; Figure 2.12. The control had a viability score of 64.0 ± 6.05% and 20.0 ± 6.97% on Day 0 and 7, respectively. These results suggest that DHA and EPA (100 µM in both fatty acids) may be cytotoxic to sperm cells.
Figure 2.11: Motility of sperm stored with ALA, DHA, EPA (all n-3), LA (n-6) or OA (n-9) at concentrations of 10 and 100 µM assessed on Days 0, 1, 3 and 7. Both a saturated fat control (PA) and a vehicle control were included. Vertical bars represent s.e.m (n = 4 replicates)
Figure 2.12: Viability of sperm stored with ALA, DHA, EPA (all n-3), LA (n-6) or OA (n-9) at concentrations of 10 and 100 µM assessed on Days 0, 1, 3 and 7. Both a saturated fat control (PA) and a vehicle control were included. Vertical bars represent s.e.m (n = 4 replicates).
2.3.2.1.2 Generation of Reactive Oxygen Species

There was an effect of Day on ROS (P < 0.0001) where ROS generation peaked on either Day 1 or Day 3 dependent on treatment (Figure 2.13). The number of live sperm positive for ROS was highest in the 100 µM LA, 10 µM EPA and 100 µM OA on Day 1 (68.6 ± 10.04%; 69.4 ± 10.04% and 67.9 ± 10.04%, respectively) and on the 10 µM OA, 100 µM OA and 10 µM ALA on Day 3 (76.9 ± 10.04%; 73.9 ± 10.04% and 73.3 ± 10.04%, respectively; Figure 2.13). The level of ROS in the control treatment reached a peak of 67.5 ± 10.04% on Day 1 and declined thereafter. PA displayed an inherent ability to keep levels of ROS in viable sperm to a minimum compared to the control on Days 1 and 3 (P < 0.01). On Day 1, most treatments showed a lower level of ROS compared to the control (67.5 ± 10.04%), only 100 µM LA, 10 µM EPA and 100 µM OA were higher (68.6 ± 10.04%; 69.4 ± 10.04% and 67.9 ± 10.04%, respectively). On Day 7 ROS generation from live sperm was highest in the 10 µM LA (68.8 ± 11.63%, P < 0.05) and ALA (59.7 ± 11.63%, P < 0.05) and 10 and 100 µM OA (68.2 ± 11.63% and 43.6 ± 11.63%, respectively) compared to the control (12.0 ± 11.63%).
Figure 2.14: Generation of reactive oxygen species (ROS) by sperm stored with ALA, DHA, EPA (all n-3), LA (n-6) or OA (n-9) at concentrations of 10 and 100 µM assessed on Days 0, 1, 3 and 7. Both a saturated fat control (PA) and a vehicle control were included, vertical bars represent s.e.m (n = 4 replicates)
2.3.2.2 Experiment 2b

Figure 2.15: Motility, viability and % live and generating reactive oxygen species (ROS) (Upper, Middle and Lower Panel, respectively) of sperm stored in the presence or absence of catalase, assessed on Days 0, 1, 3, 7 and 10. Vertical bars represent s.e.m (n = 3 replicates)
There was no significant effect of treatment by day interaction, or treatment or day on the ability of sperm to maintain PLM, viabiliy or ROS (Figure 2.15). However, on all days post Day 1, motility was numerically lower in the without catalase treatment and this reached statistical significance on Day 10 (P < 0.05; Figure 2.15, upper panel). The treatment without catalase exhibited peak ROS generation on Day 3 and Day 7 (52.6 ± 10.76% and 53.6 ± 10.76%, respectively) while peak ROS generation in the catalase treatment was delayed until Day 7 and Day 10 (56.7 ± 10.76% and 54.9 ± 10.76%, respectively), however no significant effect of treatment nor day was observed on the generation of ROS (Figure 2.15, lower panel).

2.3.2.3 Stress to Sperm Cells by \( \text{H}_2\text{O}_2 \)

As the concentration of \( \text{H}_2\text{O}_2 \) increased, the percentage of sperm cells live and generating ROS in the sample increased proportionally (Figure 2.16). There was an extremely low level of cells (events) positive for ROS in the samples that did not contain cells, suggesting that (1) there was very little auto-fluorescence of the CM-H\(_2\)DCFDA, and (2) the sperm cells themselves are a main cause of ROS generation. \( \text{H}_2\text{O}_2 \)
2.3.3 Experiment 3

2.3.3.1 Motility and Viability

PLM decreased with time in all treatments (P < 0.01), but similar to Experiment 2 the most dramatic decline in motility was in the EPA and DHA treatments (10 and 100 µM in both) having lower PLM scores on Day 7 compared to the control (3.8 ± 11.27%; 6.4 ± 13.10%; 0.0 ± 17.46%, Control: 13.2 ± 13.10%; Figure 2.17). PA maintained the highest PLM score up to Day 3 (78.8 ± 10.23%), but declined on Day 7. ALA (100 µM) and OA (both 10 and 100 µM) demonstrated the highest motility on Day 7. There was a significant effect of day on sperm cell viability, where all treatments declined significantly over the course of seven days (P < 0.01). PA had the highest number of live sperm on Day 7 (34.5 ± 4.51%) in comparison to other treatments and the control (Figure 2.18). Similar to Experiment 2, 100 µM EPA and DHA had a significantly lower number of live sperm on Day 7 compared to the control (16.6 ± 4.87%, 5.3 ± 4.51% and 26.8 ± 4.51%, respectively; P < 0.05).
Figure 2.17: Motility of sperm stored with ALA, DHA, EPA (all n-3), LA (n-6) or OA (n-9) at concentrations of 10 and 100 µM assessed on Days 0, 1, 3 and 7. A saturated fat control (PA) and a vehicle control were included. Vertical bars represent s.e.m (n = 4 replicates)
2.3.3.2 Generation of Superoxide ($\text{O}_2^-$)

There was a significant effect of Day on superoxide production ($P < 0.0001$) where the production of superoxide peaked on Day 0 (except 10 µM EPA) with all treatments having in excess of $72.7 \pm 9.55\%$ live sperm staining positive for superoxide (Figure 2.19). Superoxide levels remained greater than $59.7 \pm 9.55\%$ (value of 10 µM ALA) in all treatments of Day 1 and declined thereafter, however, the rate of decline was not dependent

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**Figure 2.18**: Viability of sperm stored with ALA, DHA, EPA (all n-3), LA (n-6) or OA (n-9) at concentrations of 10 and 100 µM assessed on Days 0, 1, 3 and 7. A saturated fat control (PA) and a vehicle control were included. Vertical bars represent s.e.m (n = 4 replicates)
on treatment. Both the 100 µM concentrations in DHA and EPA appear to be cytotoxic to cells, clearly evident in PLM and viability results. However, Day 3 levels of superoxide in the 100 µM DHA treatment (59.6 ± 10.80%) remain high compared to other fatty acids and higher than the control (54.3 ± 9.55%), whereas 100 µM EPA (36.8 ± 10.80%) led to the lowest generation of superoxide on Day 3. Following this, on Day 7, 100 µM DHA and EPA also display numerically lower levels of superoxide (25.2 ± 9.55 and 38.9 ± 9.55, respectively), similar to the levels of ROS production on Day 7, in the same treatments.

**Figure 2.19:** Generation of superoxide (O$_2$·-) by sperm stored with ALA, DHA, EPA (all n-3), LA (n-6) or OA (n-9) at concentrations of 10 and 100 µM assessed on Days 0, 1, 3 and 7.
A saturated fat control (PA) and a vehicle control were included. Vertical bars represent s.e.m (n = 4 replicates)

2.3.3.3 Stress to Sperm Cells by H2O2

There was an increase in superoxide production as the concentration of H2O2 increased, with the most dramatic increase from 100 µM to 1000 µM H2O2 (Figure 2.20). A large proportion of sperm cells were positive for superoxide without the addition of H2O2, as seen in Experiment 3, Day 0 showed the highest levels of superoxide production. These results suggest that H2O2 is fails to induce superoxide production to a similar level that it induced general ROS generation.

![Figure 2.20](image)

**Figure 2.20:** Positive control for MitoSOX Red, increasing concentration of H2O2 caused oxidative stress to sperm cells, seen in the increasing number of sperm live and generating Superoxide, most clearly seen on Day 0, n = 3
2.4 Discussion

The main findings of this thesis were that while motility and viability decreased with time, sperm cells maintained their ability to penetrate artificial mucus up to and including Day 4 (Experiment 1). ROS generation peaked on Days 1 to 3 (Experiment 2a) and declined thereafter, with OA and ALA showing a delay in the peak production of ROS, while PA maintained ROS production at basal levels over all days compared to the control. DHA and EPA proved to be detrimental to motility and viability on Days 0 and 1 of storage. While there was no effect of fatty acid on superoxide production, its production peaked on Day 0 and declined afterwards. The addition of catalase to egg yolk based diluent showed no significant effect on stored bull sperm cell motility, viability and generation of ROS.

While motility and viability did decrease significantly with incubation time (Experiment 1), there was no effect of time on the ability of sperm to penetrate artificial mucus, up to and including Day 4. This is in contrast to a recent study that demonstrated that after 2 days in storage, the ability of sperm cells to penetrate artificial mucus was significantly decreased (Al Naib et al., 2011b). In addition, there was no effect of treatment on the sperm’s ability to penetrate artificial mucus in comparison to the control. It is postulated that this lack of effect could be attributed to the egg yolk based diluent used in Experiment 1, as on Day 4, a high PLM and viability value (all treatments showed motility >69.4 ± 3.53% and viability >68.8 ± 5.88%) was allocated to all treatments, a characteristic not observed in Experiment 2 and 3 (no egg yolk used). As none of the fatty acid treatments had any effect, the egg yolk may have a buffering effect, not allowing the fatty acids to have any influence over the sperm cells performance under each parameter assessed. It has been previously reported that BSP proteins bind to the sperm cell membrane choline phospholipids after ejaculation, causing an efflux of phospholipids and cholesterol from mature sperm, triggering the initial stage of
capacitation (Thérien et al., 1999). The inclusion of egg yolk into semen extenders results in a significant decrease in the number of BSP proteins bound to sperm cells, an increase in lipid levels (phospholipids and cholesterol) and retention of high motility (Bergeron et al., 2004). It has been claimed that the low density lipoprotein fraction of egg yolk is responsible for the protective role that egg yolk plays in sperm cell storage. These low density lipoprotein fractions could carry out this action through two mechanisms, the first by disrupting the BSP proteins binding to the sperm cell membrane phospholipids and thus stopping the lipid efflux from the membrane, and the second, the lipid portion of the LDF, or in fact the whole molecule itself, becomes incorporated into the plasma membrane of the sperm cell and in doing so, preserves the membrane stability during sperm storage (Bergeron et al, 2004). It is for this reason that the majority of semen extenders are egg yolk based used in storage of sperm cells for in vitro use.

DHA and EPA were detrimental to the motility and viability of the sperm cells (Experiments 2a and 3), where higher concentrations of these long chain fatty acids caused a more rapid decline in both parameters. DHA has been reported to be the most abundant unsaturated fatty acid in ejaculated bull sperm (Kelso et al., 1997b), where poor quality sperm cells had a significantly lower amount of DHA than normal cells (Koppers et al., 2010). Despite this, the addition of DHA to sperm in vitro appears to have a detrimental effect. In agreement with the current study, Koppers et al. (2010) reported a significant decrease in progressive motility and viability of human sperm cells following 24 h incubation with 10 µM DHA. Aitken et al. (2006) stimulated ROS production after the short term exposure (15 min) of human sperm to 10 µM DHA while exposure to 115 µM DHA was cytotoxic to the sperm cells. In addition to this, DHA has been reported to be more susceptible to peroxidation than other PUFAs in human sperm, such as arachidonic acid (Lukiw & Bazan, 2008; Oborna et al., 2010) and the
in vitro addition of highly unsaturated fatty acids, like DHA, increases the amount of ROS formed by human spermatozoa (Aitken & Baker, 2006). EPA has similar effects on PLM and viability as DHA, as similar trends were observed in both parameters. It was noted however, that 10 µM EPA was not as detrimental as 10 µM DHA on motility and viability. To the best of our knowledge this is the first report of the effects of EPA on bull sperm in vitro.

In contrast to the detrimental effects caused by the addition of DHA and EPA to sperm cells in vitro, the addition of fatty acids to the diet of a range of species has proved to reap beneficial effects (Comhaire et al., 2000; Castellano et al., 2010; Gholami et al., 2010). A DHA and EPA rich nutriceutical, when added to the diets of bulls for a period of up to 12 weeks, improved sperm concentration, motility and viability (Gholami et al., 2010). Again in contrast to the majority of in vitro work, and the work of this study, the amount of DHA and EPA added to the diet was higher (10 g and 0.6 g, respectively, as part of the diet, compared to 10 or 100 µM added in vitro). The positive results from work carried out on fatty acids as part of the diet suggests that fatty acids such as DHA and EPA should be included into the diet in order for them to reap their beneficial awards, and to become incorporated into the sperm cell membrane. In addition to this, Comhaire et al. (2000) investigated the effects of the addition of essential fatty acid supplements to human diets, mainly, observing the effect of the addition of 1 g DHA. Patients were shown to show elevated levels of DHA during the treatment in comparison to before (11.16 ± 1.75 and 9.53 ± 1.34% mole, respectively), while it also proved beneficial to sperm cell concentration and acrosome reaction in oligospermic men. It is possible that the addition of DHA and similarly, EPA, to sperm cells in vitro, provides plentiful double bonds for the LPO chain reaction to take place, thus causing a rapid deterioration of motility and viability, a characteristic not seen when higher concentrations of the fatty acid are added to the diet.
In general, ALA, OA and PA maintained motility and viability up to and including Day 7 (Experiment 2a). In the ALA and OA treatments ROS generation peaked on Day 3, compared to Day 1 in the control. PA maintained ROS at the lowest levels on all days, corresponding to its good viability and motility scores (as well as rapid movement; data not shown). PA is the most prominent saturated fatty acid in bull and human sperm fractions (Kelso et al., 1997b; Lenzi et al., 2000), while OA has a high representation in both these species also. Neill and Masters (1971) demonstrated that fatty acids added in vitro, are incorporated into the membrane of the sperm cell. Thus, it is likely that the incorporation of PA (no double bonds) or OA (one double bond) into the sperm membrane would reduce the cells susceptibility to attack by ROS. Our findings are supported by Aitken et al., (2006) who demonstrated that short term exposure to PA had no effect on ROS generation by sperm.

Immature or poor quality human spermatozoa have been reported to have higher levels of unsaturated fatty acids than normal sperm cells (Ollero et al., 2001). It has been suggested by Cocco et al. (1999) that these PUFAs could inhibit specific complexes of the mitochondrial ETC, consequently allowing the leakage of electrons, followed by the formation of superoxide. It is for this reason that excessive generation of superoxide is a key indicator of poor, defective spermatozoa (De Iuliis et al., 2006) and has been reported to be the principal ROS generated by sperm cells (Lenzi et al., 1996). The current study would support this as greater than 71.7% of live sperm were positive for the superoxide anion on Day 0. This coupled with the findings in Experiment 2 would indicate that bull sperm stored in vitro are subject to the greatest oxidative stress early in the storage period, on Day 0 from superoxide and on Days 1 and 3 from other oxidising free radicals, including the hydroxyl radical and hydrogen peroxide. It has been reported that superoxide (·O$_2^-$) can gain a proton to quickly form the hydroperoxyl (HO$_2^-$) radical (Kothari et al., 2010) or can generate hydrogen peroxide
spontaneously or after breakdown by superoxide dismutase (Alvarez et al., 1987). Following this, in the presence of hydrogen peroxide and excess superoxide, hydroxyl radicals can be formed via the Haber Weiss reaction (Aitken et al., 1989). Thus, we hypothesise that the superoxide anion dominates on Day 0, and its breakdown products then form other ROS like the hydroperoxyl radical, hydrogen peroxide and the hydroxyl radical on subsequent days.

Interestingly, even though PA maintained levels of ROS at basal levels in Experiment 2a, it had no inhibitory effect on superoxide production. This is in agreement with Koppers et al. (2010) who reported that saturated fatty acids did not generate nor inhibit superoxide. The exogenous unsaturated fatty acids added in the current study did not stimulate additional superoxide anion generation over and above the control. However, this contrasts with Koppers et al. (2010) who demonstrated a high percentage of functional human sperm cells live and positive for MitoSOX Red after 15 min exposure to unsaturated fatty acids (DHA, LA, AA and OA) at 10 µM. The reason we did not observe a difference in the current study may be due to the extraordinary high number of live sperm positive for ROS in the control and therefore, the potential for further increases are limited.

Oxidative stress to sperm cells occurs when there is an imbalance between the biochemical processes that produce ROS and those processes that remove ROS (Safarinejad et al., 2010). This study found no beneficial effect of the addition of catalase (17.14 U/mL Caprogen) on motility, viability nor on ROS production of sperm cells, however, it was noted that sperm without catalase did show lower motility scores than sperm with catalase on the same day, although this did not reach statistical significance (Experiment 2b). Supporting the results seen in this study, Ball et al. (2001) showed no significant effect of catalase (100 or 200 U/mL) on motility of stallion sperm. Another study on the effect of catalase on bovine sperm,
showed no significant difference on viability and fertilising ability (Vandemark et al., 1950). In contrast, Foote et al. (2002) observed that antioxidants improved bovine sperm motility. A definitive in vivo study by Shannon et al. (1984) on the effect of the addition of catalase to semen diluents on bull sperm, reported that the inclusion of catalase (4.5 µg) to 5 and 20% egg yolk Caprogen, significantly increased the non-return rate in bulls. A possible reason for the lack of effect of exogenous catalase on bull sperm parameters observed in this study could be related to the buffering effect of egg-yolk, as seen in Experiment 1. It could also be attributed to the low concentration of catalase that actually comes into contact with the sperm cell, only 17.14 U/mL is found in the egg yolk based diluent, while in the aforementioned in vitro studies, up to 200 U/mL were used.

Currently, interest in improving AI in Ireland lies with reducing sperm number per insemination dose, thereby, maximising the number of insemination doses per ejaculate. The use of fresh semen allows a greatly reduced sperm concentration per insemination dose, which is normally stored at 5 million sperm cells per 0.25 mL dose, in comparison to frozen thawed sperm, which is stored at 80 million sperm cells per 0.25 mL dose. For fresh semen use in AI, bovine sperm is normally diluted in egg yolk based diluents, which has a protective effect on sperm by decreasing BSP proteins bound to the sperm cell and increasing lipid levels of the sperm cell membrane (Bergeron et al, 2004). Egg yolk, however, is obtained from another species and involves the introduction of a biological substance into the cow through insemination doses, which could present a worry for some dairy cattle breeders. Egg yolk is known to contain high concentrations of OA and PA (Wang et al, 2000), therefore, the inclusion of these long chain fatty acids to citrate based diluents was hypothesised to have beneficial effects on sperm cell parameters, and results from this study found that they had beneficial effects in preserving sperm cell motility and viability, in addition to reducing
sperm cell production of ROS. In contrast to this, the addition of DHA and EPA to these
citrate based diluents could cause a detrimental effect to sperm cells; DHA is present in very
low amounts in egg yolk (Wang et al, 2000). Before this step is taken, however, further work
should be carried out based on the results of this study. Although addition of OA and PA
improves sperm cell parameters such as motility, viability and reduces ROS generation, the
effect of their addition on sperm cell membrane fluidity was not assessed. As OA and PA
have one and no double bonds in their carbon chain, respectively, their incorporation into the
sperm cell membrane may reduce membrane fluidity, causing a tighter packing for the
membrane. Therefore, even though the sperm cells remain motile and viable, they may not
have the ability to fertilise an oocyte, which is dependent on the sperm cell membrane lipid
profile (Hinrichsen & Blaquier, 1980; Gatti et al., 2004) and its membrane fluidity. It is the
author’s opinion that experimental work involving the addition of fatty acids to sperm in vitro
is limited, after assessment of membrane fluidity. Presently, research is being carried out into
further reducing the number of sperm cells per fresh semen insemination doses, to
concentrations lower than 5 million per 0.25 mL dose. The main disadvantage of liquid
semen is its finite lifespan and is not used in Ireland passed 60 h post collection due to
concerns over lower fertility of aged sperm. This sudden drop in fertility could be attributed
to generation of ROS by the sperm cells themselves, followed by subsequent chain reaction
generation of ROS. A lower concentration of sperm cells could cause a reduction in ROS
generation, thereby, deterring OS, LPO and degradation of the sperm cell membrane.

Therefore, in conclusion, this study has identified that superoxide production peaks on Day 0,
with other ROS peaking on Days 1-3. The addition of DHA and EPA to bull sperm in vitro
has detrimental effects on motility and viability, while the addition of PA and OA maintains
both motility and viability. These differences may be explained by the ability of PA and OA
to minimise or delay the production of ROS. Furthermore, the use of low concentrations of catalase in chilled semen diluents seems to be of limited benefits.

2.5 Conclusions

The main findings of this thesis include:

1. Motility and Viability declined with time in all treatments, in all experiments.

2. Superoxide production peaks on Day 0 and declined thereafter. There was no effect of treatment on superoxide production on a particular day.

3. Other ROS, including $\text{H}_2\text{O}_2$, OH-, ONOO-, and ROO-, peaked on Day 1 or Day 3.

4. DHA and EPA showed detrimental effects to bull sperm motility and viability.

5. The addition of both PA and OA maintained motility and viability while also reducing and delaying ROS production peaks, respectively.

6. Superoxide production dominates on Day 0 while its breakdown products take over on subsequent days, as assessed by CM-$\text{H}_2$DCFDA

7. It is suggested that DHA and EPA have more detrimental effects on sperm cells due to their high number of double bonds (6 and 5, respectively). Already present ROS are free to attack the double bonds and a more rapid and vigorous chain reaction occurs, causing greater levels of LPO. For similar reasons, both PA and OA may have beneficial effects on sperm cells due to their lack of and only one double bond, not supplying ROS with a target to attack.
8. The addition of low levels of catalase to egg-yolk based diluent had no effect on motility, viability or generation of ROS by bull sperm.
Chapter 3 – Bibliography


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Appendices
Appendix 1 - Sperm Cell Media

Phosphate Buffered Saline (PBS)

Table 1: Composition of Phosphate Buffered Saline (PBS) media

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<th>Chemical</th>
<th>Formula</th>
<th>Supplier</th>
<th>Product Code</th>
<th>Molecular Mass (g/mol)</th>
<th>Concentration (mM/L)</th>
<th>g/L</th>
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<tr>
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<td>10.14</td>
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<td>1.76</td>
<td>0.24</td>
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The salts above were added to distilled water and made up to a 1 L solution using distilled water (Table 1). Following which, the pH was adjusted to 7.4 by adding 1 M Sodium Hydroxide (NaOH); (Fisher Scientific, Ireland – S/4920/60) or 0.1 M Hydrochloric acid (HCl); (Sigma Aldrich - 07102) accordingly. PBS was then filtered using No. 4 Whatman Filter Paper to get rid of particles. The media was stored in the fridge for 3-5 days.

Biggers, Whitten and Whittingham (BWW) media

The salts below were added to distilled water and made up to 1 L using distilled water (Table 2).
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<th>Chemical</th>
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<th>Product Code</th>
<th>Supplier</th>
<th>Molecular Mass (g/mol)</th>
<th>Concentration (mM/L)</th>
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<td>58.44</td>
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<td>Lactate</td>
<td>C₃H₅NaO₃</td>
<td>S/161/08</td>
<td>Fisher Scientific</td>
<td>44</td>
<td>112.06</td>
<td>5.94 mL</td>
</tr>
<tr>
<td>Sodium Carbonate</td>
<td>NaHCO₃</td>
<td>S/4240/60</td>
<td>Fisher Scientific</td>
<td>25</td>
<td>84.01</td>
<td>2.10</td>
</tr>
<tr>
<td>Heps</td>
<td>C₈H₁₈N₂O₄S</td>
<td>H3375-250G</td>
<td>Scientific</td>
<td>20</td>
<td>238.3</td>
<td>4.77</td>
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<tr>
<td>Glucose</td>
<td>C₆H₁₂O₆</td>
<td>G/0450/60</td>
<td>Fisher Scientific</td>
<td>5.6</td>
<td>180</td>
<td>1.01</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>KCl</td>
<td>BP366-500</td>
<td>Fisher Scientific</td>
<td>4.6</td>
<td>74.55</td>
<td>0.34</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>CaCl₂</td>
<td>C/1400/60</td>
<td>Fisher Scientific</td>
<td>1.7</td>
<td>110.99</td>
<td>0.19</td>
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<tr>
<td>Chloride</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Potassium Dihydrogen Phosphate</td>
<td>KH₂PO₄</td>
<td>20592500</td>
<td>Fisher Scientific</td>
<td>1.2</td>
<td>136.09</td>
<td>0.16</td>
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<tr>
<td>Magnesium</td>
<td>MgSO₄</td>
<td>20,809-4</td>
<td>Sigma Aldrich</td>
<td>1.2</td>
<td>120.37</td>
<td>0.144</td>
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<tr>
<td>Sulphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>C₃H₃NaO₃</td>
<td>P3663-25G</td>
<td>Sigma Aldrich</td>
<td>0.27</td>
<td>110.04</td>
<td>0.03</td>
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<tr>
<td>Bovine Serum Albumin</td>
<td>C₁₆H₁₈N₂O₄S</td>
<td>BPE9704-100</td>
<td>Fisher Scientific</td>
<td>0.3%</td>
<td>256.43</td>
<td>3</td>
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<tr>
<td>Penicillin g</td>
<td>C₁₆H₁₈N₂O₄S</td>
<td>P3032-25MU</td>
<td>Sigma Aldrich</td>
<td>5 U/mL</td>
<td>356.37</td>
<td>0.0034</td>
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<tr>
<td>Streptomycin</td>
<td>C₂₁H₃₉N₇O₁₂</td>
<td>1000953685</td>
<td>Sigma Aldrich</td>
<td>5</td>
<td>1457.58</td>
<td>5</td>
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</table>
Bovine Serum Albumin (BSA) was sprinkled on top of the media when all other components were dissolved and left in the incubator at 37 °C for 15-20 minutes, until all BSA was dissolved. Again, pH was adjusted to 7.4 by adding 1 M NaOH or 0.1 HCl accordingly. BWW was filtered using No. 4 Whatman filter paper.

**Egg Yolk Based Diluent (Experiment 1)**

The egg yolk based diluent was prepared on a weekly basis, using a method recommended by the National Cattle Breeding Centre Limited, with minor adaptions. The diluent was composed of four main constituents, Glycerol-Antibiotic Solution, Buffer Solution, Catalase Solution and Egg Yolk.

**Glycerol-Antibiotic Solution**

Penicillin g sodium (500, 000 units - 0.3345 g); (Sigma Aldrich – P3032-25MU) was dissolved in 1 mL of distilled water (diH₂O), while streptomycin sulphate (0.5 g); (Sigma Aldrich - 1000953685) was dissolved in another 1 mL diH₂O. Following this, the streptomycin sulphate solution was added to the penicillin g sodium solution and was brought to 5 mL using diH₂O. Pure glycerol (5 mL); (Fisher Scientific – G-0650/08), which acts as a cryoprotectant for the cells, was added and the solution was mixed thoroughly using a vortex. A 10 mL solution of glycerol-antibiotic solution is made up in total, which is viable for up to four weeks in the fridge.

**Buffer Solution**

Sodium citrate (Na₃C₆H₅O₇ – 3g); (Sigma Aldrich - 32320), Glycine (NH₂CH₂COOH – 1.5 g); (Fisher Scientific – G/0800/60) and Glucose (C₆H₁₂O₆ – 0.45 g); (Fisher Scientific - G/0450/60) were added to 40 ml of boiling diH₂O and allowed to dissolve using the magnetic stirrer. Refrigerated diH₂O was added immediately afterwards to bring the volume to 140 mL. Glycerol-antibiotic solution (3.75 mL) was added to the solution, following which, the
solution was brought to 150 mL with more refrigerated diH₂O. Buffer solution was prepared weekly.

Catalase Solution
Catalase from bovine liver (0.0045 g); (Sigma Aldrich – C1345-10G) was dissolved in 10 mL buffer solution and stored at 4 °C for up to 2 weeks.

Egg Yolk
The egg yolk of an organic egg was separated from egg white, leaving no excess egg white on the skin of the egg yolk. The egg yolk skin was punctured and the required inside was allowed to pour into a container. No egg yolk skin was used, as a precaution to keep out egg white.

A 5% egg yolk based diluent (100 mL) was prepared; buffer solution (94 mL) was added to a beaker, to which catalase solution (1 mL) was added and allowed to stir on the magnetic stirrer. Citric acid (C₆H₈O₇ – 0.014 g); (Fisher Scientific – C/6160/53). egg yolk (5 mL) was added as the final step and left to stir on the magnetic stirrer until all components were mixed thoroughly. The pH of the diluent was adjusted to 7.4 by adding 1 M NaOH or 0.1 M HCl accordingly. The 5% egg yolk based diluent was filtered using No. 4 Whatman filter paper and stored at 4 °C for up to four days, but remains viable for 1 week.

Citrate Based Diluent (Experiment 2a & 3)
The citrate based diluent was prepared in the same way as the 2.2.3 Egg Yolk Based Diluent, but with the omission of egg yolk. In its place, 5 mL of buffer solution was added to the solution. Again, pH was adjusted to 7.4 by adding 1 M NaOH or 0.1 M HCl accordingly.
The 5% egg yolk based diluent was filtered using No. 4 Whatman filter paper and stored at 4 °C for up to four days, but remains viable for 1 week.

**Diluent with Catalase (Experiment 2b)**

This was prepared as in 2.2.3 Egg Yolk Based Diluent

**Diluent with No Catalase (Experiment 2b)**

Diluent with no catalase was prepared as in 2.2.3 Egg Yolk Based Diluent but with the omission of catalase solution. In its place, 1 mL of buffer solution was added to the solution. Again, pH was adjusted to 7.4 by adding 1 M NaOH or 0.1 M HCl accordingly. The 5% egg yolk based diluent was filtered using No. 4 Whatman filter paper and stored at 4 °C for up to four days, but remains viable for 1 week.
Appendix 2 - Reagents

Nigrosin-Eosin Stain

Nigrosin (water soluble – 2.5 g); (BDH - 34058) and Eosin Y (water soluble - 0.5 g); (BDH - 34027) were added to 50 mL of diH₂O and dissolved with the magnetic stirrer. Sodium citrate (1.5 g); (Sigma Aldrich - 32320) was added to the solution and when no more could dissolve, it was brought to the boil on a hot plate with regular stirring to help dissolve. The stain was then allowed to cool to room temperature and filtered using No. 4 Whatman filter paper. It was stored at 4 °C for 4-5 months in a dark glass bottle.

Artificial Mucus

Sodium hyaluronate based, artificial mucus was prepared in the lab by diluting Map5 (Labstock MicroServices, Ireland) (3 mL) in PBS (2 mL), leaving a 60% sodium hyaluronate solution.

SYTOX Green

SYTOX Green (Invitrogen, Molecular Probes – S7020) was added to BWW, where serial dilutions were made to allow a working concentration of 0.25 µM in the sample. Aliquots of SYTOX Green were stored at -20 °C and remained stable for up to 1 year when stored correctly. SYTOX Green is excited at 504 and emits at 523 nm.

MitoSOX Red

The contents of one vial of MitoSOX mitochondrial superoxide indicator (Invitrogen, Molecular Probes – M36008); (50 µg) were dissolved in dimethylsulfoxide (DMSO); (Fisher Scientific – D/4120/PB08) and serial dilutions were carried out in BWW to allow a working
concentration of 2.5 µM in the sample. Aliquots of MitoSOX Red were stored at -20 °C. MitoSOX Red is excited at 510 nm and emits at 580 nm.

Propidium Iodide (PI)

Propidium Iodide (Invitrogen - P1304MP); (10 mg) was dissolved in filter sterilised diH2O, giving a final solution of 10 mg/10 mL or a 1.5 mM solution. This was further diluted to 300 µM in PBS, in order to allow a working concentration of 50 µM in the sample. PI aliquots were stored at 4 °C. PI excites at 535 nm and emits at 617 nm.

Chloromethyl-2,7-dichlorofluorescein diacetate (CM-H2DCFDA)

Chloromethyl-2, 7-dichlorofluorescein diacetate (CM-H2DCFDA); (Invitrogen, Molecular Probes – C6827) was prepared by dissolving the contents of one vial in DMSO, followed by serial dilutions in PBS, to allow for a working concentration of 100 µM. CM-H2DCFDA lower concentrations were prepared daily when assessing sperm parameters while new vials were dissolved each 2-3 weeks, depending on 1 colour fluorescent controls daily on the BD-LSR1. CM-H2DCFDA is excited at 492-495 nm and emits at 517-527 nm.

Hoechst 33342

Hoechst 33342 (Sigma Aldrich – B2261-25MG) was prepared in filter sterilised diH2O and aliquoted into 10 mg/mL concentrations. Further dilutions were made when necessary to bring Hoechst 33342 to 0.5 mg/mL. Both stocks were stored at -20 °C and were stable for up to 1 year.
Sodium Hydroxide (NaOH)

A 1 M (100 mL) solution of sodium hydroxide (NaOH) was prepared by dissolving 3.999 g NaOH in diH₂O

Hydrochloric Acid (HCl)

A 0.1 M (100 mL) hydrochloric acid (HCl) solution was prepared using 36% concentrated HCl (12 M). HCl (37% - 0.86 mL) was added to a small amount of diH₂O and then made up to 100 mL by adding more diH₂O

Hydrogen Peroxide Stocks for Stress of Sperm Cells

Hydrogen peroxide (H₂O₂ - 1 L) is approximately 1.11 kg, due to its density. Therefore, 30% H₂O₂ (1 L) is a 9.68 M solution.

Serial Dilutions for Stock Solutions of H₂O₂

- A **500 mM (0.5 M)** solution of H₂O₂ in PBS (Dilution factor – DF = 19.36) was prepared by the addition of 517 µL H₂O₂ to 9583 µL PBS (Stock 1).
- A **50 mM (0.05 M)** solution of H₂O₂ in PBS (DF = 10) was prepared by the addition of 500 mM H₂O₂ Stock 1 (1 mL) to PBS (9 mL) (Stock 2).
- A **5 mM (0.005 M)** solution of H₂O₂ in PBS (DF = 10) was prepared by the addition of 50 mM H₂O₂ (Stock 2) (1 mL) to PBS (9 mL) (Stock 3).
- A **0.5 mM (0.0005 M)** solution of H₂O₂ in PBS (DF = 10) was prepared by the addition of 5 mM H₂O₂ (Stock 3) (1 mL) to PBS (9 mL) (Stock 4).