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Improving the quality of frozen-thawed ram semen

By

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Abstract

Cryopreserved semen is widely used for assisted reproduction in livestock, however, in sheep its use is still limited. Freezing and thawing cause biochemical and physiological alterations into the sperm. These alterations such as oxidative damage caused by reactive oxygen species (ROS) and changes in heat shock proteins (HSP) appear to determine the fertilizing ability of the sperm. Seminal plasma (SP) and antioxidants have the ability to improve sperm function and reduce the effect of cryopresrvation. This study aimed to improve the integrity of frozen-thawed ram sperm, so that an increase the fertility rates could be achieved. **The first experiment** evaluated the effect of the post-thaw addition of 1.5 mg/ml of different seminal plasma (SP) protein fractions (Whole SP, >100, 30-100, and <30kDa) on the quality of frozen-thawed ejaculated and epididymal ram sperm. SP proteins fractions, particularly <30kDa improved the integrity of fresh and frozen-thawed epididymal spermatozoa and fresh ejaculated spermatozoa ($P < 0.001$), but there was no effect on frozen-thawed ejaculated spermatozoa. This reduction could be related to the effect of ROS on sperm integrity. Therefore, **the second experiment** was designed to assess the effect of the antioxidants cysteine, taurine, and vitamin C on frozen-thawed ram semen. Semen samples were treated pre-freeze (PF) and post-thaw (PT) to evaluate the optimal timing and concentration of antioxidant supplementation on frozen-thawed ram semen to improve sperm function and reduce ROS production. The addition of 0.5 and 1.0 mg/ml cysteine or taurine (PF + PT) improved the integrity of frozen-thaw ram sperm. There was no effect of vitamin C supplementation on frozen-thawed ram sperm, however, it improved penetrability and reduced ROS production. In **the third experiment**, the effect of oxidative stress induced by 5mM and 15mM hydrogen peroxide (H_2O_2) on the integrity of fresh ram sperm was assessed. ROS production, lipid peroxidation (LPO) in SP, and the expression of heat shock proteins (70 and 90) were determined. H_2O_2 has the capability to eliminate sperm functions significantly at 15µM. This effect could indicate the importance of HSP70 and HSP90 to protect sperm membrane functions, thus there is a need to maintain the function of these proteins. **The fourth experiment** identified the relationship between supplementation with 1.0 mg/ml (PF + PT) of taurine or cysteine and the expression level of HSP90 and HSP70 on frozen-thawed ram sperm. The results showed that PF or PT supplementation of antioxidants (1.0 mg/ml of cysteine or taurine) improved post-thaw ram sperm integrity and maintained the expression of HSP70 and HSP90. There was a positive relationship between the level of expression of HSP70 and HSP90 and sperm parameters such as motility, acrosome integrity, viability, penetrability, ROS concentration and the level of LPO in SP and sperm. Therefore, the findings of this thesis collectively may assist to improve the quality of cryopreserved ram semen, and effectively contribute to reproductive technologies in the sheep industry.

Declaration

I declare that this thesis has been composed entirely by myself, Ahmed Mustafa Kafi. As a part of my degree this research is original and has not been published before. Source of information and assistance have been specifically acknowledged by means of references.

Ahmed Kafi

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List of abbreviations

Symbol	Name
AI	Artificial insemination
$A^{\bullet}$	Antioxidant Radical
ADP	Adenosine diphosphate
AH	Antioxidant
AKAPs	A-kinase support proteins
ALP	Alanine aminotransferase
ALT	Aspartate amino transferase
ANOVA	Analysis of Variance
AO	Antioxidant
ASA	Anti-sperm antibodies.
AST	Alkaline phosphatase
ATP	Adenosine-5'-triphosphate
AV	Artificial vagina
BSA	Bovine serum albumin
BSPs	Bovine seminal plasma
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
Cm/h	Centimetres per hour
CR	Conception rates
DAPI	4′,6-diamidino-2-phenylindole
DHA	Docosahexaenoic
dl	Decilitre
DNA	Deoxyribonucleic acid
FAs	Fatty acids
FITC	Fluorescein isothiocyanate
FITC-PNA	Fluorescein isothiocyanate-peanut agglutinin
FN2	Fibronectin type 2

FRAP	Fluorescence recovery after photo bleaching
FSH	Follicle stimulating hormone
FT	Frozen-thawed
G6PD	Glucose-6-phosphate dehydrogenase
GPX	Glutathione peroxidase
GSSG	Oxidized GSH
H_2O_2	Hydrogen peroxide
HO	hydroxyl radical
HSP	Heat shock protein
HSP100	Heat shock protein H and
HSP60	Heat shock protein 60 D
HSP70	Heat shock protein 70 A
HSP90	Heat shock protein 90 C
HSPF1	Heat shock proteins factor 1
kDa	Kilo Dalton
KG	Kilo gram
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
LSD	Least significant difference
MDA	Malondialdehyde
ml	Millilitre
mM	Milli moles
mRNA	messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue Tetrazolium
NO	Nitric oxide
NOS	Nitrous oxide
NOX5	Nicotinamide adenine dinucleotide phosphate oxidase
^oC	Celsius scale

OD	Optical density
OHCL	Peroxyl radical
OS	Oxidative stress
P	Probability
PBS	Phosphate buffered saline
pg/ml	Picograms Per Millilitre
PGDs	Prostaglandin-D
PI	Propidium iodide
PKA	Protein kinase-A
PT	Post-thaw
PUFAs	Polyunsaturated fatty acids
r	Correlation
$R^{\bullet}$	Lipid-Free Radicals
REML	Restricted maximum likelihood
RH	Fatty Acid
RNA	Ribonucleic acid
RNE	Residual nuclear envelope
RNS	Nitrogen species
$RO^{\bullet}$	Alkoxy Radical
ROOH	Lipid Hydroperoxide
$ROOO'^{\bullet}$	Peroxyl Free Radical
ROOR	Non-Radical
ROS	Reactive oxygen species
RSP	Ram seminal plasma
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SED	Standard Error of Differences of Means
SH	Thiol group
SOD	Superoxide dismutase
SP	Seminal plasma

SPP	Seminal plasma proteins
TBARS	Thiobarbituric acid-reactive species
TCAI	Trans-cervical artificial insemination
TCG	Tris-citrate-glucose
TEMED	Tetramethylethylenediamine
WHO	World Health Organization
WSP	Whole seminal plasma
$^{\cdot\bullet}O_2$	Superoxide anion
µl	Microliter
µM	Micromole

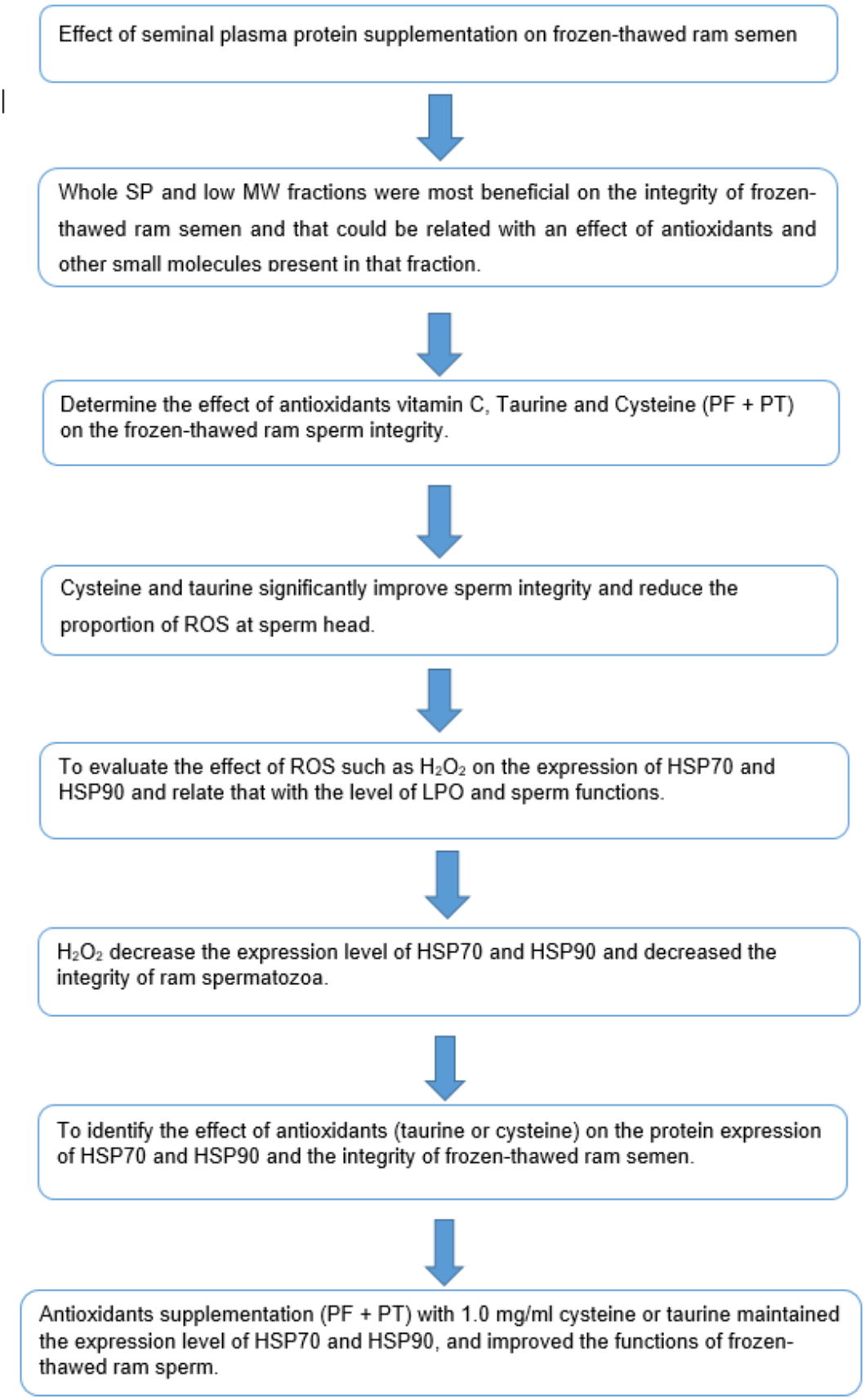


Figure 1. 1. Flow diagram demonstrating the experimental structure and main findings of the PhD thesis.

Chapter 1. Literature review

1.0. General introduction

Artificial insemination (AI) is a reproductive technology in which sperm cells are manually collected and deposited into the female reproductive tract (Evans and Maxwell, 1987). Artificial insemination increases the rate of genetic gain, facilitates cryopreservation of semen, allows easier transport, reduces the need for male animals on the farm, allows out-of-season breeding when used with oestrus synchronization technologies and provides better disease control (Cseh *et al.,* 2012). There are several methods of AI used in the sheep industry such as vaginal, cervical, laparoscopic intrauterine and transcervical insemination (TCAI). Some methods are more widely used than others.

Damage of the sperm membrane during the process of cryopreservation limits the use of frozen-thawed (FT) semen in sheep (Leahy *et al.,* 2009). Previous studies have focussed on mechanisms to induce cervical relaxation to allow the insertion of an inseminating pipette through the cervical canal. However, success has been restricted (Leethongdee, 2010).

Recent research has proposed that the interaction between seminal plasma (SP) and sperm is critical for the transit of sperm through the cervical canal (Pini *et al.,* 2016). This research exposes SP as a medium to increase spermatozoal survival because SP contains components which are essential for sperm function, metabolism, and transportation in the female tract (Juyena and Stelletta, 2012). Mammalians have two main families of SP proteins;spermadhesins and those that comprise fibronectin type-II domains (Bergeron *et al.,* 2005). These proteins seem to contribute to capacitation, improve acrosome integrity with the motility of frozen-thawed ram sperm and assist sperm-egg interaction which leads to improved fertility rates of cervical AI in ewes (Martínez-Rodríguez *et al.,* 2012). Whilst SP is known to aid sperm passage in the female reproductive tract, the role of numerous SP proteins in sperm survival and fertilizing ability is unknown. Despite advanced and improved cryopreservation protocols, the freeze-thaw process renders a large proportion of FT sperm incapable of fertilizing an oocyte through sub-lethal damage, primarily in the form of cryocapacitation and oxidative stress.

In many species, including sheep, attack of spermatozoa by free radicals causes lipid peroxidation (LPO) of the plasma membrane and disrupts DNA stability which consequently causes a loss of sperm function and integrity (Ferrusola *et al.,* 2009; Aitken *et al.,* 2009). Both a deficiency in protective enzymes and cytoplasm loss during spermiogenesis leave spermatozoa vulnerable to oxidation of polyunsaturated fatty acids in the plasma membrane resulting in peroxidative damage. Polyunsaturated fatty acids (PUFAs) in the sperm membrane (Wathes *et al.,* 2007), provide the plasmalemma with the flexibility that the

spermatozoa requires to contribute to the membrane combination events related to fertilization. However, these materials are also susceptible to attack by reactive oxygen species (ROS), with consequent loss of membrane functions, damaged cell integrity and reduced sperm motility (Lenzi *et al.,* 2002; Bucak *et al.,* 2007). From reducing the level of ROS and increasing the survival of sperm to promoting motility, various antioxidants have been recognized as beneficial in protecting male fertility (Sinclair, 2000). The oxidative stress induced by ROS has a detrimental effect on all components of the cell such as lipids, proteins, sugars and nucleic acid (Agarwal *et al.,* 2008). Antioxidants are compounds which block the harmful effects of ROS and decrease oxidative stress, thus enhancing sperm motility, viability, capacitation and the acrosome reaction (Bansal and Bilaspuri, 2008).

The antioxidant defence system in mammalian sperm is comprised of enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione reductase (GR) and catalase. Non-enzymatic antioxidants such as α-tocopherol, ascorbic acid, and methionine also play a role in defending cells against oxidative damage (Aitken, 1995; Bucak *et al.,* 2012).

However, these endogenous defense mechanisms are limited, especially given that sperm have a limited ability to biosynthesise these molecules (Aitken, 1995), and that their concentration is reduced during semen dilution. Therefore, the supplementation of antioxidants such as vitamin C, taurine, and cysteine, even in small concentrations, can enhance sperm function during cryopreservation (Allai *et al.,* 2018). These types of antioxidants have high potency as ROS scavengers with high efficiency as a water-soluble non-enzymatic antioxidant (Silva, 2006). Heat shock proteins (HSPs) are found in almost all species including bacteria, mammals and birds. HSPs are highly conserved proteins classified depending on their molecular weight (Bukau and Horwich, 1998). HSP synthesis increases in response to a sudden rise in temperature and to other stressors such as chemical and physical conditions (Welch, 1992). These proteins have a special ability to protect essential cell proteins from aggregation and denaturation. ATP-dependent chaperones such as HSP70 and HSP90 can bind unfolded or inadequately folded proteins and stimulate refolding using ATP hydrolysis as an energy source (Richter *et al.,* 2010). It has been reported that heat shock proteins HSP90 and HSP70 are molecular chaperones that collect in cytoplasm, endoplasmic reticulum, and mitochondria. These molecules have been documented to have a critical role in male reproduction, with roles as diverse as spermiogenesis, sperm-egg recognition, and reduction of apoptosis and elimination of remaining cytoplasm through sperm maturation (Redgrove *et al.,* 2012). It has been observed that these proteins may be involved in many physiological processes in the female tract, such as cell proliferation, regulation of cell death and survival, and differentiation during the involution process (Liman, 2017). Therefore, this thesis aimed to evaluate and

explore the effectiveness of SPP, antioxidants and HSPs on the integrity of frozen-thawed ram spermatozoa.

1.1. Sperm cell

1.1.1. Spermatogenesis

As shown in Figure 1.2, spermatozoa are produced from germ cells in the seminiferous tubules of the testes, where they develop from spermatogonia to spermatozoa (Evans and Maxwell, 1987). Spermatogenesis (production of spermatozoa) is a complex process which takes approximately 75 days from the start until a spermatozoa is produced into the lumen of the seminiferous tubules. Spermatogonia (type-A) are immature germ cells present within the seminiferous tubules close to the basement membrane. When these cells divide, one cell will develop into a new type-A spermatogonium, continuing the pool of immature germ cells. The other cell develops into a type-B spermatogonium; a cell dedicated to propagation that enters meiosis and splits into primary spermatocytes.

These primary spermatocytes undergo a meiotic division to produce secondary spermatocytes. A further meiotic division of these secondary spermatocytes results in the production of four round, haploid spermatids, which change to become elongated spermatids, and finally mature spermatozoa. The process of elongation and variation from round spermatids to spermatozoa is termed spermiogenesis. This process comprises nuclear condensation, acrosome formation, the development of the sperm tail, and a decrease of cytoplasm. Spermatozoa, when released from the sertoli cells, are transported over the rete testis to the epididymis – the site of maturity and increased motility. Spermatozoa reside in the epididymis for a mean of 10–14 days and are sometimes stored for about one month. After that, the cells are ejaculated or reabsorbed. Spermiogenesis and maturation in the epididymis are temperature sensitive processes (Bearden *et al.,* 2004).

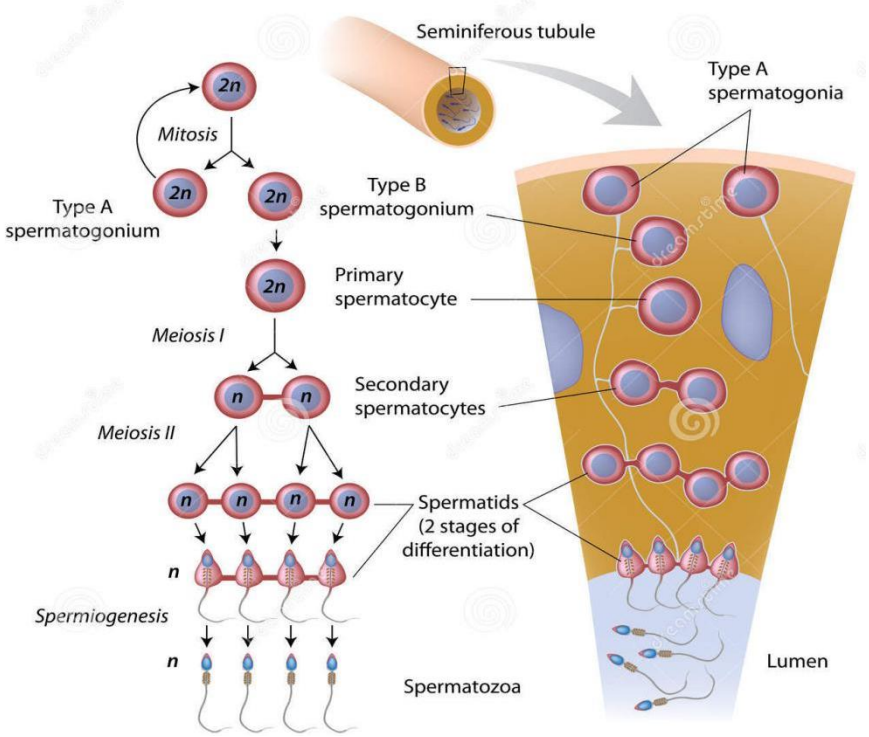


Figure 1. 2. Process of spermatogenesis (adapted from Cheng, 2018).

1.1.2. Sperm structure

Sperm have a specialized structure, and each sperm comprises three major parts named the head, the middle piece and the tail (Figure 1. 3). The sperm head includes the plasma membrane and the nucleus with chromosomes, which are responsible for the transport of genetic material. The frontal part of the head is covered by a specialized cap called the acrosome which contains enzymes responsible for the penetration of the oocyte membrane (Corselli and Talbot, 1987). Sperm plasma membranes are similar to other cell membranes which contain lipids and proteins including heat shock proteins (HSPs), a class of proteins that have the ability to protect cells from any stress including oxidative damage (Beere and Green, 2001). The middle piece is comprised of a fibrous sheath that contains mitochondria, which are the source of energy for tail movement to mobilise the sperm (Bearden *et al.,* 2004). Spermatozoa transit through the epididymis which is composed of three parts: the head (caput), the body (corpus) and the tail (cauda). The epididymis contains about 20 to 40 x 10^9 spermatozoa (Evans and Maxwell, 1987). During transit through the epididymis, spermatozoa gain the ability to fertilize oocytes. The epididymal spermatozoa transit time is approximately ten days with changes depending on the structure of spermatozoa and the surface of the plasma membrane (Parks and Graham, 1992). Another change is an increase

in the ratio of cholesterol to phospholipid which binds glycoproteins of various molecular weights to the sperm's surface (Silva and Smith, 2015).

Although not fully matured, epididymal spermatozoa are capable of fertilisation (insert citation here) and therefore the cryopreservation of epididymal spermatozoa is considered a suitable method to preserve the genetics of deceased and castrated males. The collection of caudal epididymal spermatozoa from slaughtered animals is considered an alternative cheap, rapid method compared with ejaculated samples (Ehling et al., 2006). In addition, if sperm collection cannot be completed immediately, it is possible to store ram epididymides at 5°C for up to 24 hours before collection and cryopreservation (Kaabi et al., 2003).

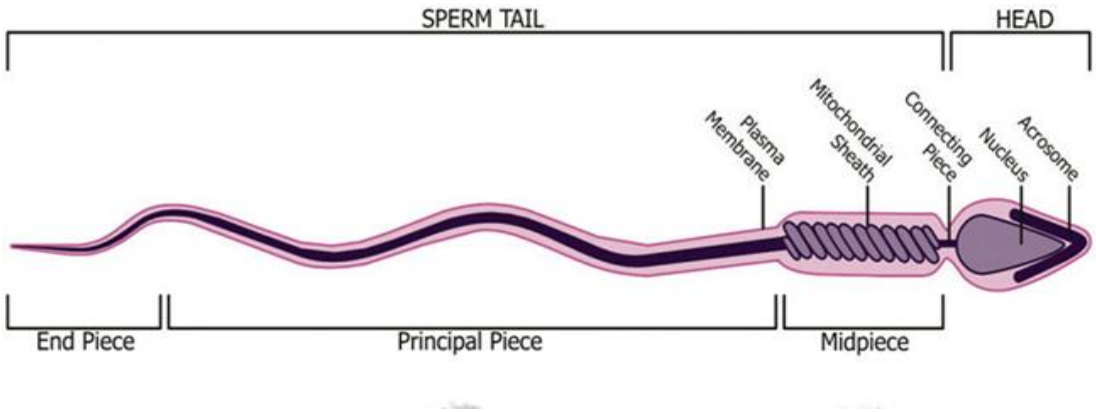


Figure 1. 3 The structure of the spermatozoa (adapted from Bearden *et al.,* 2004).

Ram semen is composed of spermatozoa and seminal plasma produced by the accessory sex glands (seminal vesicles, prostate gland, and bulbourethral gland) (Bearden *et al.*, 2004). The volume of ram semen is very limited, about 0.5 to 2 ml per ejaculate, with high concentrations of spermatozoa, at about 2-5 billion per ml (Evans and Maxwell, 1987). This small volume of semen in rams compared to other animals, such as bulls, which can produce up to 6 ml, is related to the presence in rams of a large vesicular gland and relatively small, or absent, prostate and bulbourethral glands. Semen volume in rams is also related to age, breed and season (Bearden *et al.,* 2004).

1.1.3. Changes in the proteomes of sperm

During epididymal transfer the lipid, protein and sugar composition of spermatozoa is altered. Considering that spermatozoa are translationally silent, it has been thought that proteins required for spermatozoa development in the epididymis are created by the epididymal epithelium before being transported to sperm cells where they are combined (Hintonand Palladino, 1995). The mechanism by which epididymal proteins are transported

to spermatozoa has not been fully elucidated. However, there are three possible hypotheses:

1. Proteins are transferred through the absorption of soluble proteins released into the epididymal fluid by epithelial cells (Cuasnicú *et al.,* 2002).
2. Proteins are secreted from the epithelium in exosomes or epididymosomes. Epididymosomes contain non-coding RNA, proteins and diverse groups of lipids, which are transported to the spermatozoa while they are transported through the epididymis (Sullivan and Saez, 2013; Belleannée, 2015; Sharma *et al.,* 2016).
3. High-resolution images show that interactions between spermatozoa and the apical surface of epididymal epithelial cells occur (Păunescu *et al.,* 2014). The authors consequently propose that these interactions result in the transfer of molecules from the epithelium to sperm cells.

Of these proposed mechanisms of protein transmission from the epididymal epithelial to sperm cells, epididymosomes have received increased attention in recent years. Epididymosomes are theorised to be the main mechanism of transporting molecules to maturing spermatozoa (Sullivan *et al.,* 2007). However, the complete mechanism by which epididymosomes successfully transport molecules to spermatozoa has not yet been revealed (Sullivan and Saez, 2013).

The epididymal epithelium includes several epithelial cell types comprising of principal cells, clear cells, narrow cells, and basal cells (Hermo and Robaire, 2002). These cell types have a particular structure and function that differs depending on their localization along the epididymis (Breton *et al.,* 2016). Additionally, these cell types interact to produce an epididymal lumen microenvironment which is conducive to the storage and maturation of spermatozoa (Shum *et al.,* 2011). Through the merocrine and apocrine mechanisms epididymal epithelial cells secrete proteins into the epididymal lumen (Hermo and Robaire, 2002).

Merocrine secretion is the process by which cells expel their secretions by fusing their cell membranes with those of Golgi-derived vesicles which results in secretion of vesicle contents into the extracellular space (Farkaš, 2015). Apocrine secretion is achieved by collecting free cytoplasmic molecules or vesicles in an apical protrusion of the cell membrane. This protrusion can bud off, releasing itself into the extracellular space (Farkaš, 2015). It is thought that epididymosomes are released by apocrine secretion from principal cells (Sullivan *et al.,* 2007). There is a high degree of heterogeneity in epididymosome content and size within different sections of the epididymis (Rejraji *et al.,* 2006), which could explain the difference in protein transmission to the spermatozoa.

It has been suggested that different proteins are transferred to spermatozoa in different sections of the epididymis (Sullivan and Saez, 2013). Some of these acquired proteins are associated with sperm function such as motility (Frenette *et al.,* 2004; Murta *et al.,* 2016), capacitation (Krapf *et al.,* 2012), acrosome reaction (Joshi *et al.,* 2013), the interaction between sperm and zona pellucida (Montfort *et al.,* 2002), and fertilization (Gibbs *et al.,* 2010; Caballero *et al.,* 2012). Since many of these functional changes are related to changes in surface glycans composition, it has been proposed that sperm maturation in the epididymis is linked to changing lipid homeostasis in spermatozoa.

Evidence for changes in lipid homeostasis has been described in spermatozoa (Saez, 2011). In most species, the cholesterol to phospholipid ratio changes between anatomic sections of the sperm with the highest levels found in the caput plasma membrane and the lowest in the cauda plasma membrane (Saez, 2011). This change in the cholesterol to phospholipid ratio increases the fluidity of the sperm plasma membrane evidenced by fluorescence recovery after photobleaching (FRAP) assays (Christova *et al.,* 2004).

Membrane fluidity is an essential determining factor in a mature sperm's ability to fulfil various actions during fertilization including the acrosome reaction and membrane fusion with the oocyte. Wathes *et al.* (2007) reported that cholesterol and phospholipid concentration affects the fluidity of the sperm membrane as these polyunsaturated fatty acids are more susceptible to oxidative stress. There is a shutdown of lipid synthesis within the sperm membrane after leaving the seminiferous tubules; however, sperm undergoes post-testicular alterations of lipid composition of their membrane through both maturation in the epididymis (Parks and Hammerstedt, 1985) and capacitation in the female reproductive tract (Evans *et al.,* 1980). The high ratio of cholesterol to phospholipid in the composition of lipid sperm membrane could play a significant role in capacitation and the acrosome reaction (Langlais and Roberts, 1985). A large portion of sperm plasma membrane lipids are not free to diffuse in somatic cell plasma membranes (Wolf *et al.,* 1988).

1.1.4. Lipids of sperm membrane

The sperm membrane is comprised of long chain polyunsaturated fatty acids (PUFAs) which contribute to the fluidity of the membrane fusion actions relating to fertilization (DuTeaux *et al.,* 2004; Macías *et al.,* 2011). There is a considerable quantity of PUFAs in the spermatozoa of most mammalian species (Macías García *et al.,* 2011). There are many types of PUFAs (Table 1.1) and specifically phospholipid in ram sperm (Table 1.2), which have an important role for sperm resistance to cold shock and membrane integrity, such as linolenic, docosahexaenoic (DHA), and eicosapentaenoic acids (EPA) (Estienne *et al.,* 2008). The high concentration of DHA in both ejaculate and during spermatozoa stages in the epididymis is positively correlated with the motility of human sperm (Zalata *et al.,* 1998).

Connor *et al.* (1998) reported that DHA could be involved in membrane fluidity essential for the motility of sperm tails. Spermatozoa from oligozoospermic men showed lower levels of DHA than those of normozoospermic men (Zalata *et al.,* 1998). Moreover, ram and bull spermatozoa use FAs as an endogenous metabolic fuel source (DuTeaux *et al.,* 2004). Lower oxidizable FAs in spermatozoa may contribute to a reduction in motility, and oxidation of phospholipid-bound DHA has been reported to be one of the primary reasons *in vitro* that reduce the duration of sperm motility (Aksoy *et al.,* 2006).

During cryopreservation, there is damage to sperm by peroxidation of the cell membrane that lead to decreases in membrane fluidity, loss of acrosome integrity and mitochondrial damage (Martin-Muñoz *et al.,* 2015). Consequently, these factors are related to reduced sperm motility, viability and fertilizing ability (Peña *et al.,* 2004; Bucak *et al.,* 2008; Aitken *et al.,* 2012). Several studies reported that lipid peroxidation of the sperm membrane in different species causes a reduction in the integrity of cooled or cryopreserved spermatozoa (Aurich, 2005; Aurich, 2016). The ability of sperm to resist the harmful effects of cryopreservation appears to be associated with the lipid composition of the sperm membrane (Ortega- Ferrusola *et al.,* 2009).

Table 1.1. Lipid components in human sperm cells (adapted from Alvarez and Storey, 1995).

Component	**nmol/10^8 cells**
Phospholipid	138.3
Saturated fatty acids	141.4
Unsaturated fatty acids	198.8
Cholesterol	133.0
Glycolipids	6.4

Table 1.2. Phospholipid composition of ram spermatozoa (µg/1.8 X10^9 sperm) before and after Freezing (adapted from Hinkovska-Galcheva *et al*., 1989).

Phospholipids	Pre-freeze	Post-thaw
Sphingomyelin	58	62.45
Phosphatidylcholine	159	153.73
Phosphatidylserine	8.80	9.13
Phosphatidylinositol	6.60	8.31
Phosphatidylethanolamine	49.09	41.18
Phosphatidylglycerol	15.50	14.05
Diphosphatidylglycerol	26.80	20.85
Cholesterol	0.769	0.630

1.2. Capacitation

Mammalian sperm are not instantly capable of fertilizing an oocyte after their deposition in the reproductive tract of the female and have to undergo a preparation period called capacitation (Parrish *et al.,* 1989). The mixing of de-capacitating factor (proteins in SP) with ejaculated spermatozoa reduces the ability of the sperm to fertilize (Dacheux and Paquignon,1980). The period of capacitation differs between species and ranges in sheep from 1-2 h (Austin, 1970). During capacitation there are biochemical and biophysical changes such as: changes in concentration of intracellular ions increasing calcium levels and metabolism (Yanagimachi, 1994); increases in the membrane fluidity (Harrison and Miller, 2000); increases in cyclic adenosine monophosphate (cAMP) concentration (White and Aitken, 1989); increases in the concentration of protein tyrosine phosphorylation (Visconti *et al.,* 1995) and increases of reactive oxygen species (ROS) concentration (Aitken, 1995). Capacitation and hyperactivation of spermatozoa are affected by amounts of ROS, which regulate protein tyrosine phosphorylation and are essential for adequate sperm function (Baker and Aitken, 2004). The fibrous sheath AKAP3 and flagella calcium binding protein are extremely tyrosine-phosphorylated during sperm capacitation leading to sperm hyper-activation (McCauley *et al.,* 1999). It has been reported that capacitation is considered as a prerequisite for stimulation of the acrosomal reaction in intact cells (Saling, 1989).

1.2.1. Main types of sperm proteins

Sperm proteins are classified into three groups:

1. Proteins associated with energy production via mitochondrial enzymes and glycolytic pathways. Previous studies of sperm protein expression profiles led to the designation of proteins which are related with the metabolism of sperm energy (McCauley *et al.,* 2001).
2. Composition proteins of thick outer fiber and A-kinase support proteins (AKAPs) in the flagella. Actin is considered a main cytoskeletal protein, and has important cellular functions, such as cell motility, organelle and vesicle movement, biological processes, and cell signalling, with the formation and repair of cell structure and cell shape (Miao *et al.,* 1998). Actin polymerization could work as an essential regulative pathway that is connected to tyrosine phosphorylation in sperm (Mohanarao and Atreja, 2011). Therefore, restructuring of the actin cytoskeleton prompted by osmotic stress contributes to sublethal sperm flagellar and the decline of motility (Correa *et al.,* 2007).
3. Signal transducers proteins such as protein kinase-A (PKA) and serine-threonine-tyrosine kinase/phosphatases. These are believed to exert a significant role in development, maintenance and regulation of sperm motility by post-translational modifications of the proteins convoluted in the process.

   Cyclic adenosine monophosphate (cAMP) is driven by the signal of tyrosine phosphorylation during sperm capacitation, which is in turn controlled by the spermatozoa's redox status (De Lamirande and Gagnon, 1998). Tyrosine phosphorylation has been shown to increase ROS production in spermatozoa from several species including humans (Aitken *et al.,* 1998; Villegas *et al.,* 2003), bulls (O'Flaherty *et al.,* 2005), rats (Lewis and Aitken, 2001), mice (Ecroyd *et al.,* 2003), boars (Boerke *et al.,* 2013), and horses (Baumber *et al.,* 2003). An increased amount of thiol on certain sperm proteins has been linked to the capacitation process *in vivo* (O′Flaherty, 2015), with increasing thiol abundance thought to be a result of oxidative stress (Kralikova *et al.,* 2017). This could reflect a protective response controlled by an increase in nicotinamide adenine dinucleotide phosphate (NADPH) production, associated with capacitation, driven by the hexose monophosphate shunt (Urner and Sakkas, 2005).

It has been proposed that cAMP production is upstream of bicarbonate in tyrosine phosphorylation regulation and free radical generation. Bicarbonate deficiency prevents tyrosine phosphorylation and this can be reverse by supplementation of spermatozoa with cAMP(Ecroyd *et al.,* 2003). This observation is consistent with the concept that ROS has a

dual role in capacitation including reducing the action of tyrosine phosphatase and stimulating cAMP production (Figure 1.4).

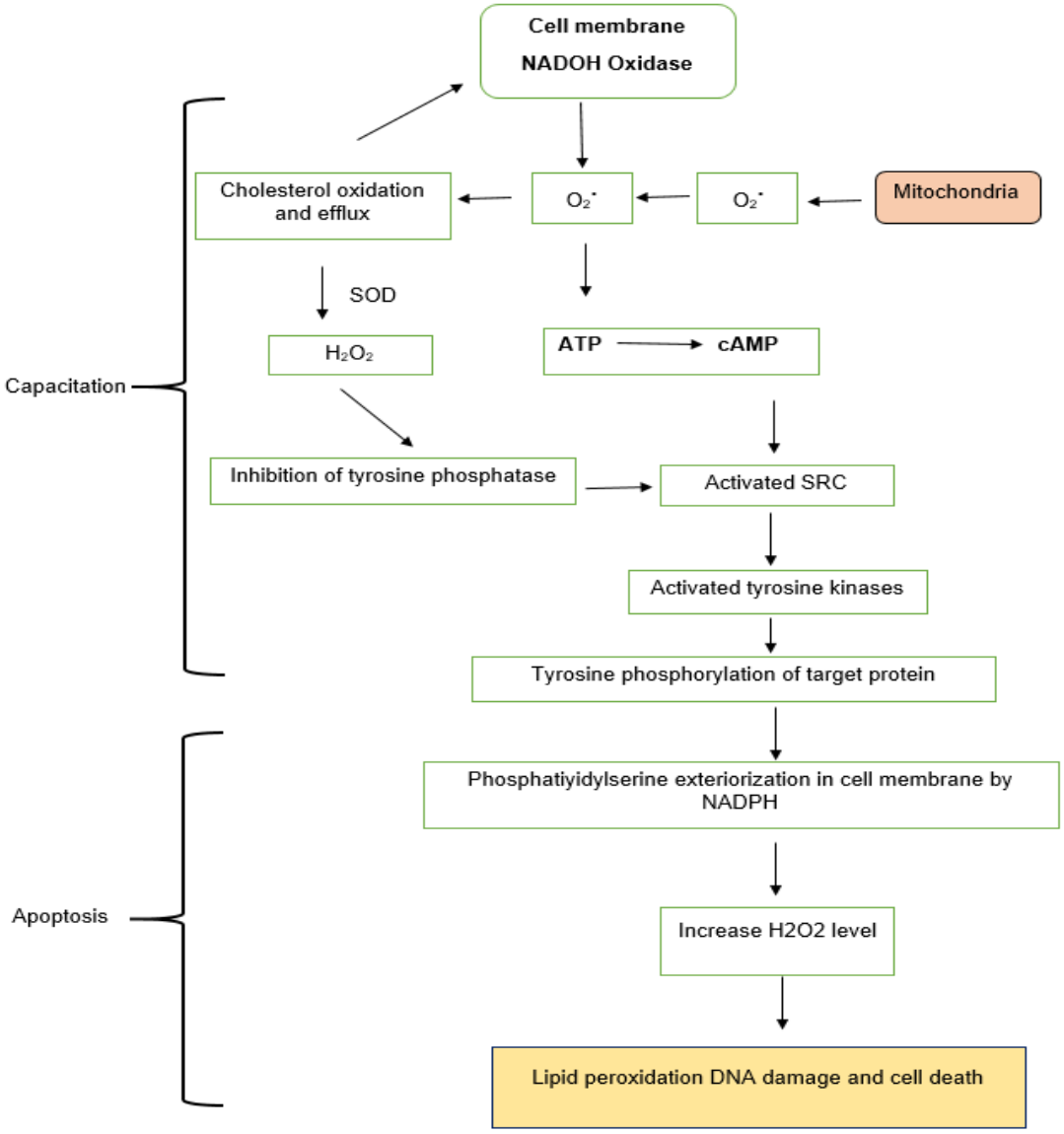


Figure 1. 4. The stages of capacitation process of sperm and the effect of oxidative stress on sperm cell (adapted from Urner and Sakkas, 2005)**.**

1.3. Acrosome reaction

The acrosome is a membrane-related organelle covering the frontal part of the head of the sperm. It contains the hydrolytic enzymes hyaluronidase and acrosin which are emitted by exocytosis through the normal acrosome reaction (Corselli and Talbot, 1987). The acrosome has an important role during interaction with the oocyte. Abou-Haila and Tulsiani (2000) reported that effective fertilization includes numerous serial steps. During fertilization, acrosome-intact sperm bind the plasma membrane, which is covering their head, to a glycoprotein in the zona pellucida, which is the extracellular coat of the ova. The

bound sperm then starts the acrosome reaction, which results in contact of the internal acrosomal membrane, increasing levels of free [Ca^{2+}] and pH, penetration of the zona pellucida by the sperm head, and combination with the ova plasma membrane and ultimately penetration of the ova. The acrosome-reacted sperm needs to remain bound to the ova, regardless of the loss of plasma membrane from the frontal region of the head and contact of the internal acrosomal membrane (Bleil *et al.,* 1988). The cryopreservation process impairs the integrity and role of the acrosome, nucleus and sperm plasma membrane (Üstüner *et al.,* 2015). Research related to the post-thaw acrosome defects has reported that the freeze-thaw process causes about 45–65% of the acrosome to be non-intact. This means the acrosome loses its ability to penetrate the zona pellucida of the oocyte (Soylu *et al.,* 2007).

1.4. Insemination process

Artificial insemination was pioneered by the Russian scientist Ilya Ivanov in the early 1900s (Salamon and Maxwell, 2000). This method is more widely used in cattle than in sheep and goats due to high fertility rates achieved in cattle and has been associated with increased production and economic benefits (Valergakis *et al.,* 2010). Methods of AI used in sheep are cervical, laparoscopic intrauterine, vaginal and transcervical insemination (TCAI) (Table 1. 3). Laparoscopic AI is the most effective and dependable technique, albeit one achieved through a surgical method under sedation with local anaesthesia of the abdominal wall to allow insertion of a laparoscope and insemination pipette into the abdominal cavity and insertion of a needle into the uterine wall for the placement of semen into each uterine horn (Evans and Maxwell, 1987).

Using the laparoscopic intrauterine method, fresh semen can produce pregnancy rates as high as 83% (Evans and Armstrong, 1984). Sanchez-Partida *et al.* (1997) reported that similar results could be achieved using frozen-thawed semen with pregnancy rates of about 70% to 75%, illustrating that, when deposited directly into the uterine horn, frozen-thawed semen achieves high fertilization rates. However, laparoscopic AI is costly, time-consuming, requires practical ability, restricts the number of ewes which can be used for breeding, and is not considered welfare friendly (Evans and Maxwell, 1987). Accordingly, laparoscopic intrauterine AI is not accepted as an appropriate method for routine use in several profitable sheep breeding programmes.

Cervical insemination is considered a less challenging technique in which semen is deposited into the cervix of the ewe through the vagina. Donovan *et al.* (2004) stated that the pregnancy rate with cervical AI with fresh semen was 76%. However, when using frozen-thawed semen, it was only 36%, which is not commercially viable. Therefore, the use of cervical AI with frozen-thawed semen is limited in sheep breeding programmes. It

has been illustrated that as the depth of insemination into the cervix increases, pregnancy (Halbert *et al.,* 1990) and lambing rates were improved (Salamon and Maxwell, 1995). However, when frozen-thawed semen is placed into the cervical os (the opening to the cervical lumen) lambing rates are on average 19% (Salamon and Maxwell, 2000).

The inseminating pipette is hardly inserted more than 1mm (Evans and Maxwell, 1987), and this is due to the convoluted structure of the ovine cervix, and the fact that the second and third cervical rings are often misaligned with the others (Kershaw *et al.,* 2005), preventing passage of the pipette (Leethongdee *et al.,* 2010). In addition, there is no ability to manipulate the cervix per rectum, as in bovine AI (Robinson *et al.,* 2011).

There is a progressive and positive correlation between the lambing rates and depth of penetration of insemination, superficial cervical 0.5-1.5 cm, midcervical 1.5-2.5 cm and intrauterine which are 39%, 47% and 61% respectively (Salamon and Maxwell, 1995) (Figure 1.5.) Therefore, an enhanced method of artificial insemination with frozen-thawed semen in ewes is TCAI in which the inseminating pipette is inserted through the cervix into the uterine cavity where semen is placed (Salamon and Maxwell, 2000). This method could overcome welfare, economic and practical constraints associated with laparoscopic AI. The great differences in the length of the cervix (3.7cm to 7.3cm) between breeds could explain the variation in the success of TCAI in ewes (Kershaw *et al.,* 2005). There have been many attempts to improve fertility using this technique (TCAI) in sheep such as an increase in the deposition numbers of spermatozoa deposition (Colas and Guerin, 1981), using hormones such as oxytocin and prostaglandin to increase relaxation of the cervix and contraction of the female reproductive tract (King *et al.,* 2004), and using a modified inseminating pipette (Buckrell *et al.,* 1994) Table 1.3.

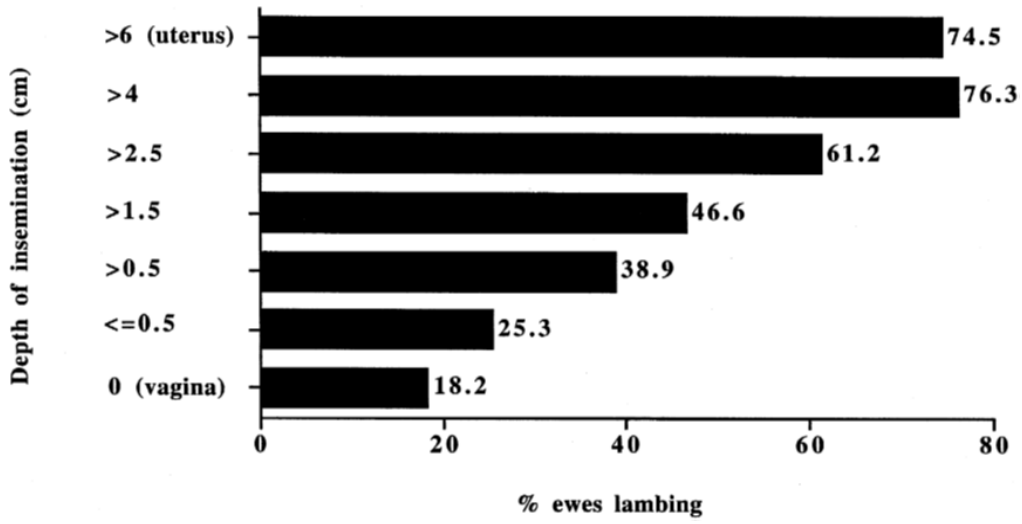


Figure 1. 5. Effect of depth of insemination on the lamping rates of frozen-thawed ram semen introduced through the cervix (adapted from Salamon and Maxwell, 2000).

Anatomically, the female reproductive tract of ewes has some factors that could decrease fertility rates. The cervix of the ewe is approximately 3-7cm in length with a diameter of 2-7mm. The lumen of the cervix is highly complex with the presence of 4-7 cervical rings, which act as a natural barrier to external pollutants (Fukui and Roberts, 1978)

Table 1.3. Comparison of conception rates (CR) in sheep using different methods of AI with fresh and frozen semen.

Method of AI	CR with fresh semen (%)	CR with frozen-thawed semen (%)	Volume and spermatozoa concentration of the inseminate	References
Vaginal	30-50	5-15	0.2ml at 400×10^6	Evans and Maxwell, 1987; Anel *et al.*, 2005.
Cervical	35-78	25-35	0.2ml at 200×10^6	Anel *et al.,* 2005.
Transcervical	35-75	30-70	0.5ml at 20×10^6	Candappa and Bartlewski, 2011.
Laparoscopic	65-95	60-80	0.5ml at 20×10^6	Salamon and Maxwell, 1995; Shipley *et al.,* 2007.

1.5. Seminal plasma

Seminal plasma (SP) has a complex and variable composition in different animal species. SP has different biochemical components which are secreted by the accessory sex glands of males (Mann and Lutwak-Mann, 1981). The three accessory sex glands are the seminal vesicles, prostate, and the bulbourethral gland, all of which produce SP proteins at ejaculation (Metafora *et al.,* 1989).

The main components of the secretion of the accessory sex glands are fructose, prostatic sulphates, citric acid, phosphor choline and proteins (Juyena and Stelletta, 2012). Artificial insemination (AI) using cryopreserved semen in which SP has been either diluted or removed so reducing the sperm concentration, leads to a decrease in fertility rates compared to natural insemination (Tummaruk *et al.,* 2000). SP components are involved in sperm maturation, fertilization, and embryo development in the female genital tract by maintaining osmolality and metabolism of sperm (Evans and Maxwell, 1987). Sheep and goats have a greater match of SP composition than with other, more distantly related,

species like pigs and horses. While differences between species could be explained by phylogenetic relationships, they may also be a result of different reproductive strategies. Research in ruminant species has confirmed that there is a similar reproductive procedure of a low volume of semen with a high concentration of spermatozoa deposited in the vagina. This differs from the boar and stallion, which deposit a high volume of semen, with a consequently reduced sperm concentration directly into the uterus. Therefore, differences in the requirement for spermatozoa to interact with the cervix during cervical migration may have resulted in protein variations between the species (Druart and de Graaf, 2018). While there are exceptions in some species such as dogs and camelids, the main composition of seminal plasma in other mammalian species is related to seminal vesicles and prostatic secretions with 2000 such proteins identified in humans (Rolland *et al.,* 2012; Milardi *et al.,* 2013; Gilany *et al.,* 2015).

Bergeron *et al.* (2005) reported that the main component of ram SP are proteins including four fibronectin type 2 (FN-2) proteins namely RSP (ram seminal protein) 15, RSP16, RSP 22 and RSP 24 KDa. Muiño-Blanco *et al.* (2008) reported that the main effect of SP in repairing and protecting sperm membranes from cold shock is due to the proteinaceous components. Table 1.4 shows the main types of ram SP proteins and their corresponding functions.

Table 1. 4. The main types of ram seminal plasma proteins.

Protein name	Function	References
RSP 14kDa, 12-16kDa, RSP 20kDa, 21.5kDa 29kDa, 31kDa, 72.45kDa, 80kDa, TIMP2, GPX5, PGDS and BSP5.	To protect the membrane integrity of spermatozoa from cold shock, to stabilize the sperm plasma membrane and involvement in gamete interaction.	Bergeron *et al.,* 2005; Domínguez *et al.,* 2008; Bernardini *et al.,* 2011; Goularte *et al.,* 2014; Souza *et al.,* 2012.
RSP 17kDa.	To improve motility of sperm.	Gatti *et al.,* 2000.
15, 16, 19, 25, 36, 41 and 46kDa	To prompt cholesterol efflux of the sperm membrane.	Leahy *et al.,* 2010; Mehr and Sharafi, 2013.
RSP 13kDa, 72.45kDa	To improve parameters of sperm metabolism and protect sperm membranes from oxidative damage.	Schöneck *et al.,* 1996; Yue *et al.,* 2009.
17and23kDa, Epididymal secretory protein1	To control proteins of the epididymis in the ram and directly integrate into specific domains of the sperm plasma membrane.	Dacheux *et al.,* 2016; Moura *et al.,* 2010.
Bodhesin-2, TIMP2, RSVP14, BSP5, GPX5, and PGDS.	To assist stabilization of the acrosome or contact between spermatozoa and the oviduct or oocyte.	Souza *et al.,* 2012.
lacto transferrin	Stabilising sperm membranes	Sylvester *et al.,* 1994

1.5.1. Function of Seminal Plasma (SP)

Several studies report the effect of SP on sperm function in numerous species, both in fresh and cryopreserved semen, demonstrating the adverse and beneficial results according to the species.

Through the physiological processes of the reproductive system, there is an interaction, for a short period, between spermatozoa and SP. Consequently, it would be valid to assume that proteins with a beneficial effect on sperm function must enact these effects in the shortest amount of time possible. Therefore, a prolonged interaction between undiluted SP and spermatozoa, as occurs in unprocessed semen, would cause the functional ability of spermatozoa to quickly deteriorate. Conversely, sperm harvested from the epididymis can be maintained in undiluted epididymal fluid for 4 hours while maintaining their ability to fertilize ova (Forouzanfar *et al.,* 2010; Bergstein-Galan *et al.,* 2017). Semen dilution, and consequent dilution of SP reduces the negative effect of SP. Instead, the presence SP in a final concentration which differs between species is often found to be beneficial for semen preservation under a variety of temperature conditions (Maxwell *et al.,* 2007; Kershaw-Young and Maxwell, 2011; Mata-Campuzano *et al.,* 2015). However, different studies examining the effectiveness of SP in semen preservation have produced inconsistent results (Leahy and de Graaf, 2012). These inconsistencies may be explained by individual variation in SP composition, such as small molecules, proteins or ions, between ejaculates or individual males. The effect of SP on ram spermatozoa resistance to cryopreservation has been shown to be related to the source of the SP (Rickard *et al.,* 2016).

The influence of ram SP on the functional integrity and motility of ram spermatozoa has received considerable attention. The main function of SP in natural insemination is associated with spermatozoa and subsequent survival in the female reproductive tract (Evans and Maxwell, 1987). It also has many functions such as maintaining motility, inhibition of premature activation through physical transport of spermatozoa with plasma membrane stabilization and inhibition of capacitation. It provides the best osmotic and nutrient medium (Villemure *et al.,* 2003), and protects spermatozoa from phagocytosis (Alghamdi *et al.,* 2004). It supports the interactions of sperm-ovum (Souza *et al.,* 2008; Leahy and Gadella, 2011), and stores sperm in the female reproductive tract (Talevi and Gualtieri, 2010). SP improved fertility (from 28% without SP to 51% with SP) after cervical insemination using frozen-thawed semen (Maxwell *et al.,* 1999). The role of SP proteins has been divided into influence, in the form of either dilution or cryopreservation. This provides a suitable interaction related to capacitation and acrosome reaction, to allow oocyte penetration (Maxwell et al., 2007b).

SP increases the ability of sperm to penetrate the cervical mucus by increasing the motility and velocity of ram spermatozoa (Robayo *et al.,* 2008). However, some research has reported that the SP increased spermatozoa penetration of cervical mucus without increasing motility or velocity (Rickard *et al.,* 2014). Another function of ram SP proteins is the protection of the sperm membrane through *in vitro* processing of spermatozoa due to biophysical and biochemical properties of the proteins (Leahy and de Graaf, 2012). SP has an important role in increasing fertilization rates. It was confirmed *in vivo* that addition of SP to the epididymal spermatozoa improved pregnancy rates (from 3% without SP to 17% with SP) after cervical insemination (Rickard *et al.,* 2014). Obviously, knowledge of these inhibitory and stimulatory functions of SP could lead to better sperm handling technologies and identify the sperm protecting factors. Additionally, Juyena and Stelletta (2012) stated that the variances in concentrations of some elements in SP might be due to differences in exposure from feeding, detection methods, management, the metabolic activity of spermatozoa suspended in SP, and the action of enzymes present in SP. Values of the most significant elements of ram SP are given in Table 1.5.

The addition of SP before cryopreservation protects spermatozoa from the damage induced by cold shock with improved motility percentage of 55% eight hours post-thaw, compared with the control which was 35% (Leahy *et al.,* 2010), and maintained the functionality of mitochondria in cryopreserved ram sperm (Del Valle *et al.,* 2017). There are many types of SP proteins (as mentioned in Table 1.4) that have an important role in decreasing the effect of cryopreserving spermatozoa and which could be due to their antioxidant capacity (Muiño-Blanco *et al.,* 2008). However, they reduced the acrosome reaction of the sperm membrane to approximately 35% through *in vitro* capacitation (Barrios *et al.,* 2005). The understanding of the mechanisms of sperm interactions with SP proteins may provide the means to regulate cryoinjury, which in turn might aid the formulation of improved diluents for cryopreservation of spermatozoa for utilization in reproductive technologies.

Recently, Paul *et al.* (2017) found that antioxidants reduced lipid peroxidation (LPO) in spermatozoa and maintained sperm motility and plasma membrane integrity. They have an important role in the protection of proteins that bind to the plasma membrane at 48 and 72 hours of storage. Pini *et al.* (2018) reported that bovine SP BSP1 and BSP5 in the ram have the ability to prevent alterations that are associated with freezing in membrane lipid disorder. These results propose that BSPs could significantly improve freezing consequences of ram spermatozoa (Figure 1.6).

Table 1.5. Components of ram seminal plasma (values are mg/dL).

Content	Ram
Total proteins, g/dL	2.30–2.50
Total lipids	254–396
Testosterone, pg/mL	25–375
Prostaglandins, ng/mL	500–20 000
ALP	14 895–40 818 mU/mL
AST	190–256 mU/mL
ALT	39–148 mU/mL
LDH	968–1697 mU/mL
Citric acid	110–260
Fructose	150–600
Glucose	0.9–1.6
Glutamic acid	4.5–5.2
Na	120–258
P	4.8–12.0
Cl	86
Mg	2–13
Zn	56–179
K	50–140

AL, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase (Adapted from Juyena and Stelletta, 2012).

1.6. Cryopreservation

Cryopreservation of spermatozoa is one of the most widely used and influential biological methods in animal reproduction. It allows the storage of semen for long periods at -196 ºC using liquid nitrogen, and effective distribution of the highest quality genetics (Aitken *et al.,* 2015).

Cryopreservation induces cryo-capacitation in which spermatozoa exhibit a capacitated like state, similar to that observed prior to fertilisation (see section 1.2). These changes in the features of capacitation which are present in the motile spermatozoa reduce the life span and capacity of frozen-thawed sperm, to interact with the female tract and therefore decreases fertilization (Medeiros *et al.,* 2002). Cryopreservation is a biotechnology method that has been utilized to preserve germplasm and improve genetics in different fields such as domestic species, aquaculture, agriculture, and conservation of threatened species, with practical and economic benefits (Holt, 2000; Andrabi and Maxwell, 2007; Gibbons *et al.,* 2011). It has been reported that, although the fertility of cryopreserved ram semen has not improved over a 27 year period, it could possibly improve the preservation of genetic resources (Salamon and Maxwell, 2000; Barbas and Mascarenhas, 2009). Currently, there are developments in the banks of semen for rare domestic breeds of cattle, goat, sheep and pig as well as for non-domestic species (Hagedorn *et al.,* 1997). Cryopreservation is an important process for the management of genetic material. It has been calculated that storing 1000 doses of semen from 15 different males would be enough to safeguard the existence of one rare species of *Bovidae* (Comizzoli *et al.,* 2000). In modern biology, the cryopreservation of semen can be applied to a variety of situations including conservation and medicine.

1.6.1. Semen cryopreservation

1.6.1.1. Extenders

Semen extender is a liquid, which is used as a medium added to semen to preserve the fertilizing ability of sperm. It plays an important role in reducing their metabolism and toxic byproducts and protects the sperm cells from cold shock and osmotic stress during chilling and transport (Salamon and Maxwell, 1995). Semen extender, which is also used as a freezing extender, acts as a cryogenic preservative of sperm for long periods and also to increase the number of insemination doses from a ejaculate (Salamon and Maxwell, 1995).

In order to adequately protect sperm during semen preservation, semen extenders must fulfil several characteristics including a suitable osmolality and pH (with sufficient buffering capacity to maintain this) which help to reduce cryogenic damage to sperm cells (Salamon

and Maxwell, 2000). The semen extender must include a cryoprotectant capable to diffuse across the sperm plasma membrane and exert an intracellular effect (like dimethyl sulfoxide, glycerol or ethylene glycol), a non-permeating cryoprotectant (like egg yolk or milk), one or more sugars (lactose, glucose, saccharose, raffinose, or trehalose), a buffer (Test or Tris), salts (sodium citrate, citric acid) and antibiotics (streptomycin, penicillin) (Evans and Maxwell 1987).

In frozen semen extenders used for rams, the tris–glucose base is hypertonic. Dried skimmed milk diluents are commonly used as sugar sources (Evans and Maxwell, 1987). Mammalian semen has a pH of 7.2-7.8 with semen extenders maintaining a similar pH between 6.75 and 7. Since larger sugar molecules such as lactose, raffinose, sucrose, trehalose and dextran are unable to pass through the plasma membrane, their presence creates an osmotic gradient that results in the efflux of water from sperm cells. This cellular dehydration reduces the formation of intracellular ice.

Generally, monosaccharides have a superior cryoprotective effect to disaccharides when combined with Tris (Molinia *et al.,* 1994). Trehalose is widely used in ram and goat semen extender. Aboagla (2003) reported that the addition of high concentration trehalose to semen extender has the ability to protect post-thawed sperm parameters such as motility, acrosome integrity and thermal resistance. In addition, the research stated that trehalose results in a reduction in freeze-thaw damage to sperm cells due to an increased membrane fluidity before cryopreservation.

Many different types of extenders have been used to preserve mammalian spermatozoa, and they can be grouped in the sequence of their development and use (Salamon and Maxwell 2000). These extenders such as citrate-sugar based diluents, milk diluents, lactose-based diluents, saccharose based diluents, raffinose based diluents, and the main extender used which is Tris based diluents, are regularly used for freezing semen in rams, bulls, and bucks (Purdy, 2006). The concentration of Tris varies between species with ram spermatozoa able to tolerate a concentration of 250 to 400mM. It has been found that glucose is the best sugar to use in Tris medium, over fructose, raffinose and lactose (Salamon and Maxwell 2000). Fiser *et al.* (1987) showed that motility and acrosomal integrity post-thaw was best preserved by a Tris medium containing 2% egg yolk and with an osmolarity of 375 Osmol/kg. This extender was found to produce superior results to lactose-yolk and saccharose-lactose-yolk media extenders *in vitro*. An addition of 2% bovine serum albumin also enhanced protection of acrosome integrity. Reasonable fertility levels in sheep can be attained (30-70%) by using semen cryopreserved in Tris diluent for transcervical insemination (Candappa and Bartlewski, 2011). Purdy (2006) reported that

Tris based extenders used for ram and buck semen freezing generally comprise fructose or lactose, at lower concentrations compared to other extenders.

1.6.1.2. Cryopreservation methods

In general, two methods are used for cryopreservation: vitrification and slow freezing. Vitrification, a fast method of freezing designed to reduce cold shock, is generally unsuccessful with semen samples since the heat transmission properties of spermatozoa are inadequate to allow fast freezing (Arav *et al.,* 2002). It has been stated that the concentration of cryoprotectant such as glycerol causes damage to sperm integrity (Salamon and Maxwell, 2000). Slow freezing is a more successful method and relies on cryoprotectants, which reduce osmotic shock and chemical toxicity.

Generally, the cryopreservation method incorporates four steps: decreases the temperature, dehydrates cells, freezes the sample and thaws before use (Medeiros *et al.,* 2002). The decrease in temperature from body temperature to 4ºC results in a reduction of metabolic activity within spermatozoa and an increase in their life span. Cryopreservation temporarily inhibits cellular activity but allows them to resume regular functioning on thawing (Mazur, 1984).

The rapid cooling in domestic species from 30 to 0 ºC causes cell damage in some sperm cells known as ‘‘cold shock’’, although the degree to which cells suffer depends on the rate of cooling and the temperature interval (Gilmore *et al.,* 1998; Watson, 2000). Cold shock causes change in the selective permeability of sperm membranes to calcium, thus leading to excessive intracellular levels, which reduce motility and functions (Robertson *et al*, 1990). Watson (2000) stated that slow cooling rates of semen at 0.5–1ºC/min reduces the stresses on sperm membranes, and that could be related with the alterations in lipids bilayer and changed functional state of membranes. The permeability of the sperm membrane after cooling is increased and this may be a result of increased membrane leakiness, specific protein channels and the regulation of calcium involved in cell death. These alterations during cooling stimulate the fusion of the plasma and acrosomal membranes. Cold shock decreases the ability of water and solutes to permeate the cell membrane and damages the acrosomal membranes (Purdy 2006).The differences among species in the sensitivity of their sperm to cooling are mostly attributable to compositional variable of the sperm plasma membranes. The sensitivity of the plasma membrane to undergo lipidphase transitions during cooling is inversely related to the proportion of cholesterol present (Drobnis *et al*, 1993). Lower cholesterol levels are present in bull and ram sperm, which are considered to be sensitive to cooling, than in rabbit and human sperm, which are less susceptible. Moreover, the effectiveness of glycerol as a cryoprotectant is partially attributed to its ability

to prevent some of the phase transitions during cooling by increasing the water permeability and fluidity of sperm plasma membranes (Noiles *et al*, 1995).

The primary site of damage induced by cryopreservation is the sperm plasma membrane (Watson, 1995). It has been showed a important membrane permeabilization after sperm were exposed to a high salt concentration followed by restoration of osmotic equilibrium as would be generated during a freeze-thaw cycle induce enormous change in cell water volume, which present considerable mechanical stress on the cell membranes (Noiles *et al,*1995). Through the physiologic temperatures of bull sperm, freeze fracture electron micrographs of head plasma membranes show a random distribution of membrane proteins (de Leeuw *et al*, 1991). It has been reported that these ultrastructural changes are not fully correctable on rewarming, and cryopreserved sperm have reduced head size compared with unfrozen sperm, possibly reflecting permanent modification of membrane architecture (Gravance *et al*, 1998). Cryopreservation has a sever affect in composition and organization of the plasma membranes lipid in ram sperm (Hinkovska-Galcheva *et al*, 1989). Ultrastructural damage to membranes due to cryopreservation change these elements, predisposing sperm to gross morphologic defects like an abnormal acrosomes and may play as a reason in the poor fertility of cryopreserved sperm. Post cryopreservation, surviving sperm contain more intracellular calcium than before, reflecting impaired membrane selective permeability mechanisms (Bailey and Buhr, 1994). The elevated intracellular calcium levels of cryopreserved sperm and their reduced capacity to maintain normal concentrations of this cation (McLaughlin and Ford, 1994), may also partly explain the poorer fertility of sperm post-thaw. Moreover, Trinchero *et al.* (1990) determined that frozen-thawed sperm are more easily peroxidized than fresh sperm. Recent researches show that cryopreservation decreases the antioxidant defenses system of whole semen.

If the freezing process occurs too quickly, water will not have enough time to leave cells via osmosis, resulting in intracellular ice formation and permanent cell damage (Fiser and Fairfull, 1986; Aitken *et al.,* 1995). Most ice crystal creation is related to osmotic pressure alterations in the unfrozen solution (Medeiros *et al.,* 2002). Damage to sperm cells such as cytoskeletal impairment, cytoplasmic fracture and damage to genetic material can result from intracellular ice formation, osmotic pressure and chilling injuries during cryopreservation (Isachenko *et al.,* 2003)..

Consequently, freezing can induce biochemical, functional and ultrastructural changes which weaken sperm and reduce their survival through the female reproductive tract with a subsequent reduction in the fertility (Salamon and Maxwell, 2000). It has been reported that the ultrastructural damage to the mitochondrial sheath, axoneme and acrosome membranes is greater in ram sperm compared to bull spermatozoa (Salamon and Maxwell, 2000). The morphological integrity of frozen-thawed sperm is less preserved than its

motility. Biochemical alterations have been shown in the form of damage to lipoproteins and amino acids, reduction in phosphatase activity, decline in loosely bound cholesterol protein, decreases in potassium and increases in sodium content, reduction in activity of hyaluronidase and acrosin enzyme, damage to prostaglandins, decreases in ATP and ADP synthesis and reduction in acrosomal proteolytic activity (Salamon and Maxwell 1995). It has been stated that sperm sensitivity to cold shock is variable between species, with the greatest sensitivity in boar sperm, high sensitivity in ram, bull and stallion; slight sensitivity in dog and cat and least sensitivity in human, rabbit and rooster sperm (Watson, 2000). Therefore, the contents of cryopreservation media act to decrease the chemical and physical stresses resulting from cooling, freezing and thawing of sperm cells (Gao and Critser, 2000; Purdy, 2006).

Cryoprotectants are defined by their ability to penetrate the cell membrane and therefore whether they exert their effects intracellularly or extracellularly. Penetrating cryoprotectants, such as glycerol, ethylene glycol, dimethyl sulfoxide and propylene glycol cause the rearrangement of proteins and lipid membranes leading to a subsequent improvement in membrane fluidity. This increases spermatozoa resistance to cryopreservation by removing water from cells at low temperatures and so decreasing intracellular ice formation (Holt, 2000). Furthermore, penetrating cryoprotectants are diluents that increase the solubility of salts and sugars in the cryopreservation medium (Purdy, 2006). Who also stated that glycerol is commonly used as a cryoprotectant for ram semen during cryopreservation; however, it has a concentration-dependent toxic effect on spermatozoa. A cryoprotectant media with 4-6% glycerol obtained the best results with a freezing state of 10–100 °C/min (Byrne *et al.,* 2000; Anel *et al.,* 2003).

Non-penetrating cryoprotectants, such as egg yolk, trehalose, sucrose, non-fat skimmed milk, amino acids and dextran cannot cross the plasma membrane and must, therefore, exert their effects extracellularly (Aisen *et al.,* 2005). Non-penetrating cryoprotectants may exert their effect on the plasma membrane or by reducing the medium's freezing temperature so reducing the chance of extracellular ice formation (Amman, 1999; Kundu *et al.,* 2002). When preparing semen extenders, a complete extender including glycerol can be added to the semen after collection (the one-step method). Alternatively, extender without glycerol is mixed with semen before refrigeration. The second fraction of extender with glycerol is then added before semen freezing (the two-step method). Evans and Maxwell (1987) have suggested that one step addition of glycerol at 30°C is a useful and commonly used method for ram semen freezing. A short contact time of just 5-10s has been shown to provide effective cryoprotection for ram semen although a longer contact time of 0 to 5 minutes is recommended for bull and boar semen. This observation suggests that diffusion of glycerol across the sperm membrane is not required for glycerol to have a

protective effect. It has been stated that there was no effect on lambing rate when glycerol was removed from thawed semen by centrifugation or by dialysis (Salamon and Maxwell 2000).

Egg yolk is considered a widely used normal component in semen extenders, as it protects the sperm cell against cold shock during freezing and thawing. The active components of egg yolk are thought to be phospholipids, such as lecithin, and low density lipoproteins (Medeiros *et al.,* 2002; Purdy, 2006). Egg yolk has a greater effect on the cell membrane of bull rather than ram spermatozoa and is used in freezing ram semen with a concentration of 15–17% (Salamon and Maxwell, 2000). Commonly in domestic species, there is about 50% fewer motile frozen-thawed sperm than in the equivalent fresh sample; however, the dose of sperm per insemination varies between species. In ram frozen-thawed semen, approximately 40-60% of cells are motile, while only 20–30% remain biologically functional (Barbas and Mascarenhas, 2009). Therefore, it is worthwhile stating that despite all the research, frozen-thawed ram sperm motility and functionality is still impaired and that there are still advances that must be made to improve the integrity of frozen-thawed ram sperm and consequently improve fertility rates.

1.6.1.3. Use of frozen-thawed semen

In mammals, the use of a large number of spermatozoa for insemination is recommended to overcome decreased fertility due to the cryopreservation process (Watson 2000). Using frozen-thawed semen for cervical AI in sheep has been shown to reduce fertility rates to 21.8% (O' Meara *et al.,* 2008). Many factors may play a role in this, such as the effect of the freeze-thawing process, reduced passage, reduced viability and capacitation of sperm, embryonic mortality and ewe management such as heat detection and timing of insemination (Anel *et al.,* 2005; Aisen *et al.,* 2005). Intrauterine AI has been shown to be more effective than posterior cervical insemination in sheep. Maxwell (1986) showed that a dose of 200 million motile thawed spermatozoa per insemination was required to achieve a fertility rate of 50% with cervical insemination in ewes. Due to the reduced viability and function of live sperm, insemination with cryopreserved spermatozoa can be expected to result in reduced fertility rates in domestic species (Watson 2000; Salamon and Maxwell, 2000). Laparoscopic insemination has been shown to be the superior method of insemination with frozen-thawed semen in terms of fertility rates when compared to transcervical or cervical AI (Anel *et al.,* 2005; Fair *et al.,* 2005); however, this method involves the use of special and costly equipment and may result in reduced animal welfare (Paulenz *et al.,* 2005; Fair *et al.,* 2005). Barbas and Mascarenhas, (2009) stated that fertility

rates using transcervical AI ranged between 17.5% and 30.6% using frozen-thawed ram semen.

1.6.1.4. Effect of cryopreservation on sperm

Cryopreservation has four main influences on sperm:

1. It removes the coating of extracellular components and associated coating of lipids and proteins from the cryoprotective diluent (Bergeron *et al.,* 2005).

2. The decreased temperature leads to cross-phase separation of lipids and thus a lateral reorganisation of membrane components (Meyers, 2006), and reduction in the membrane and acrosomal integrity due to the formation of intracellular ice causing damage to the sperm membrane (Salamon and Maxwell, 1995).

3. The sperm surface permeability to cryoprotectants, water and ions is changed (Oldenhof *et al.,* 2010).

4. There is a weakening of the cell that decreases its capability to resist more stress (Guthrie and Welch, 2006).

Because of the stress caused by freezing, lipid peroxidation is stimulated on the membrane of the sperm. This allows SP to completely interact with sperm before exposure to cervical mucus (Maxwell *et al.,* 1999). The membrane of ram spermatozoa has a low ratio of cholesterol to phospholipids, and this causes it to become sensitive to cold shock and osmotic stress (Muiño-Blanco *et al.,* 2008). This damage, called the dilution effect, is caused by a decrease in SP concentration due to excessive dilution (Mann, 1964). This effect can be decreased by the addition of SP through delayed alterations in the membranes of frozen-thawed spermatozoa during thawing (Ghaoui *et al.,* 2007). Preservation methods of ram semen need to use diluents as they have a suitable osmolarity to protect sperm from cryogenic injury (Figure 1.6). Figure 1.6 Scanning electron microscopy shows the detrimental effect of cold shock on the sperm membrane (Fig 1.6.B) whereby the pixilated appearance illustrates membrane damage. This damage is partially reverted by the protective effect of SPP.When cells were incubated with 0.7 mg of seminal plasma proteins, approximately 65% of damaged spermatozoa showed the sperm plasma membrane surface restored to its original appearance (Fig 1.6C). Furthermore a beneficial effect of SPP was observed when sperm exposed to cold shock were incubated with low molecular weight SPP in that SPP fractions reversed the structural damage induced by the cold shock (Fig 1.6D), restoring the original appearance of the sperm plasma membrane surface. After

incubating sperm cell with 0.7 mg of low molecular weight SPP, approximately 50% of damaged spermatozoa showed repaired surface.

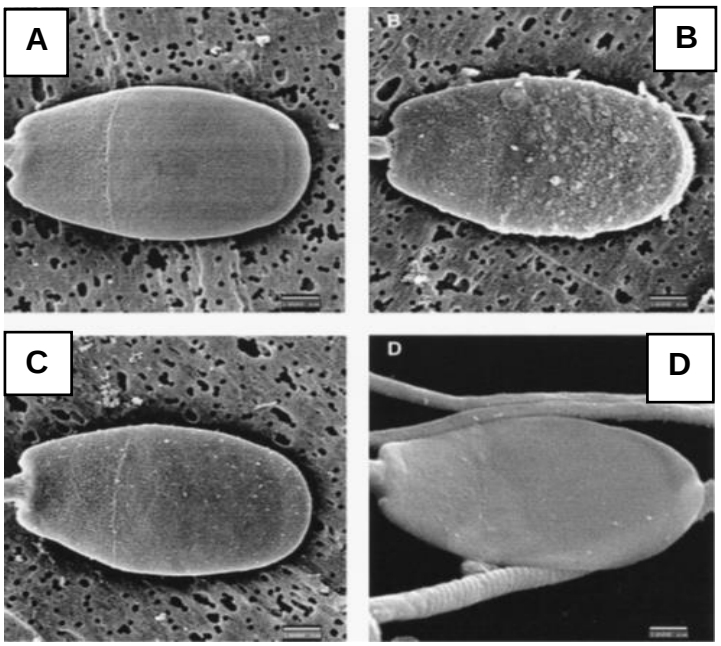


Figure 1. 6. Ram spermatozoa scanned by electron microscope (A) Gained by swim-up; (B) exposed to cold shock; (C) exposed to cold shock and incubated with 0.7 mg SP proteins; (D) exposed to cold shock and incubated with 0.7 mg of 20kDa (low molecular weight) SPP (adapted from Barrios *et al.,* 2000).

1.7. Free radicals

Free radicals are intermediates from chemical reactions, which have a short reactive life and comprise unpaired electrons (Sanocka and Kurpisz, 2004; Kefer *et al.,* 2009). They have a high ability to attack nearby molecules to gain an electron and damage the cellular structures (Agarwal *et al.,* 2008). This results in the oxidation of cellular components such as lipid membranes or amino acids in proteins (Ochsendorf, 1999). Free radicals are also involved in intracellular signalling processes essential for normal development such as cell division, differentiation and development (Aziz *et al.,* 2004; Ford, 2004). The free radicals have a dual purpose in that they can control various reproductive functions in the reproductive tract (Lampiao and Du Plessis, 2008). High production of free radicals commonly induces the release of spermatozoa with an abnormal level of cytoplasmic retention from the germ epithelium during spermiogenesis (Sanocka and Kurpisz, 2004). Free radicals include two main types: reactive oxygen species (ROS) and reactive nitrogen species (RNS). The best examples of ROS include hydrogen peroxide (H_2O_2), the superoxide anion (O_2), peroxyl radical (HO_2) and hydroxyl radical (OH) (Sikka, 2004), while

RNS, such as nitric oxide (NO), Nitrous oxide (N_2O), peroxynitrite (NO_3) and peroxynitrous (HNO_3), are considered to be a subclass of ROS (Sharma *et al.,* 2000; Agarwal, 2005).

1.7.1. Reactive oxygen species (ROS)

During the normal reactions of enzymes, ROS are formed as required by-products of inter- and intra-cellular signals. Mammalian spermatozoa can generate ROS such as the superoxide anion ($•O_2^-$), hydrogen peroxide (H_2O_2), hypochlorite radical (OHCl•), and the hydroxyl radical (OH•) during incubation in aerobic environments. ROS can rapidly associate with other molecules due to their extremely reactive nature and produce oxidation that leads to changes in function and structure, resulting in cellular damage (Guérin *et al.,* 2001; Agarwal, 2005).

However, the main mechanism by which ROS influences sperm capacitation has not yet been found. NADPH has the ability to stimulate tyrosine phosphorylation and sperm capacitation in humans (Aitken *et al.,* 1995), bulls (Rivlin *et al.,* 2004), horses (Baumber *et al.,* 2003) and rats (Lewis and Aitken, 2001) by stimulating redox reactions, which drive these processes. The presence of catalase can reduce NADPH's stimulatory effects since this enzyme breaks H_2O_2 down into water and oxygen (Rivlin *et al.,* 2004; Kefer *et al.,* 2009) (Figure 1.7).

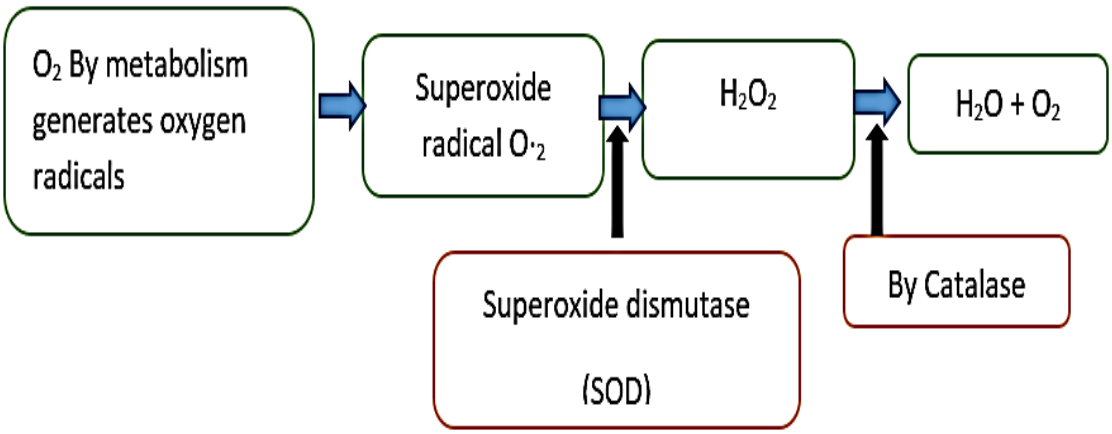


Figure 1. 7. The process of converting free radicals to H_2O and O_2 (adapted from Kefer et al., 2009).

1.7.1.1. Mechanisms for the regulation of ROS in spermatozoa

There are five important aspects of ROS regulation (production and inhibition) in sperm cells:

1. Glucose is required for the tyrosine phosphorylation cascade which is related to sperm capacitation and ROS production in human and murine spermatozoa (Niwa and Iritani, 1978; Hung and Suarez, 2012).
2. The presence of 2-deoxyglucose, a non-metabolizable glucose analogue, blocks ROS creation and tyrosine phosphorylation during capacitation. This effect can be inverted by adding NADPH (Aitken *et al.,* 1998; Urner and Sakkas, 2005).
3. Sperm capacitation can reduced hexose monophosphate shunts and this is reversed by the addition of NADPH (Urner and Sakkas, 2005).
4. The production of ROS and glucose-6-phosphate-dehydrogenase by spermatozoa are associated together (Baumber *et al.,* 2000).
5. ROS production in human spermatozoa is driven by NADPH oxidase (NOX5) (Musset *et al.,* 2012).

Another potential source of ROS in spermatozoa is L-amino-acid oxidase which is found in human (Houston *et al.,* 2015), ram (Upreti *et al.,* 1998), and horse spermatozoa (Aitken *et al.,* 2015). This enzyme contributes to oxidative deamination of aromatic amino acids like tryptophan, tyrosine and phenylalanine. Aitken (2017) reported that spermatozoa kept in a substrate containing phenylalanine produce higher ROS levels in live spermatozoa than dead spermatozoa.

This biochemical property of sperm has practical implications for cryopreservation, especially where egg yolk is used as an extender. If enough dead spermatozoa are present, central ROS production might be stimulated since egg yolk contains enough free aromatic amino acids to stimulate L-amino-acid oxidase (Aitken *et al.*, 2015a).

Since considerable numbers of non-viable spermatozoa are normally present in cryopreserved samples, their presence may be sufficient to induce oxidative stress of live spermatozoa through stimulation of L-amino-acid oxidase (Aitken *et al.,* 2015). Hereng *et al.* (2011) stated that glycolysis is a driving force for sperm capacitation in human spermatozoa, with the mitochondrial influence of this process (Stendardi *et al.,* 2011). However, glycolysis was found to be essential for monkey spermatozoa capacitation while oxidative phosphorylation was not required for hyperactivated sperm motility, and other sperm functions such as acrosome reaction and sperm-zona binding (Hung *et al.,* 2008). Equally, the presence of inhibitors of respiration, such as electron transfer inhibitors, or the absence of respiration substrates in cell storage media do not affect phosphorylation and subsequent sperm capacitation (Travis *et al.,* 2001). Conversely, evidence has been

presented to show that sperm mitochondria play a central role incapacitation of boar sperm, which, incidentally, rely heavily on oxidative phosphorylation for metabolic processes (Ramió-Lluch *et al.,* 2011). As can be seen above, there are many sites of ROS production in spermatozoa (Vernet *et al.,* 2001); however, there appears to be a wide species variation in the contribution of ROS from these sites and the requirement for ROS to stimulate capacitation (Aitken, 2017).

1.7.1.2. Negative effects of ROS

Excessive exposure to ROS has a number of deleterious effects including decreased motility and ability of sperm to fertilize. Additionally, the cold shock, which results from exposure to other stressors, influences membrane moulding and dynamics in both male and female gametes (Bucak and Tekin, 2007). The sperm membrane contains a high amount of polyunsaturated fatty acids (PUFAs). These molecules are sensitive to ROS attack and subsequent lipid peroxidation (Figure 1.8). ROS damage to the sperm membrane through this process can result in reduced sperm motility due to axonemal injury following the loss of intracellular ATP; decreased sperm viability and prevention of capacitation and acrosome reaction following morphological changes to the mid-sperm region (Bansal and Bilaspuri, 2008). Thus, ROS may be used as indicators of infertility (Bucak *et al.,* 2010).

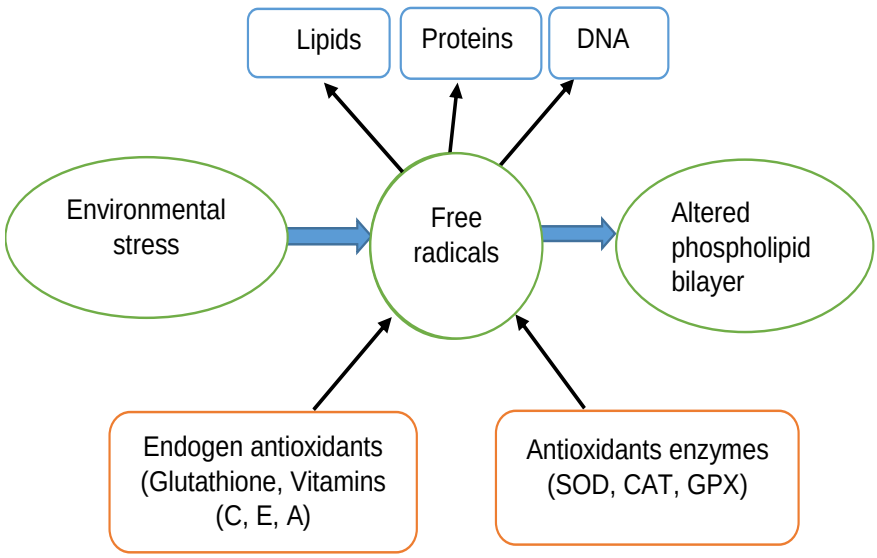


Figure 1. 8. Environmental heat stress which increase the production of free radicals and consequently antioxidants are organised to scavenge the produced radicals. When antioxidant system fails to maintain the stabilizing concentration of the oxidant species, nucleic acids, proteins, lipids, and sperm membranes are altered (adapted from Belhadj Slimen *et al.,* 2016).

1.7.1.3. Source of ROS in the male reproductive tract

ROS that eventually finish up in the seminal plasma and exert their effects on spermatozoa can be produced by various endogenous or exogenous sources. The endogenous sources of ROS start from semen which contains diverse types of cells such as immature and mature spermatozoa, epithelial cells and leukocytes such as neutrophils and macrophages (Hendin *et al.,* 1999). The seminal vesicles and prostate have the ability to make leukocytes peroxidase positive (Connor *et al.,* 1998), which produce high levels of ROS (Tremellen, 2008). Leukocyte production of ROS increases specifically during disease through the nicotinamide adenine dinucleotide phosphate (NADPH) system (Blake and Lunec,1987; Aitken and Baker, 2006), and decreases the levels of antioxidant superoxide dismutase (SOD) leading to ROS production (Blake and Lunec, 1987). These states of high levels of ROS due to infections in the epididymis and testis leads to a reduction in defence mechanisms by antioxidants and reduced sperm function (Ochsendorf, 1998; Cocuzza *et al.,* 2007), such as motility and fertilizing ability (Wolf *et al.,* 1990). It has been shown that the level of ROS is increased in immature spermatozoa during development (Whittington and Ford, 1998), and leukocytes produce 1000 times more ROS than human spermatozoa (Plante *et al.,* 1994). Spermatozoa produce ROS by two mechanisms: 1) through the plasma membrane via the NADPH oxidase system, 2) through the mitochondria by NADH oxidoreductase which is considered to be the main source of ROS in infertile men (Plante *et al.,* 1994). Amiri *et al.* (2007) stated that ROS levels increased with reduced semen parameters and increased DNA fragmentation by H_2O_2 in infertile men. In an *in vivo* study, it has been reported that antioxidant therapy reduced DNA damage of sperm and apoptosis and decreased the level of seminal oxidative stress (Agarwal *et al.,* 2008). In another study of antioxidants (such as taurine) were shown reduce mitochondrial damage and apoptosis of sperm (Aitken and De Iuliis, 2009). ROS also caused fragmentation and cross-linking of proteins with carbohydrate injury causing an alteration in external receptors (Potts *et al.,* 2000; Aitken, 2017; Saraswat *et al.,* 2014), and affected sperm maturation (Gangwar *et al.,* 2015) Figure 1.9.

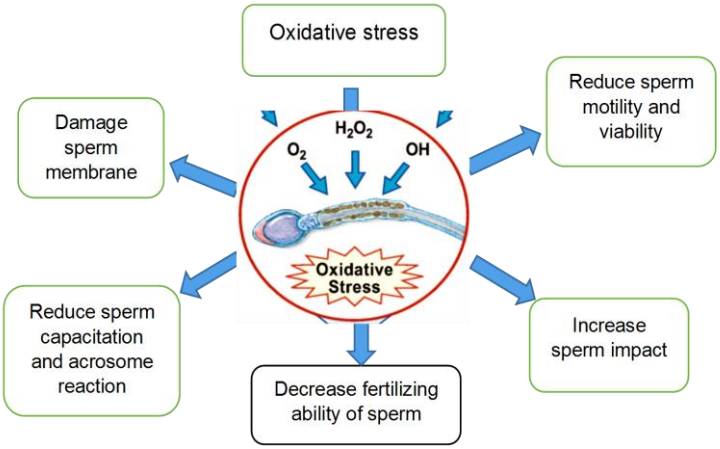


Figure 1. 9. The effect of ROS on sperm functions (adapted from Agarwal *et al.,* 2008).

1.7.2. The effect of lipid peroxidation on the sperm membrane

Lipid peroxides are naturally produced in the plasma membrane of sperm from where they are released by phospholipase A2. They have the ability to induce damage to DNA and reduce the fertility rate of stored semen (Aitken *et al.,* 1998). Peroxides are not just normally related to reduced sperm viability and functions, but they also increase the capability of spermatozoa to bind to homologous and heterologous elements of the zona pellucida (Aitken *et al.,* 1995).

LPO damage can be measured by measuring levels of malondialdehyde (MDA), a constant by-product of LPO (Agarwal, 2005; Aitken *et al.,* 1989) using the spectrophotometric thiobarbituric acid test. The amount of MDA is considered an indicator of the degree of decrease of sperm motility and sperm-oocyte fusion (Lampiao and Du Plessis, 2008). It has been reported that antioxidant defence mechanisms coupled with the tight packing of DNA in semen provide greater protection and resistance to DNA damage in sperm cells when compared to myoblastoid and germ cells (Fraczek and Kurpisz, 2007).

1.8. Antioxidants

Antioxidants are the compound that prevent the creation of ROS or oppose their actions. Thiol groups also act as a defence against oxidative stress in sperm cells by detoxifying and antioxidising ROS, so maintaining the intracellular redox status (Bansal and Bilaspuri, 2008). Previous studies have shown that inclusion of antioxidants in cryopreservation extenders increases factors such as motility and membrane integrity after thawing in human, ram, goat, bull, canine and boar sperm. Supplementation with antioxidants prior to the cryopreservation process may improve cryopreservation methods used in the sheep industry (Bucak *et al.,* 2010).

The antioxidant defence system in mammalian sperm cells is formed of a variety of enzymes including superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase as well as non-enzymatic components like α-tocopherol, methionine, and ascorbic acid (Mann and Lutwak-Mann, 1981; Kantola, 1988; Aitken *et al.,* 1995; Bucak *et al.,* 2012). The limited ability of sperm to biosynthesise (Aitken, 1995), coupled with the reduction in antioxidant concentrations present in the semen after dilution, reduces the constructive effect of these endogenous antioxidative defences. Therefore, even a small addition of antioxidants to cryopreservation media may have a positive effect on sperm function (Allai *et al.,* 2018).

1.8.1. Classification of antioxidants

1.8.1.1. Synthetic antioxidants

Synthetic antioxidants are chemical compounds that are produced with the aim of generating greatly effective antioxidants that remain stable under a varied range of pH, at a low cost and using simple production methods (Karre *et al.,* 2013). Previous research has observed the effect of adding different types of synthetic antioxidants at different concentrations to diluted semen prior to cryopreservation in order to determine the effects post-thawing. In addition, these compounds may be enzymatic or non-enzymatic (Table 1.6). The main parameters measured to determine the effect of these compounds are sperm integrity, motility, viability, acrosome integrity, penetrability and sperm morphology.

1.8.1.1.1. Enzymatic antioxidants

Enzymatic antioxidants also considered natural antioxidants which play an important role in preventing damaging to the structure of the cell by neutralizing the excess ROS. Enzymatic antioxidants include of glutathione peroxidase (GPx), catalase, superoxide dismutase (SOD), and glutathione reductase (GR) which reduce hydrogen peroxides to alcohol and water (Agarwal *et al.,* 2005). SOD catalyses the conversion of oxygen free radicals to hydrogen peroxide and oxygen (Sikka, 2004), as shown in the equation below:

$$2(O^{-}_{2}) + 2H \xrightarrow{\text{By SOD}} H_2O_2 + O_2,$$

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + 1/2\ O_2.$$

The SOD enzyme is present in ram sperm (Martí *et al.,* 2003; Kasimanickam *et al.,* 2006; Bucak *et al.,* 2008; Marti *et al.,* 2008) in the mitochondria (Mn-SOD) and in the cytoplasm (Cu, Zn - SOD) where it is responsible for facilitating the binding of two superoxide anion molecules (O_2^-) to form hydrogen peroxide (H_2O_2). Hydrogen peroxide can then be broken down to water and oxygen by catalase (CAT) and glutathione peroxidase (GSH Px) (Amidi *et al.,* 2016). The principal active enzyme in fresh semen is SOD, while levels of other antioxidant enzymes are relatively low (Marti *et al.,* 2008). However, Bucak *et al. (*2007) detected that trehalose supplementation did not affect the activity of GSH and GSH-Px of frozen-thawed ram semen. Silva *et al.* (2011) showed that SOD improved preservation of the mitochondria and conserved the integrity of the acrosome. Catalase (CAT) was previously used as an additive in semen extenders because it has the ability to preserve the functional integrity of spermatozoa (Maxwell and Stojanov, 1996; Upreti *et al.,* 1998; Maia *et al.,* 2010). It has been reported that 200 U/ml was considered a toxic dose to sperm. Glutathione peroxidase (GSH) is the most common enzymatic system that controls levels of cellular peroxide. In conjunction, glutathione (GSH) acts to convert hydrogen peroxide to

water as well as reducing lipoperoxides to less harmful alkyl alcohols (Amidi *et al.,* 2016). The oxidized GSH OR GSSG (Glutathione disulphide) can be reduced back to its original form by glutathione reductase (GSR), whose activity is induced by the presence of oxidative stress. Bucak *et al.* (2008) and Silva *et al.* (2011) found that the addition of GSH or GSSG to semen extender in frozen-thawed ram sperm resulted in increased GSH concentrations and GSH-Px activity; however, there was no effect on lipid peroxidation or sperm integrity. Antioxidant enzymes present in seminal plasma and produced by spermatozoa reduce oxidative stress (Chi *et al.*, 2008). This leads to increased motility and could be beneficial in terms of male infertility (Bansal and Bilaspuri, 2008). Antioxidants decrease oxidative stress by disrupting the oxidative chain reaction (Kumar and Liang, 2001).

1.8.1.1.2. Non-enzymatic antioxidants

Non-enzymatic antioxidants are the second type of synthetic antioxidants or dietary supplements such as vitamins (vitamin E, vitamin C) and minerals (zinc), and amino acids (glutathione, hypotaurine and taurine) (Agarwal, 2005). Antioxidants such as vitamin E have been proposed as the main chain breaking antioxidant as they are able to immediately reduce free radicals like peroxyl and alkoxyl which are created by ferrous ascorbate prompted LPO (Bansal and Bilaspuri, 2009) (Table 1.6).

Glutamine (5mM) has been shown to provide a cryoprotective effect through enhancing parameters such as membrane integrity, motility and CAT action in ram semen post-thawing (Bucak *et al.,* 2009).

Frozen-thawed sperm that were stored in an extender containing inositol had improved parameters such as motility, acrosome integrity, and intact morphological rates. Inositol acts as a molecule with antioxidative properties resulting in greater antioxidant GSH action (Bucak *et al.,* 2010). Numerous vitamins such as vitamin E, C, B12 have an important role in promoting sperm quality. Vitamin E (alpha-tocopherol or Trolox) is found in high concentrations in the sperm membrane making it a primary antioxidant (Aitken *et al.,* 1995). Vitamin E is lipophilic and as such plays a significant role in the sperm membrane protecting PUFAs from peroxidation (Halliwell, Barry, 2015). Different forms of vitamin E can improve sperm integrity when added to extender used to store and preserve ram semen (Kheradmand *et al.,* 2006; Amini Pour *et al.,* 2013; Azawi and Hussein, 2013).

Vitamin C (Ascorbic acid) has a vital role in maintaining sperm integrity by inhibiting oxidative damage to genetic material in sperm (Fraga *et al.,* 1991; Azawi and Hussein, 2013). Amidi *et al.* (2016) reported that ascorbic acid has a role as a pro-oxidative agent when transition metal ions are also present. Amino acids such as taurine, hypotaurine, cysteine, proline, glycine, glutamine and histidine are present with high concentrations in

SP, where they have an important role as non-enzymatic hunters with antioxidant features. Several studies have reported benefits in terms of post-thaw ram sperm integrity and fertility when extenders are supplemented with amino acids (Sanchez-Partida *et al.,*1997; Bucak *et al.,* 2013).

Cysteine has a low molecular weight and contains thiol groups, which participate in glutathione biosynthesis. Cysteine has a vital role in preventing lipid peroxidation and maintaining the integrity of post-thawed ram sperm. Previous studies found that cysteine and glutathione prevented membrane loss and protected the acrosome integrity of post-thawed ram sperm (Bucak, Mustafa Numan, 2008; Çoyan *et al.,* 2011; Sharafi *et al.,* 2015).

Taurine is an amino acid which is found in both epididymal and oviduct fluid. Previous studies found that it is one of the most important methods of free radical scavenging. Taurine has been shown to protect post-thawed ram sperm from the damaging effects of ROS on lipids (Rather *et al.,* 2016; Banday *et al.,* 2017) (Table 1.6).

1.8.1.2. Natural antioxidants

Natural antioxidants are compounds that are obtained from natural sources such as herbs, seeds, vegetables, spices and fruits. These natural antioxidants are often phenolic compounds or vitamins that have an appropriate molecular structure allowing them to chemically scavenge free radicals (Velasco and Williams, 2011; Liang *et al.,* 2012). In plants, phenolic compounds have numerous biological roles such as antioxidants, anti-bacterial and anti-inflammatories (Manthey and Grohmann, 2001). Antioxidants obtained from natural sources such as green tea and rosemary have the ability to enhance sperm integrity, which minimizes the damage that occurs during storage (Table 1.6).

1.8.2. Mechanism of action of antioxidants

Antioxidants inhibit oxidation through three different mechanisms: scavenging of free radicals, chelating metals, and reducing singlet oxygen (Reische *et al.,* 2002). Through the initiation and propagation stages, antioxidant substances can reduce lipid oxidation by interacting with free radicals to produce constant and non-free-radical products (Huang *et al.,* 2005). Reische *et al.* (2002) stated that antioxidants provide hydrogen molecules to the peroxy- and oxy-free radicals, which may be formed through the autoxidation circulation step. Additionally, through the initiation stage, the lipid radicals are converted by antioxidants into non-radical products as shown in Figure 1.10.

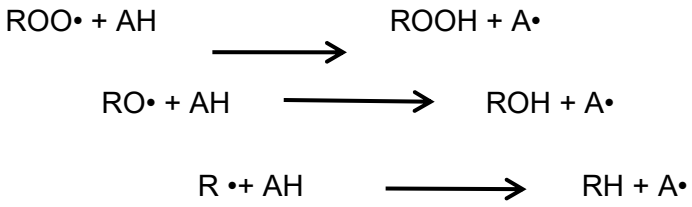


Figure 1. 10. Summary of chemical reactions between antioxidants and free radicals controlling lipid oxidation. ROO• - peroxyl free radical, AH - antioxidant, ROOH - lipid hydroperoxide, A• - antioxidant radical, RO• - alkoxy radical, R• - lipid free radicals, RH - fatty acid (adapted from Reische *et al.,* 2002).

Antioxidants may also act by chelating metals, that can occur in different oxidation phases, and the additional oxidised stages can cause lipid oxidation by a redox-based in which the metal is decreased, and the lipid transform is oxidised (Decker, 2002). Lipid peroxidation can be prevented by antioxidants through reduction of the metal's redox potential and avoiding the interaction between metals and lipids by creating a metal-antioxidant complex. Moreover, different non-conventional antioxidants, like phosphoric and citric acid can inactivate metals (Reische *et al.,* 2002). These combinations have the capacity to decrease oxidization of lipids by metals over contact metals with their several carboxylic acid groups and producing an organometallic compound.

Table 1.6. Synthetic and natural antioxidants used in semen extenders in various animal species with different doses.

Antioxidants	Concentration	Species	Semen Parameter Assessed	Effect	Reference
Glutathione disulphide Bovine serum albumin Cysteine lycopene	(5 mM), (20 mg/ml), (10 mM) (800 µg)	Ram	Motility, morphology, acrosome integrity, viability	Improved	Bucak *et al.,* 2007
Trehalose	76 g/L	Ram	Motility acrosome integrity	Improved	Aisen *et al.,* 2005
Rosemary	0.15 g	Ram	Post-thaw quality of ram semen, motility	Improved	Motlagh *et al.,* 2014

Antioxidants	Concentration	Species	Semen Parameter Assessed	Effect	Reference
Cysteamine	(2, 4, 6 and 8 mM),	Ram	Post-thaw quality of ram semen	Improved	Najafi *et al.,* 2014
Green tea	0,2,4,6mg/ml	Ram	Volume, Motility, viability	Improved	Mehdipour *et al.*, 2016
Curcumin and dithioerythritol	(0.5 and 2 mm)	Bovine	Motility, viability	Improved	Bucak *et al.*,2012
Zinc sulphate	0.288 mg/L	Buffalo	Motility, acrosome integrity, viability	Improved	Dorostkar *et al.*, 2014
Methionine curcumin and ellagic acid	(1mM), (1 and 2mM(1 and 2mM))	Ram	Motility, acrosome integrity	Improved	Omur and Coyan, 2016
Vitamin C	0.45,0.9 g/l	Equine	Motility, acrosome integrity, viability	Improved	Aurich *et al.,* 1997
Curcumin	2.5mM	Goat	Motility acrosome integrity	Improved	Bucak *et al.,* 2010
athione(GSH Oxidized glutathione	5 mM	Ram	Motility acrosome integrity, Viability	Improved	Buck *et al.,* 2008
Glutamine hyaluronan	2.5; 5 mM 500;1000 µl/ml	Goat	Motility acrosome integrity	Improved	Bucak *et al.,* 2008
Vitamin E	1.0 mg/ml	Bovine	Superoxide dismutase activity	Improved	Beconi *et al.*,1993
Catalase	100 and 200 U/ml	Ram	Motility Viability lipid peroxidation	Improved	Budai *et al.,* 2014
Melatonin	0.1, 1, 3 mM	Ram	Motility	Improved	Ashrafi *et al.*,2011

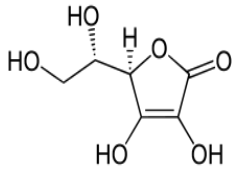
Antioxidants	Concentration	Species	Semen Parameter Assessed	Effect	Reference
Curcumin	1, 2, 4mM	Ram	Motility acrosome integrity, Viability	Improved	Omur and Coyan, 2016
Fennel	2.5 g	Boar	Motility acrosome integrity, Viability	Improved	Malo *et al.*, 2012
Methionine	1, 2, 4mM	Ram	Motility acrosome integrity, Viability	Improved	Omur and Coyan, 2016
Ergothioneine	1, 2, 4 mM	Ram	Motility acrosome integrity, Viability	DNA Integrity Improved	Bucak *et al.,* 2014
Superoxide dismutase (SOD),	100 U / ml	Ram	Motility acrosome integrity, Viability	Improved	Budai *et al.* 2014
Glutathione peroxidase (GPX)	2 - 5 mM	Ram	Motility acrosome integrity, Viability	Improved	Budai *et al.*, 2014
Pyruvate Resveratrol	5 to 20 µg/ml	Ram	Motility acrosome integrity.	Improved	Budai *et al.,* 2014

1.8.3. Antioxidants regulate the proteins of the sperm membrane

Supplementation of several antioxidants to semen extender was found to be useful in preserving sperm motility and viability through hypothermic storage of semen from different species (Ashrafi *et al.,* 2011; Câmara *et al.,* 2011). The motility of ram and bull sperm was maintained with the addition of a superoxide dismutase (SOD) and catalase (CAT) mixture (Stojanov *et al.,*1994), glutathione peroxidase (Pomares *et al.,*1994), BHT and Desferal (Vishwanath and Shannon, 2000), melatonin (Ashrafi *et al.,* 2011), CAT and reduced glutathione (GSH) (Câmara *et al.,* 2011) and SOD and GSH (Silva *et al.,* 2011).

Paul *et al.* (2017) reported that antioxidants play an important role by scavenging ROS produced by dead spermatozoa and thus avoiding ROS-mediated lipid peroxidation of the plasma membrane of spermatozoa. In addition, lipid-protein interactions in the plasma membrane may be protected by antioxidants that regulate protein anchorage to the lipid bilayer and could be impacted by the lipid peroxidation through hypothermic preservation of ram spermatozoa. Antioxidants protect sperm membrane integrity by protecting hydrophobic interactions in the bilayer. Moreover, as proteins in the bilayer are held in between lipids mostly through hydrophobic interactions with the lipids, the protection of these interactions by antioxidants could also maintain their anchorage to the bilayer (Paul *et al.,* 2017).

1.9. Types of antioxidants used in this thesis



1.9.1. Vitamin C C6H8O6

1.9.1.1. Vitamin C structure synthesis and function

Vitamin C is generally distributed through the tissues in all species of animals which are able to synthesize ascorbic acid or obtain it from the diet. Ruminants and some other species are able to endogenously produce ascorbic acid through the action of their gut flora, and therefore no recommended dietary requirement has been published by the National Research Council. In turkeys, however, supplementation of 150 mg/kg improved semen volume by 31% per ejaculate. This result can be explained by vitamin C's ability to stimulate testicular activity through its role in steroid hormone synthesis (Dobrescu, 1987).

It has been found that vitamin C is essential in several biochemical methods that involve electron donation. In this way, vitamin C functions as a reversible oxidiser able to reduce other molecules. Stocker *et al.* (1991) reported that vitamin C is considered as the most significant antioxidant in extracellular fluids and has the ability to reduce potentially harmful peroxyl radicals before they can initiate the process of lipid peroxidation in the cell membrane (Mukhopadhyay *et al.,* 1995).

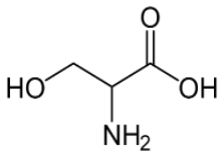
1.9.1.2. Effect of vitamin C on sperm

Vitamin C has the ability to protect cells and tissues in the body from oxidative damage by ROS, by decreasing circulating glucocorticoids (Degkwitz, 1987). Vitamin C is also able to reduce tocopheryl free radicals, thus regenerating the antioxidant tocopherol (Jacob, 1995). The concentration of vitamin C found in seminal fluid is ten times higher than that of serum (Fraga *et al.,* 1991; Agarwal and Sekhon, 2010). Declining levels have produced non-specific sperm agglutination, and so confirm the positive effect of vitamin C in protecting sperm from oxidative stress (McDowell, 2000). Ascorbic acid (vitamin C) is an antioxidant

substance, which is found in the seminal plasma and epididymal fluid as a protective vitamin in the epididymis (Chinoy, 1972). Vitamin C can play an important role in defending against ROS damage to sperm (Nour *et al.,* 2008), mainly by preventing oxidative damage to DNA (Fraga *et al.,* 1991) in numerous species including the ram.

Vitamin C also supports spermatogenesis through its ability to maintain an active state of antioxidation. A GSH-dependent dehydroascorbate reductase found at high concentrations in the testes maintains vitamin C in a reduced state. Vitamin C has been found to progress sperm motility and improve semen quality and fertility in rats (Fraga *et al.,* 1991). Vitamin C contributes about 65% of the antioxidant compounds of sperm plasma in fertile men (Agarwal and Prabakaran, 2005). It has been confirmed that vitamin C is one of the essential treatments for male infertility (Agarwal and Sekhon, 2010). Extracellular antioxidants of sperm contribute considerably to protecting the sperm membrane against oxidative stress (Showell *et al.,* 2011). The positive effect of vitamin C on frozen-thawed spermatozoa in several species was widely researched using different concentrations but did not identify the effect of optimal timing that can be used to protect and improve the integrity of frozen-thawed spermatozoa.

1.9.2. Cysteine C3H7NO2S



1.9.2.1. Cysteine structure synthesis and function

Cysteine is a semi-essential amino acid with the chemical formula HO_2CCH (NH_2) CH_2SH (Basu *et al.,* 2014). Cysteine has a thiol group chain, which contributes to enzymatic reactions. The thiol group is sensitive to oxidation, which performs an essential structural role in many proteins. It is mainly present in types of foods such as dairy products, meat, eggs, and whole grains. Cysteine biosynthesis begins in animals with the amino acid serine.

The sulfhydryl group in cysteine is nucleophilic and easily oxidized. Reactivity is improved when the thiol is ionized, and cysteine containing proteins have values close to neutrality (Figure 1.11); therefore, they are often in their reactive thiolate form in the cell which aids numerous biological functions (Bulaj *et al.,* 1998), due to the presence of the sulfhydryl group of cysteine (Del Valle *et al.,* 2017). Cysteine has properties as an antioxidant, which is classically known as tripeptide glutathione, found in humans and other organisms. Systemic oral glutathione (GSH) has a low oral bioavailability; consequently, it must be biosynthesized from its essential amino acids: cysteine, glutamic acid and glycine. Cysteine acts as an important source of sulphide in human metabolism. Cysteine provides the sulphide in iron-sulphur bonds and nitrogenase although it is converted to alanine during the reaction (Lill and Mühlenhoff, 2006).

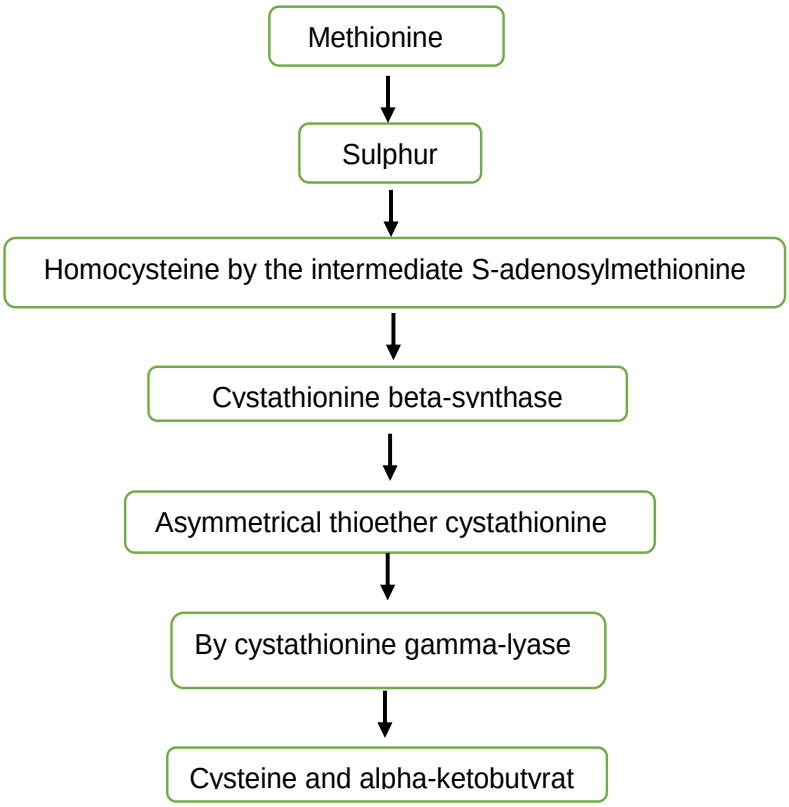


Figure 1. 11.Cysteine synthesized process from methionine (adapted from Hell *et al*., 1997).

1.9.2.2. Effect of cysteine on sperm

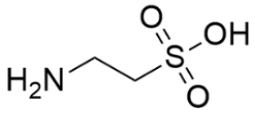
Cysteine has the ability to enter the membrane of mammalian cells easily and improves intracellular glutathione biosynthesis both *in vivo* and *in vitro* (Mazor *et al.,* 1996). It has been reported that cysteine can avoid losses of motility, viability and membrane integrity during liquid frozen sperm storage, with improved oocyte maturation and fertilization *in vitro* using porcine cells (Byeong-Seon Jeong, 2001). Cysteine is able to maintain intracellular glutathione levels and reduce ROS through its ability to stimulate glutathione synthesis (Bansal and Bilaspuri, 2011). Moreover, cysteine prevents loss of sperm activity during liquid storage of sperm or in cryopreservation (Bucak and Uysal, 2008).

In addition, thiol groups in cysteine can separately penetrate sperm where they function as non-enzymatic antioxidants (Çoyan *et al.,* 2011; Topraggaleh *et al.,* 2014). It has the ability to regulate electron transport through evolution and provides support in enzymatic catalytic reactions (Piste, 2013). It also has a cryoprotective effect on bull semen with improved viability, reduced acrosome damage, and reduced loss of motility in freeze-thawed sperm (Uysal and Bucak, 2007). Cysteine has been shown to enhance the post-thawed sperm function of rams (Bucak *et al.,*2007), bulls (Sariözkan *et al.,* 2009), cats (Thuwanut *et al.,*

2008), and dogs (Michael *et al.,* 2007), as well as providing the protection against DNA damage in bull sperm (Tuncer *et al.,* 2010).

It has also been reported that cysteine has a cryoprotective effect against acrosome damage and viability (Bucak *et al.,* 2008). Cysteine also reduces the total abnormality count and improves sperm motility in freeze-thawed goat semen (5 mM) (Uysal and Bucak, 2007), bull semen (5 mM) (Uysal and Bucak, 2007), and during liquid storage of ram semen (5 mM) (Bucak and Tekin, 2007; Tonieto *et al.,* 2010). All this research confirms the positive effect of cysteine on frozen-thawed spermatozoa of different species at different concentrations. However, they did not determine the optimal timing of the effect of cysteine for the protection and improvement of spermatozoa during and after cryopreservation.

1.9.3. Taurine C2H7NO3S



1.9.3.1. Taurine structure synthesis and function

Taurine, (2-aminoethanesulfonic acid) is one of the most widely distributed low molecular compounds in humans and animals, including in the testes and spermatozoa (Buff *et al.,* 2001; Schuller-Levis and Park, 2003). It is regarded as an essential or semi-essential nutrient (Knopf *et al.,* 1978; Gaull, 1989; Bouckenooghe *et al.,*2006). Taurine is a semi-essential amino acid that plays an important role in metabolic and protein synthesis, is highly contained in different tissues and can be synthesized by several tissues such as the liver, retina, kidneys, central nervous system, and mammary gland. Additionally, taurine was found in the male reproductive system in endothelial cells, Leydig cells, blood vessels, and other testicular interstitial cells in rats (Lobo *et al.,* 2000). It has been determined that taurine can be synthesized by male reproductive organs (Li *et al.,* 2006).

Taurine synthesis is achieved by the aminolysis of isethionic acid (2-hydroxyethanesulfonic acid), which in turn is obtained from the chemical reaction of ethylene oxide with aqueous sodium bisulphite. A direct approach includes the reaction of aziridine with sulphuric acid (Kosswig, 2000). By the alkylation of ammonia with bromoethanesulfonate salts, taurine can be produced in the laboratory. It has been reported that taurine is synthesised from cysteine and that the pancreas is responsible for taurine synthesis in mammals through the cysteine sulfinic acid pathway (Ripps and Shen, 2012) Figure 1.12. Taurine is bound by its amino-terminal group with chenodeoxycholic acid and cholic acid to form the bile salt sodium taurochenodeoxycholate and sodium taurocholate. It has an important role as an antioxidant and protects against toxicity to different substances such as cadmium and lead (Sinha and Mukherjee, 2008). Additionally, taurine supplementation has been shown to protect against oxidative stress induced by exercise (Li *et al.,* 2004).

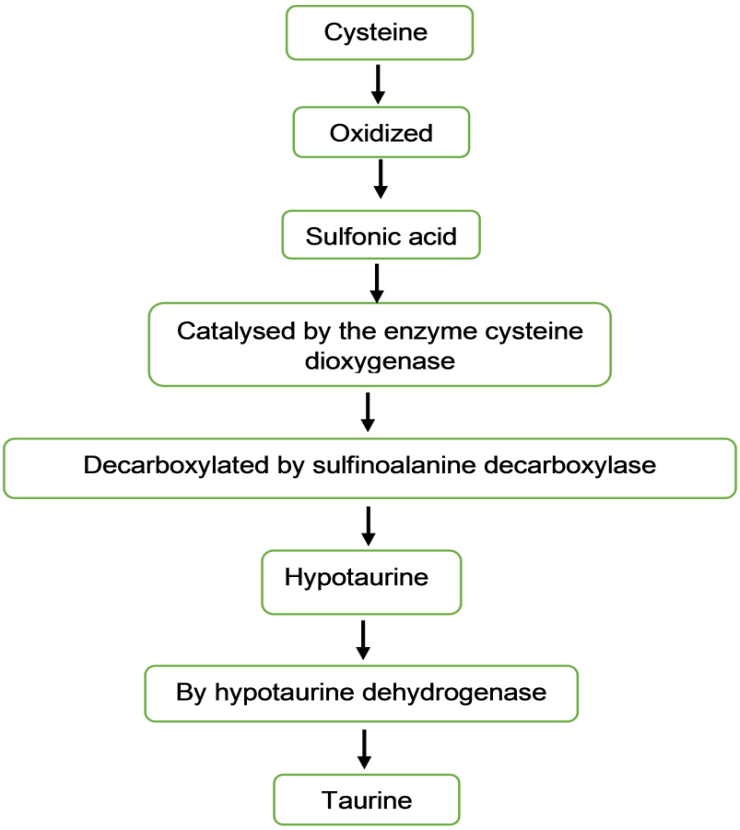


Figure 1. 12. Taurine synthesis in mammalian through the pathway of cysteine sulfinic acid (adapted from Ripps and Shen, 2012).

1.9.3.2. Effect of taurine on sperm

Taurine has the ability to maintain magnesium and potassium inside the cell while keeping extracellular sodium out because it assists in the movement of calcium, potassium and sodium across the plasma membrane (Kirk and Kirk, 1994). Taurine is known as the main free amino acid of spermatozoa and seminal plasma. Consequently, taurine acts as an antioxidant, membrane stabilization factor, capacitation factor, and sperm motility factor (Yang *et al.,* 2010). It can also promote secretion of testosterone *in vivo* and *in vitro* (Yang *et al.,* 2010). Moreover, taurine has been stated to be a part of the antioxidant defence system of cells (Green *et al.,* 1991). Studies in stallions (Ijaz and Ducharme,1995), cats (Baran *et al.,* 2009), donkeys (Dorado *et al.,* 2014), and rams (Bucak *et al.,* 2007) report that taurine supplementation of mammalian semen avoids the loss of frozen-thawed spermatozoa motility or viability *in vitro*.

The role of taurine as an antioxidant is explained by Dawson and Meldrum (1992) through either the detoxification of reactive intermediate metabolites such as hydrogen peroxide, hypochlorous acid and nitric oxide or incorporation into the plasma membrane. Taurine anti-oxidative action was also demonstrated by Yue *et al.* (2013) through mitochondria

protection against superoxide radical production when taurine organized synthesis of mitochondrial proteins. Taurine is a necessary compound for sperm capacitation, fertilization, and embryo development (Bucak *et al.,* 2007). Guérin and Ménézo (1995) quantified levels of taurine in spermatozoa and seminal fluid in numerous species and showed that taurine has a positive effect on mammalian spermatozoa. Taurine has a positive effect on cryopreservation through non-enzymatic antioxidant activity and reduces sperm damage.

The addition of taurine results in improved rabbit sperm motility (Alvarez and Story, 1983), and improved motility of rat spermatozoa (Sanchez-Partida *et al.,* 1997). Taurine has been added to cryopreservation media for human (Agarwal and Allamaneni, 2004), ram (Bucak *et al.,* 2008), goat (Bucak and Uysal, 2007), boar (Gutiérrez-Pérez *et al.,* 2009; (Hu *et al.,* 2009), (Paál *et al.,* 2018), dog (Martins-Bessa *et al.,* 2009), buffalo (Reddy *et al.,* 2010; Chhillar *et al.,* 2012), bull (Uysal and Bucak, 2007), and turkey spermatozoa (Slanina *et al.,* 2018) with a view to improving particular spermatozoa characteristics after thawing and consequently improving fertilizing ability.

It has been stated that taurine has the ability to protect ram sperm membranes against lipid peroxidation that reduces sperm motility (Uysal *et al.,* 2005; Rather *et al.,* 2016). Taurine is an important non-enzymatic scavenger that has a vital role in defence of spermatozoa against ROS, during the cryopreservation process and under aerobic conditions (Holmes *et al.,*1992; Chen *et al.,* 1993). The addition of some types of antioxidant has been shown to improve sperm motility. However, in some studies, taurine is not considered a classical scavenger or a controller of antioxidative defences; nevertheless, it has an important role as a regulator of mitochondrial protein synthesis, protecting the mitochondria against excessive superoxide production (Jong *et al.,* 2012). The most vital observation of the above studies was that which identified the effect of taurine on frozen-thawed spermatozoa from rams and other species. However, these studies did not determine the optimal timing and concentrations of taurine that can protect sperm from the harmful effects of cryopreservation and improve sperm integrity.

1.10. Heat shock proteins

Heat shock proteins (HSPs) are a class of proteins, which protect cells from oxidative stress and injury (Beere and Green, 2001). The essential processes for life are involved in maintaining intracellular protein homeostasis. HSPs decrease levels of unfolded, aggregation-prone proteins (Tyedmers *et al.,* 2010). HSPs are highly conserved proteins of mammalian species, and are classified depending on their molecular weight: HSP27, HSP40, HSP60 (HSPD) HSP70 (HSPA), HSP90 (HSPC), HSP100 (HSPH) and (HSPB) (Buchner, 1996; Bukau and Horwich, 1998). HSP90 is involved in progesterone signalling,

which induces the sperm acrosome reaction and hyperactivation in humans (Lui *et al.,* 2014; Sagare-Patil *et al.,* 2017), and also induces phosphorylation during capacitation in mouse and human sperm (Ecroyd *et al.*, 2003).

HSP synthesis increases in response to a sudden rise in temperature and to other stressors such as chemical and physical conditions (Welch, 1992). These proteins have a special ability to protect essential cell proteins from aggregation and denaturation; HSPs achieve a variety of functions also in unstressed cells (Mjahed *et al.,* 2012). ATP-dependent chaperones, such as HSP70, and HSP90 can bind unfolded or inadequately folded proteins and stimulate refolding using ATP hydrolysis as an energy source (Richter *et al.,* 2010). There is also a group of small heat shock proteins that are ATP-independent chaperones (Haslbeck *et al.,* 2016), which bind unfolding substrate proteins which are unstable or liable to continue unfolding thus helping to maintain a folding capable state.

It has been reported that heat shock protein HSP90 and HSP70 are molecular chaperones that collect in the cytoplasm, endoplasmic reticulum, and mitochondria. These molecules have been documented to have a critical role in male reproduction, with roles as diverse as sperm-egg recognition, transportation and packaging of protein, substitution of histones by protamine through spermiogenesis, reduction of apoptosis and elimination of remaining cytoplasm through sperm maturation (Kovanci *et al.,* 2001; Redgrove *et al.,* 2012). Numerous studies have exposed a possible association between HSPA2 and male factor infertility (Redgrove *et al.,* 2012; Motiei *et al.,* 2013).

1.10.1. Heat shock protein 70 (HSP 70)

In the HSP family, HSP70 has the ability to stabilize unfolded protein precursors before they are folded and combined into proteins. Their principal roles are to maintain protein conformation and move proteins across intracellular membranes (Arispe *et al.,* 2002; Tsvetkova *et al.,* 2002; Welker *et al.,* 2010). HSP70 seems to play an essential role in sperm function post ejaculation, and it was detected in proteins extracted from boar (Huang *et al.,* 2000) and bull sperm (Kamaruddin *et al.,* 2004). Further research has shown that these proteins, in particular the 70 kDa HSP (HSP70), seem to be related to the quality of boar semen (Huang *et al.,* 2000) and also could play an important role in fertilisation and in the development of embryos (Matwee *et al.,* 2001; Spinaci *et al.,* 2005). The possibility that male infertility is caused by an immune response to HSP70 has been proposed (Bohring and Krause, 2003; Shrum *et al.*, 2010). Furthermore, this protein has been shown to have a positive role on boar and bovine sperm capacitation and acrosome reactions during the sperm-oocyte interaction (Matwee *et al.,* 2001; Spinaci *et al.,* 2005).

Heat shock proteins (HSPs) 70 and 90 are abounding proteins of both eukaryotic and prokaryotic cells. Mammalian HSP70 has been recognised in male germinal cells through spermatogenesis in mice (Allen *et al.,* 1988), rats (Brien *et al.,* 1989), bulls (Raab *et al.,* 1995),humans (Kamaruddin *et al.,* 2004; Huang *et al.,* 2005) and in mature sperm in mice, humans, bulls and boars (Kamaruddin *et al.,* 2004; Spinaci *et al.,* 2006). Sperm-specific antigens present opportunities for study in cases of immune-mediated human infertility and for the identification of alternative diagnostic method for numerous mammalian species.

HSP70 has been recently recognised as one of the sperm membrane antigens for anti-sperm antibodies (ASA) evidenced in the SP of infertile men (Bohring and Krause, 2003). HSP70 has an important role through the sperm-oocyte interaction during *in vitro* fertilization of bovine (Matwee *et al.,* 2001) and swine ova (Spinaci *et al.*, 2005)

HSP70 has been indicated to interact with the lipids of the cell membrane and conserve their integrity and functions under stress (Broquet *et al.,* 2003).

1.10.2. Heat shock protein 90 (HSP90)

HSP90 is a highly conserved molecular chaperone (Dun *et al.,* 2012), which plays diverse and important roles in cell protein development such as supporting transcription factors and kinases, as well as signal transduction and changes in protein structure, be that folding or degradation (Taipale *et al.,* 2010). While the exact mechanism of HSP90 action is yet to be confirmed, nevertheless it has an important role in sperm function. It has been found that the level of HSP90 protein was 36% higher in fresh sperm than sperm left to cool for an hour, suggesting that a great reduction in HSP90 is related to reduced sperm motility (Huang *et al.,* 2000). The role of HSP90 has been studied in the sperm of various mammals including mice, rats, rabbits, stallions, cats, dogs, macaques, boars, and humans. HSP90 is found in the testis during rat sperm development (Itoh and Tashima, 1991).

HSP90 has an ATPase activity that is stimulated by mouse sperm nuclear protein (autoantigenic sperm protein) (Alekseev *et al.,* 2005; Grad *et al.,* 2010). Sperm lacking in HSP90b1 (HSP90 beta member 1) exhibit large heads and abnormal middle pieces, and have less fertilizing ability (Audouard and Christians, 2011). Levels of HSP90AA1 (alpha member A1) protein in boar ejaculate are dependent on the cryopreserved temperature (Casas *et al.,* 2010). Biggiogera *et al.* (1996) reported that levels of HSP90 vary depending on the developmental stage of spermatozoa with high levels during finding the extension phase while almost none was detected in late spermatids and mature sperm of mice.

HSP90 levels and motility of human sperm decrease during cryopreservation (Cao *et al.,* 2003), as HSP90 is tyrosine phosphorylated during capacitation (Ecroyd *et al.*, 2003a). HSP90 expression in the neck, middle piece, and tail regions of the human, cat, and boar

sperm is increased during capacitation (Mitchell *et al.,* 2007; Li *et al.,* 2014), and found in the tail region of stallion and dog sperm (Volpe *et al.,* 2008). These regions control sperm motility, so this finding is consistent with HSP90's role in sperm motility and hyperactivation (Flores *et al.,* 2008). A positive relationship between the motility of frozen-thawed bull sperm and expression of HSP90 has been identified (Zhang *et al.,* 2015). This subsequently has an effect on the integrity of the sperm plasma membrane and the acrosome (Wang *et al.,* 2013).

HSP90 has a significant role in sperm fertilizing ability due to its localization in the sperm tail (Wang *et al.,* 2014; Calle-Guisado *et al.,* 2017). In addition, some types of proteins such as serine-threonine kinases and tyrosine kinases are related to HSP90 (Pratt, 1998) which influences sperm motility (Huang *et al.,* 2000; Casas *et al.,* 2010; Wang *et al.,* 2014). The level of ROS affects the mechanism of HSP90 synthesis (Li *et al.,* 2014), and reduces HSP90 function (Padmini and Rani, 2011; Chen *et al.,* 2012; Zhang *et al.,* 2015).

Zhang *et al.* (2018) observed the conversion of cysteine residues to sulphenic acids and sulphenyl-amide linkages and internal disulphides or disulphides with glutathione. These changes have been associated with sensitivity of HSP90 to H_2O_2 evidenced by morphological changes such as larger sperm heads and abnormal middle pieces. Therefore, the mechanism of HSP90 against ROS spontaneous phospholipid oxidation still needs to be further investigated. The reasons for differences in HSP90 expression need to be clarified, but could be due to the differences between species or even due to various biological processes in the testes, epididymis, and ejaculate. In addition, the variations could also arise because of the various origins of antibodies used to detect HSP90.

Previous studies have focussed on the effect of cryopreservation and ROS on frozen-thawed ram sperm integrity. However, studies related to the role of HSP70 and HSP90 on protection against and reducing this damage are very limited. Further studies are required to determine the relationship between the addition of antioxidants and the expression of HSP90 and HSP70 and could provide a new approach to cryopreservation and improve the integrity of frozen-thawed ram sperm.

1.11. Summary of Literature Review

Cryopreservation including cold shock, freezing and thawing of semen is essential for a successful AI programme in sheep industry. However, semen cryopreservation leads to a reduction in sperm integrity such as reducing the percentage of motility, acrosome integrity, viability, and the ability of sperm to penetrate the mucus during transit through the cervix. Through cryopreservation, ram sperm membrane loses the protective mechanism by SPP

particularly of the low molecular weight proteins and antioxidants. The sperm membrane of the ram has high concentrations of unsaturated fatty acids, which make it more susceptible to oxidative stress. Furthermore, phospholipids and unsaturated fatty acids undergo oxidation by free radicals produced through cryopreservation. Whole SP and SPP fractions supplementation, particularly low molecular weight proteins have a protective effect against cold shock in frozen-thawed ram spermatozoa. The presence of antioxidants in semen extenders prior to cryopreservation can improve the post-thaw integrity of ram spermatozoa and reduce the percentage of ROS generation. Antioxidants such as taurine, cysteine and vitamin C have the potential to reduce LPO and protect sperm function and integrity.

1.12. Hypotheses

The aim of this thesis is to investigate the effect of seminal plasma proteins and antioxidants on the function and integrity of frozen-thawed ram spermatozoa, and to determine the effect of oxidative stress and antioxidants on the protein expression of heat shock proteins. The development of robust protocols for the cryopreservation of ram semen, that reduce spermatozoa damage, will aid the development of reproductive technologies in the sheep industry.

1.13. Objectives

a) **To** evaluate the effect of whole seminal plasma and seminal plasma protein fractions on the post-thawed ejaculated and epididymal ram spermatozoa functions.
b) **To** evaluate the ability of ram sperm preserved in extenders containing whole SP and SPP fractions to penetrate cervical mucus.
c) **To** determine the effect of antioxidant supplementation on sperm integrity and to optimise the timing and concentration of antioxidants for enhanced sperm function.
d) **To** evaluate the effect of antioxidants on the reduction of the percentage of ROS production and level of lipid peroxidation on the ram sperm and SP.
e) **To** determine the effect of oxidative stress by H_2O_2 on the protein expression level of HSP70 and HSP90 in fresh ram semen.
f) **To** investigate the impact of antioxidants on the expression of HSP70 and HSP90 in frozen-thawed ram semen.
g) **To** evaluate the relationship between the expression of HSP90 and HSP70 on the sperm integrity.

Chapter 2. General Materials and Methods

The Harper Adams University Ethics Committee approved the protocols of this study.

2.0. Animals

2.1. Animal training for semen collection

Six mature Texel rams (2–3 years old), with a body score condition of 3–4 (Kenyon *et al.*, 2014) were used for semen collection. Rams used in this experiment were obtained from the Harper Adams University flock. Rams were housed individually in metal pens (2 x 2 m) with straw bedding during the period of the study. Rams were fed approximately 1 kg/d fresh weight concentrate feed specific for rams that had 18% crude protein and 3.75% crude oil and fats (Wynnstay Ram Master Coarse Mix 2281) with *ad libitum* access to straw and fresh water. Rams were trained to ejaculate using an artificial vagina (AV) which usually involves a preliminary training period before starting a collection of samples. The collection with an AV is the stimulation of natural mating because it provides the correct temperature and pressure to erect the penis. The liner of the AV was rinsed with 70% alcohol and dried before filling half with warm water (about 42 to 45°C) for the semen collection and 37°C for the collecting glass. The AV was held in the right hand in a kneeling position at an angle of 45° with the open end facing towards the male (Evans and Maxwell, 1987). The training period lasted three weeks using six teaser ewes to stimulate the rams during May.

2.2. Preparation of teaser ewes

Six, 2–4 year old Mule ewes, with a body condition score of 2–3 (Kenyon *et al.,* 2014), were divided into two groups of 3 ewes and fed approximately 350 g/d fresh weight concentrate designed for ewes that had 4.75% crude oil and 19% crude protein (Wynnstay Ewes Master Coarse Mix) with *ad libitum* access to clean water and hay. Teaser ewes were prepared at

2.3. Collection of semen samples

The ejaculates were collected according to the protocol outlined by Evans and Maxwell (1987). The AV was prepared by adding 70 ml of 45°C water and replacing the screw lid. The collecting glass (Minitube, Germany) was sealed at one end of the AV liner, and the AV was filled with air using the valve until it exerted pressure but still allowed penetration. A small amount of non-spermicidal lubricant (United Farms Ltd., UK) was applied to the the beginning of May by clipping the excess long wool around the perennial region. They were treated with intra-vaginal sponges containing Flugestone acetate (20 mg/sponge) (Intervet Ltd., UK), for 12 days and injected with 200 IU/im PMSG hormone (pregnant mare serum gonadotropin, Intervet. Ltd., UK) at the time the sponges removal. The ewes came in to oestrus 48 hours after the injection. The collecting glass was warmed then insulated with a

thermal sock to minimise cold shock. Since the rams had been previously trained, the teaser ewe was not required to be in oestrus. However, she was restrained using a yoke. The person collecting the sample crouched beside the rear end of the teaser ewe within the collecting pen, the ram was then released into the collecting pen and guided towards the teaser ewe. The ram was encouraged to mount the ewe. On mounting, the person collecting deflected the ram's penis into the AV. Once ejaculation had been achieved the ram was guided back into the holding pen and a brief visual assessment was carried out to ensure sufficient volume and concentration of the ejaculate. An effective sample was considered to be of at least 1.0 ml with a milky-creamy consistency, which suggests at least 3.0 x 10^9 sperm/ml (Evans and Maxwell, 1987; Avdi et al., 2004) (Table 2.1). The collection glass containing the ejaculate was covered with parafilm, labelled with the ram ID and ejaculate number, and then placed in a thermal container to maintain the temperature at approximately 35°C. Ejaculates were transported to the Harper Adams University laboratory within 10 minutes of collection.

Table 2.1. Ram semen concentration assessed by consistency

Score	Consistency	Number of Sperm (x10^9)/ml	
		Mean	Range
5	Thick creamy	5.0	4.5–6.0
4	Creamy	4.0	3.5–4.5
3	Thin creamy	3.0	2.5–3.5
2	Milky	2.0	1.0–2.5
1	Cloudy	0.7	0.3–1.0
0	Clear	Insignificant	0

(Evans and Maxwell, 1987)

2.4. Fresh analysis

Once transported to the laboratory, the collecting glasses were immediately placed in a test tube rack in a water bath maintained at 37°C, ensuring that the semen was submerged. Ejaculates were individually assessed and processed for experimentation if they met the following criteria: a volume of 0.75–2.0ml and a concentration of ≥ 3.5 × 10^9 spermatozoa/ml was assessed using hemocytometer (Agar Scientific Ltd, Parsonage Lane, Essex, UK), with motility ≥ 80% and sperm abnormalities ≤ 10%. Abnormalities were assessed on eosin-

nigrosin stained semen smears and using a light microscope (Olympus CX31RBSF, Tokyo) under 40× magnification as described by Evans and Maxwell (1987). The percentage of sperm displaying progressive motility, defined as moving progressively forwards, was analysed as described in section 2.9.1 Table 2.2.

Table 2. 2. Assessment of mass sperm motility.

Class	Characteristics	Score
Dead	Non-motile	0
Very poor	Few spermatozoa with weak movements (<20%)	1
Poor	Some motile spermatozoa (20-40%) without wave movement	2
Intermediate	Slow wave movement (40-60%) motile spermatozoa	3
Good	Rapid wave movement without whirlpools (60-80%) motile spermatozoa	4
Very good	Very rapid wave movements with clear whirlpools (<80%) motile spermatozoa	5

Adapted by Avdi *et al.* (2004).

2.6. Sperm concentration

Sperm concentration for fresh and frozen samples were assessed using a haemocytometer. Ten µl of semen was diluted in 90µl of 3% sodium chloride then vortexed. Ten µl of diluted semen was pipetted on to each side of a haemocytometer (Agar Scientific Ltd, Parsonage Lane, Essex, UK) and allowed to settle for 5 min. Sperm were observed at 40x magnification under a light microscope. The number of sperm in each sample was counted. The total number of sperm per ml was calculated.

2.7. Preparation of frozen-thawed sperm

Depending on the experiment, following collection and assessment, spermatozoa were diluted dropwise into a 1:4 dilution (semen: cryodiluent, v: v). The cryodiluent was composed of 300 mM Tris base, 94.7 mM citric acid, 27.8 mM D-Glucose, 0.0058g penicillin, 0.005g Streptomycin (Sigma Aldrich, UK) with an addition of 15% egg yolk, and 5% glycerol

at 37°C up to the final volume of 100 ml prior to use. Samples were cooled at 5°C for 2 hour in a water jacket and then frozen in pellets (200 µl) on dry ice (-79°C) according to Evans and Maxwell (1987). The pellets of semen samples were allowed to remain there for 3 minutes and then stored in liquid nitrogen for the subsequent analysis.

2.8. Post-thaw analysis

2.8.1. Thawing

All pellets from each sub-sample were removed from the liquid nitrogen store using tweezers and placed into labelled test tubes. The test tubes were submerged in a 37°C water bath and shaken vigorously for up to 1 minute until the pellets were fully thawed. The post-thaw treatments were conducted depending on the experiment.

2.9. Sperm parameters

2.9.1. Proportion of motile spermatozoa

Semen samples were assessed for total motility (Evans and Maxwell, 1987; Avdi *et al.,* 2004). Samples were evaluated for motility by placing 10 µL on a pre-warmed (37°C) slide, covering with a 22 x 22 mm pre-warmed coverslip and subjectively assessed using a phase contrast microscope at 40x magnification (Olympus CX31, Tokyo, Japan). Samples were assessed for percentage motility (%) to the nearest 5% at the incubation time.

2.9.2. Acrosome integrity and viability

2.9.2.1. Preparation of positive control for propidium iodide (PI)

Semen samples (200µl) were washed using tris citrate glucose (TCG) by centrifugation at 300×g for 3 minutes and re-suspended in 200µl of 96% methanol for 5 min at room temperature. Methanol permeabilises the cell membrane allowing the PI stain to enter inside each cell and bind to the DNA (as PI is a nuclear stain). Consequently all spermatozoa should stain positive with PI stain following methanol treatment. Spermatozoa were centrifuged at 300xg for 3 minutes to remove the methanol and re-suspended in phosphate buffered saline (PBS) with a final concentration of 25 x 10^6 sperm/ml. A 50µl aliquot of the sample was stained with PI and examined under a fluorescence microscope (Leica LEITZ DMRB, Leica Microsystems, Wetzlar GmbH. Germany) at 40x magnification. As the positive control, 100% of spermatozoa were stained red.

2.9.2.2. Preparation of negative control for PI

Semen samples (200µl) were washed using tris citrate glucose (TCG) by centrifugation at 300×g for 3 minutes and re-suspended in 1 ml of TCG. Spermatozoa were fixed using 2 µl of formaldehyde with a final concentration of 0.1% (Sigma-Aldrich) and 5 minutes allowed before evaluation. Formaldehyde quickly penetrates the cell and forms cross links between peptides in the cell membrane. Consequently this prevents the penetration of the sperm cell by PI, and all cells should appear unstained. Following centrifugation at 300×g for 3 minutes to remove all of the formaldehyde, spermatozoa were resuspended in PBS with a final concentration of 25 x 10^6 sperm/ml. The samples (50µl) were stained with propidium iodide and examined under a fluorescence microscope (Leica LEITZ DMRB, Leica Microsystems, Wetzlar GmbH. Germany). As the negative control, sperm appeared unstained.

2.9.3. Assessment of sperm acrosome integrity and viability

Acrosome integrity and viability of the sperm membrane were examined for all samples simultaneously according to (Beilby et al., 2010) using fluorescein-conjugated peanut agglutinin (FITC-PNA; Sigma-Aldrich, USA) and propidium iodide (PI; Sigma-Aldrich). Aliquots of 40µl of spermatozoa were incubated with 10 µl of FITC-PNA (final concentration of 40µg/ml) and 0.5µl of 2.4 mM PI for 5 min at 37°C. Sperm samples 200ul were then fixed by adding 2µl of formaldehyde with a final concentration of 0.1% (Sigma-Aldrich) and incubated for 5 minutes before evaluation. A 20µL aliquot of each fixed sample suspension was placed on a glass slide under a coverslip (24 x 50 mm) and observed using 40x magnification (Leica LEITZ DMRB, Leica Microsystems, Wetzlar GmbH. Germany). A fluorescence microscope, with a filter (excitation filter 460–490 nm, emission filter 510–550 nm bandpass filter, Chroma Technology Corporation, USA) was used to examine 200 cells per sample. Sperm showing a fluorescent green acrosome cap were considered to have a non-intact acrosome while sperm that did not stain were considered to be acrosome intact. Sperm that stained red with PI were considered non-viable and damaged (El-Kon *et al.,* 2009). Acrosome integrity and viability was determined depending on the experiment.

2.10. Penetration of cervical mucus or gel

2.10.1. Cervical mucus collection

Cervical mucus was collected from 11 Mule ewes aged 2-4 years in September and October (2016) during their natural breeding season when they were all cycling naturally. The body condition scores were 3–4 (Kenyon *et al.,* 2014). During the experiment, the animals were housed indoors on straw, provided with hay and water *ad libitum* and fed a commercial concentrate diet (approximately 350 g/d as fresh weight). The concentrate was designed for ewes and included 4.75% crude oil and 19 % crude protein (Wynnstay Ewes Master

Coarse Mix). All the experimental procedures on animals were conducted in accordance with the ethics committee of Harper Adams University. Oestrus was synchronised with intravaginal sponges, containing 20mg of flugestone acetate, (Chronogest, Intervet, Ltd., UK) for 12 days. Cervical mucus samples were collected 48 post sponge removal, and samples from any ewe with visible vaginal infections were excluded from the use in the lab. At the time of mucus collection ewes showed oestrus behaviour such as standing to be mounted by other ewes, rapid tail movements or raised their tail in the presence of a ram. Physical manifestations of oestrus included a reddened and swollen vulva. The ewes that showed no oestrus signs were excluded from the collection of mucus. The mucus was collected by flexible plastic tubing connected to a 50 ml plastic syringe. Using a vaginal speculum, the tube was inserted through the vulva up to the cervical os from where the mucus was aspirated. Mucus samples were centrifuged at 1300×g for 15 minutes to remove contaminants then were stored at -80°C in aliquots (500 µl) for subsequent use.

2.10.2. Preparation of synthetic media (artificial mucus/gel) for a penetration test

Acrylamide gels (1.6%) were prepared to test sperm penetration ability according to Martínez-Rodríguez *et al.* (2012). These artificial media were prepared from 30% liquid acrylamide (Sigma Aldrich, UK) diluting it to the corresponding volume with 1.5M Tris (pH 8.8) in 400ml distilled water. Once mixed, a solution of 2% ammonium persulphate up to 0.03% of final volume and 0.05% Tetramethylethylenediamine (TEMED, Sigma-Aldrich, UK) was added to induce acrylamide polymerisation (Anilkumar *et al.*, 2001). The mixture (pH 7.2) was left at room temperature for 4 h and then stored at 4°C until use. The gel was used in capillary tubes.

2.10.3. Penetration test

The penetration test was performed using either cervical mucus (chapter 3) or artificial mucus (chapter 4 and 6). The penetration of spermatozoa was assessed using a cervical migration test according to (Kremer, 1965). Samples were incubated at 37°C in a polypropylene microcentrifuge tube (500 µl, Elkay, Switzerland) and examined at 0 and 60 minutes. The mucus or gel was loaded into capillary tubes 0.3 x 0.3 x 7.5 cm (Micro haematocrit tubes, Hawksley, London, UK). Then each filled capillary tube was sealed at one end with Crista Seal (Hawksley, London, UK), and placed in the incubator to warm while the sperm sample was prepared. The tube was positioned vertically and immersed in a 0.5 ml, warm Eppendorf tube with a hole pierced in the lid. Each Eppendorf tube contained 200µl of the sperm sample (an aliquot of each washed sample was diluted to a concentration of 50 x 10^6 using PBS). The unsealed end of a loaded capillary tube was passed through this hole until the end was slightly submerged in the sample and co-incubated at 37°C for one h to allow the sperm to migrate up through the cervical mucus or

gel (Figure. 2.1 and 2.2). Following incubation, the capillary tube was sealed at the other end then incubated at -20°C for 2 min to halt sperm migration. The tube was examined

under the light microscope at 40x magnification (Olympus CX31, Tokyo. Japan), and the distance reached by the furthest spermatozoon (vanguard distance) was measured in centimetres (Figure 2.3).

Figure 2. 1. Capillary tube in a vertical position illustrates the sperm penetration test.

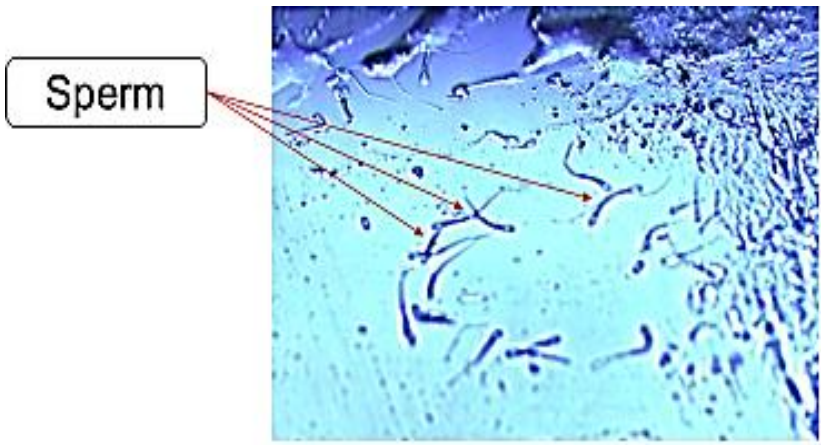


Figure 2. 2. Method of measuring distance (Vanguard) in a sperm penetration test.

Figure 2. 3. Spermatozoa penetration through the cervical mucus (40x magnification).

2.10.3.1. Validity of penetration test

A preliminary experiment was conducted to determine the variability of spermatozoa penetration through cervical mucus or artificial mucus and to determine if there was any significant difference in penetration between cervical mucus and artificial gel. Five semen samples were collected from 2 rams (n = 2 or n = 3/ram) as described in section 2.3 and cryopreserved as described in section 2.7. The penetration test was performed on frozen-thawed samples as described above.

Table 2.3. Penetration distance showing how far the sperm travelled up the capillary tube in both the gel and the mucus (Cm/h).

Sample	Penetration distance artificial mucus test 1	Penetration distance gel test 2	Penetration distance cervical mucus test 1	Penetration distance cervical mucus test 2
Ram 1 S1	1.6	1.2	1.1	1.0
Ram1 S 2	1.5	2.0	1.7	1.3
Ram 2 S1	2.2	1.9	1.6	1.4
Ram 2 S 2	1.3	1.6	1.2	1.3
Ram 2 S 3	2.7	1.8	2.2	2.6

The intra-assay coefficient of variation (CV %) for cervical mucus was 18.7%, and the CV (%) for artificial mucus was 10.5%. A two-tailed T test was performed in Genstat (version 17) to determine the difference between natural and artificial mucus. (Table 2.4). There was no significant difference in distamce travleled by spermatozoa between the two mucus types (*P* = 0.28).

Stats	Artificial mucus	Natural mucus
Mean	17.8	15.4
Variance	19.95556	25.82222222
Observations	10	10
df	18	
***P* (T<=t) two-tail**	0.28	

2.11. ROS detection by Nitro blue tetrazolium (NBT) suspension in PBS

The presence of ROS was assessed using Nitroblue Tetrazolium (NBT, Sigma Aldrich, UK) which causes the formation of formazan in the presence of ROS as described previously (Esfandiari *et al.*, 2003). A 0.1% NBT solution was prepared by stirring 10mg of NBT powder with 100 ml of PBS (pH 7.2), at room temperature for 1 hour then filtered with a 0.2mm filter. Semen samples (100µl) were incubated with an equal volume of 0.1% of NBT for 30 minutes at 37°C. The samples were centrifuged at 250×g for 5 minutes, the supernatant discarded and the pellet re-suspended in 200µl of PBS. Smears for each sample were prepared on glass slides and left to dry at room temperature 37°C. The air-dried smears were stained with Wright stain 1.0 mg/ml for 3 minutes (Sigma Aldrich, UK). Smears were washed with tap water then air-dried at room temperature. The slides were covered by a coverslip using mounting media, and 200 spermatozoa were scored under 40x magnification with a light microscope (Olympus CX31, Tokyo. Japan). The proportion of the sperm head that showed the presence of Formazan granules was graded depending on the experiment as described previously (Esfandiari *et al.,* 2003). The proportion of ROS in the sperm head was assessed depending on percentage of formazan formation in the sperm head between (0, <50, >50, and 100%) Figure 2.4.

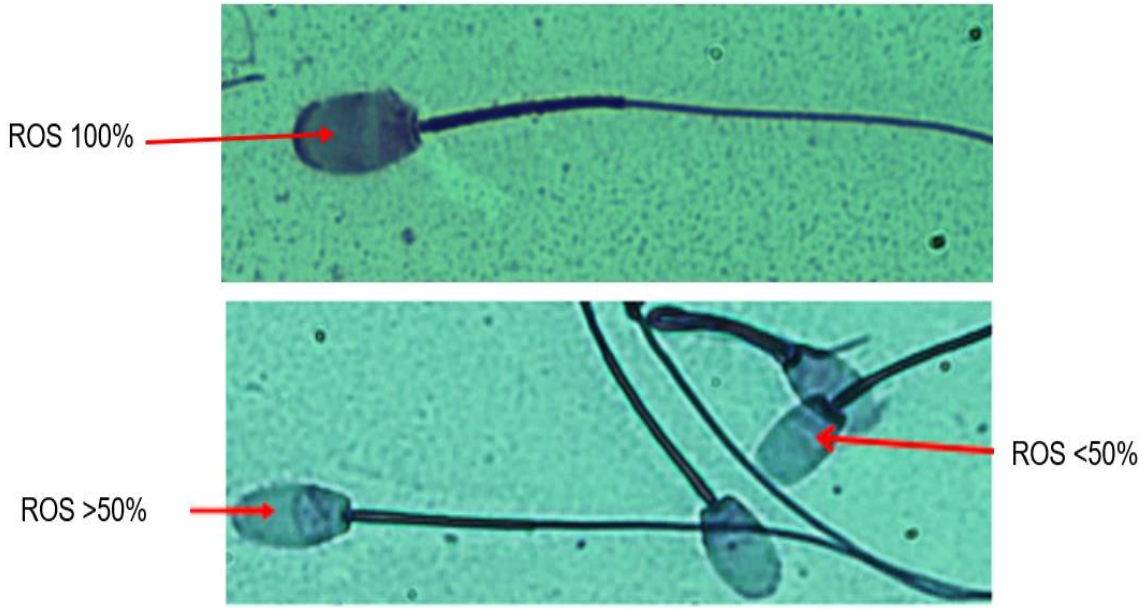


Figure 2. 4. Different proportions of formazan formation in the sperm head (0, <50 %, > 50% and 100%).

2.12. Measurement of lipid peroxidation (LPO)

Lipid peroxidation (LPO) of the ram semen was measured on the basis of malondialdehyde (MDA) level using the thiobarbituric acid-reactive species (TBARS) method as described previously (Banday *et al.*, 2017). The MDA levels were measured both in SP as well as in spermatozoa. Semen samples were thawed at 37°C. The thawed semen (0.5 ml) was then centrifuged at 1500×g in a cooling centrifuge (Eppendorf, Notholor, Hamburg, Germany) to obtain SP and a spermatozoa pellet for the assessment of lipid peroxidation. SP was frozen after centrifugation until assessed to determine the level of LPO.

2.12.1. Lipid peroxidation of seminal plasma (SP)

Seminal plasma samples were thawed at 37°C and centrifuged for 7 minutes at 1500×g (Eppendorf, Notholor, Hamburg, Germany). Then 100µl of seminal plasma was diluted in 900µl of distilled water with 2.5 ml of thiobarbituric acid reagent (Sigma-Aldrich, UK). This reagent was composed of 0.367g of 2-thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 g sodium hydroxide (Na OH) (Sigma-Aldrich, UK) and 100 ml glacial acetic acid (Fisher Scientific, UK). This reagent was prepared at room temperature and stored at 4ºC. The samples were heated with the reagent for 1h in a boiling water bath (95 ºC) then put on ice until cool. The samples were centrifuged for 10 minutes at 4,000×g and 1.0 ml of the supernatant was placed within a cuvette (Fisher brand, Fisher Scientific, UK).

The absorbance of samples was measured using a spectrophotometer (Jenway 6305, Bibby Scientific Ltd, UK) at 534 nm against a blank 1.0 ml of deionised water and 2.5 ml of TBARS stock solution The MDA concentration of sperm and SP was determined according to Banday *et al.* (2017), by the specific absorbance coefficient:

MDA level (nmol/ml) = (OD/156) $\times 10^3$ (total volume/sample volume).

2.12.2. Lipid peroxidation of sperm

Spermatozoa samples were obtained following centrifugation of semen (described above 2.12.1). The concentration of sperm was determined using a haemocytometer to obtain a concentration of 25 x 10^6 sperm/ml after sample dilution with PBS. Samples were then placed in a sonicator (Grant Instruments, Cambridge Ltd, UK) at 20,000 shock waves for two minutes and 15-second intervals in an ice-cold bath to complete disruption of spermatozoa and reduce localised heating. The MDA concentration was determined as per the procedure described under the section of seminal plasma (SP) and expressed as nmol/10^6 sperm.

2.13. Immunofluorescence staining of HSP90 and HSP70

The primary antibody, mouse monoclonal [AC88] to HSP90 (Abcam, UK) has been validated for use in immunofluorescence and cross reacts with sheep amongst a range of other species (abcam website https://www.abcam.com/hsp90-antibody-ac88-ab13492.html). It has been used previously in boar sperm (Spinaci *et al*., 2006) and is also suitable for use in frozen sections for immunohistochemistry, suggesting this is a suitable antibody for use in cryopreserved ram spermatozoa. The antibody binds to amino acid residues 604-697 of the 732 amino acid human HSP90 sequence.

The mouse monoclonal HSP70 antibody [N27F3-4; ab47454)] is suitable for use in immunofluorescence and in frozen sections for immunohistochemistry. The antibody shows cross-reactivity with a range of species including sheep (abcam website https://www.abcam.com/hsp70-antibody-n27f3-4-ab47454.html). It has been used successfully in mouse sperm (Winklhofer *et al*., 2001) and is therefore an appropriate antibody to detect expression of HSP70 in ram spermatozoa by immunofluorescence.

Washed sperm were diluted in PBS to make a final concentration of $25x10^6$ spermatozoa/ml. 20µl of diluted sperm was pipetted onto glass slides, smeared and air dried. The slides were then fixed with 100% methanol for 10 minutes at -20°C. The slides were washed with PBS in coplin jars for 5 minutes repeated three times with fresh PBS and blocked with 100µl of 1 % bovine serum albumin (BSA) (Sigma-Aldrich, UK) in PBS for 30 minutes. The primary antibody, mouse monoclonal [AC88] to HSP90 and mouse monoclonal [N27F3-4] to HSP70 antibody (Abcam, UK) was diluted at 1:100 in 1% BSA. 100µl of diluted (AC88) for HSP90 and (N27F3-4) for HSP70 were pipetted separately onto each slide and gently spread to the edges by tilting the slide. Nunc Bio Assay dish (Scientific Laboratory Supplies Ltd., UK) was used to make an humidified chamber (4x10 ml serological pipettes fixed into two rows), used to incubate the slides, and damp paper towel soaked with water was placed in the bottom to maintain the humidity for the slides. The humidified chamber was used to incubate the slides for 16-18 hours at 4°C, and all steps were completed in the dark. Then each slide was washed using PBS three times for five minutes, and the PBS at each time was changed. The slides were then incubated with sheep-anti-mouse secondary antibody (Abcam, Cambridge, UK) (1:200) in 1% BSA for 1 hour under dark conditions at room temperature. The slides were washed with PBS for 5 minutes, three times (renewing the PBS each time) and mounted by one drop of Fluorshield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Abcam.UK). A 22mm X 55mm coverslip was gently placed on top allowing diffusion of the Fluoroshield to cover all sperm. Control slides were treated as the other slides; however, instead of using primary antibody AC88 and N27F3-4, negative control slides were incubated in the moistened chamber with 100µl of 1% BSA at 4°C for 18-20 hours. Negative control slides were

produced to confirm there is no non-specific binding of the secondary antibody (Burry, 2011). The slides were evaluated under 400× magnification on a Leitz Fluorescence photomicroscope (Leica LEITZ DMRB, Leica Microsystems, Wetzlar GmbH. Germany). From each slide of HSP90 or HSP70, 200 sperm cells were assessed; the percentage of fluorescing cells was recorded. Furthermore, the analysis for each of the 200 sperm in each sub-sample, the intensity of HSP70 and HSP90 expression in each location was scored on a scale of 1 to 3 with 0 displaying no expression, 1 being very weak and 3 being very intense, as shown in Figure (2.6). The percentage of sperm expressing HSP70 and HSP90 and the fluorescence intensity (score/3) was used to generate an index score using the following calculation:

A score of expression = (number of sperm which have an expression on specific region X degree of brightness in this region) /100.

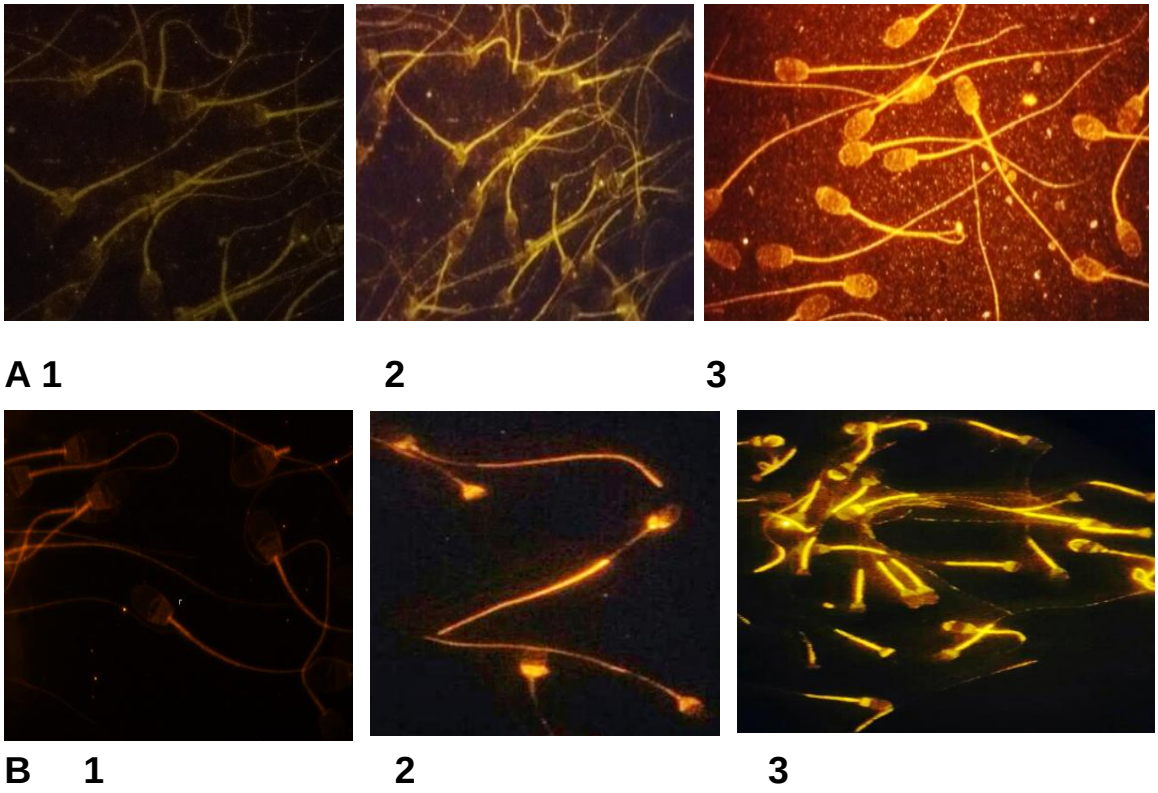


Figure 2. 5. The intensity of HSP70 (A) and HSP90 (B) expression at each location of the sperm regions (Acrosome, post acrosome, middle piece and tail) scored on a scale of 1 to 3 with 0 displaying no expression, 1 being very weak and 3 being very intense (100x magnification).

Chapter 3. The Effect of Seminal Plasma Proteins on the Integrity of Ejaculated and Epididymal Ram Spermatozoa

3.1. Introduction

Semen cryopreservation induces sperm damage, causing loss of sperm membrane components, lipids and proteins (Bergeron *et al.,* 2005). The ratio of cholesterol to phospholipids is low in ram spermatozoa increasing its sensitivity to cold shock and osmotic stress during cryopreservation (Muiño-Blanco *et al.,* 2008). This limits the use of frozen-thawed (FT) ram semen due to post-thaw reduced sperm motility, viability, acrosome integrity and fertility rates (Salamon and Maxwell, 2000). This reduced fertility has been associated with an inability of FT spermatozoa to achieve complete migration through the female reproductive tract, and the increased time required for FT spermatozoa to reach the oviduct (Soleilhavoup *et al.,* 2016).

Seminal plasma (SP) is a medium for protection, maturation and metabolism of sperm cells (Said *et al.,* 2010). Mammalian SP contains two main groups of proteins namely spermadhesins also termed as heparin-binding proteins, and bovine seminal plasma proteins(BSPP) that are characterized by the presence of tandem fibronectin type-II domains (Manjunath *et al.,* 2009). Both types of proteins have been shown to play an essential role in the promotion and prevention of capacitation (Plante and Manjunath, 2015). Ram SP (RSP) contains 2.30-2.50 g/dL protein (Juyena and Stelletta, 2012), which includes four fibronectin type II proteins namely RSP-15kDa, RSP-16kDa, RSP-22kDa, and RSP-24kDa (Bergeron *et al.,* 2005). The addition of SP to FT ram sperm enhances sperm motility, acrosome integrity and viability (El-Hajj Ghaoui *et al.,* 2007; Robayo *et al.,* 2008; Domínguez *et al.,* 2008; Bernardini *et al.,* 2011), and improves the ability of sperm to penetrate the natural cervical mucus of the ewe *in vitro* (Arienti *et al.,* 1999). SP protein fractions with low molecular weight have a significant function, preserving sperm viability (Leahy and de Graaf, 2012; Barrios *et al.,* 2005), as well as the decapacitated state of fresh sperm (Mendoza *et al.,* 2013).

Unlike ejaculated spermatozoa, epididymal spermatozoa do not have the coating components derived from SP in their membrane (Rath and Niemann, 1997), and consequently, they do not undergo the efflux of choline phospholipid and cholesterol from the membrane that induces capacitation (Bergeron *et al.,* 2005; Ehling *et al.,* 2006). The cryopreservation of epididymal sperm enables the preservation of genetic material from incapacitated and deceased males, via extraction of epididymal spermatozoa from the testes. The membrane of epididymal spermatozoa is sensitive to cryopreservation because it does not have the protection of SP components including proteins and antioxidants (Maxwell *et al.,* 2007). The addition of SP to FT epididymal spermatozoa improved

pregnancy rates following cervical insemination from 3% without SP to 17% with SP (Rickard *et al.,* 2014), signifying the importance of SP in sperm function and a potential role in aiding sperm migration through the reproductive tract.

The mechanism of migration and interaction of ram sperm through the cervix of the ewe has not been fully elucidated. The sperm enters the cervical canal and rapidly encounters the cervical mucus of the cervix (Katz *et al.,* 1997; Bigelow *et al.,* 2004). This is considered one means of sperm selection because the cervical mucus is a more significant barrier to the abnormal sperm with low motility to succeed (Yudin *et al.,* 1989; Katz *et al.,* 1997). SP increase the ability of FT sperm to penetrate the cervical mucus possibly by increasing the motility and velocity of these spermatozoa (Robayo *et al.,* 2008), although the SP components involved in this improved sperm function have not been identified.

From the literature cited above, it is clear that seminal plasma proteins (SPP) have an integral role in assisting sperm function and preserving sperm integrity during cryopreservation. Whilst numerous seminal proteins have been identified, their effect on cervical penetrability through cervical mucus is unknown. Furthermore, the effect of SPP on FT epididymal sperm is not fully elucidated yet. An understanding of the role of SPP on sperm function and integrity including penetrability of cervical mucus may aid the development of cryopreservation protocols for both ejaculated and epididymal spermatozoa to reduce sperm damage.

The aim of this study was therefore to investigate the effect of different fractions of SPP on the motility, acrosome integrity, viability and cervical mucus penetrability on four defined sperm populations:

1) Fresh ejaculated sperm

2) Frozen-thawed ejaculated sperm

3) Fresh epididymal sperm

4) Frozen-thawed epididymal sperm

3.2. Materials and methods

3.2.1. Experimental Design

Ejaculated samples (n = 8) were collected (see section 2, 3) and each sample was split into two aliquots; fresh and frozen. Both of them were subjected into 5 treatments; control, whole SP, > 100kDa, 30-100kDa and < 30kDa SP fractions. In addition, epididymal samples (n = 8) were collected from a local abattoir (see section 3.2.4) and divided into two aliquots; fresh and frozen then both of them subjected to 5 treatments as for ejaculated samples (Figure 3.1). All samples were incubated at 37°C and examined for the following parameters:

1. Motility, acrosome integrity and viability of spermatozoa were measured at 0, 30, 60,180 and 360 minutes.
2. Penetration test for each treatment at 0 and 60 minutes of incubation.

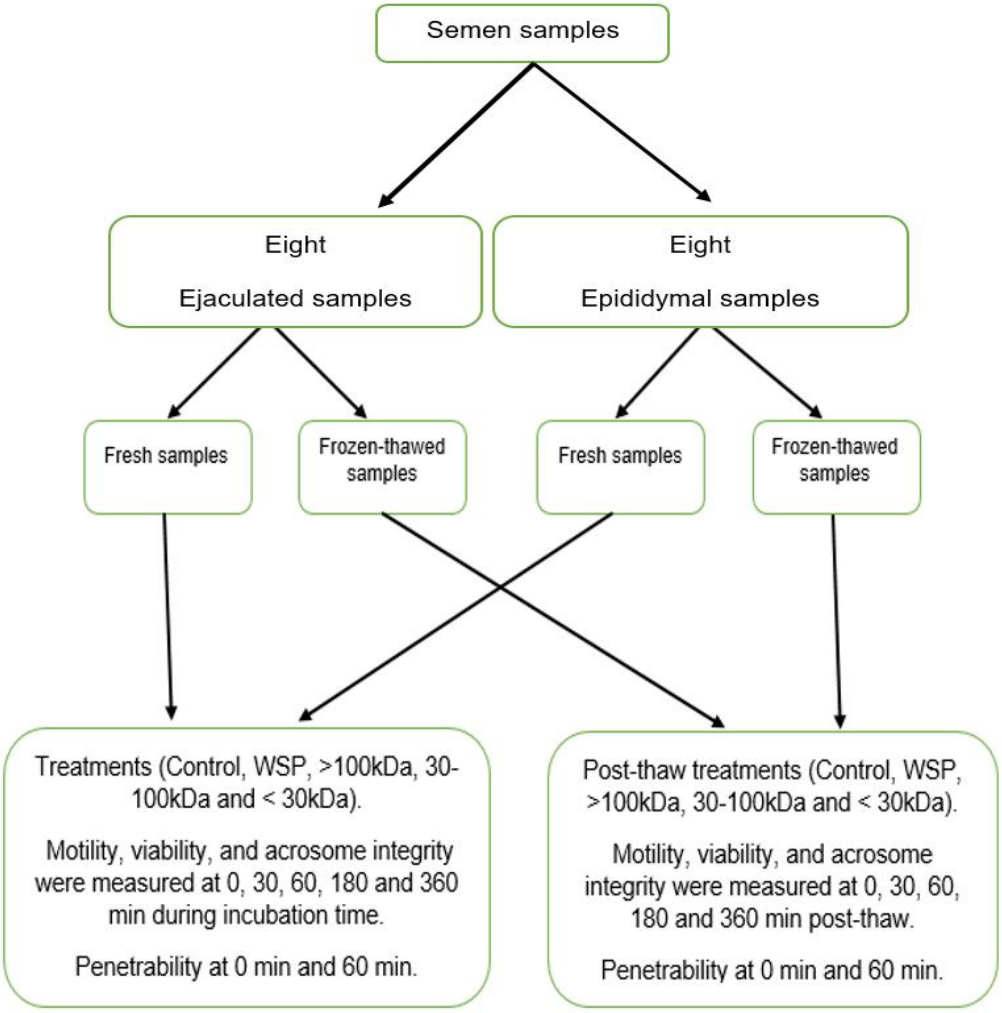


Figure 3. 1. Experimental design: The effect of seminal plasma proteins on ejaculated and epididymal ram spermatozoa.

3.2.2. Preparation of seminal plasma protein fractions

3.2.2.1. Collection of seminal plasma

Samples for the experiment were collected over 12 weeks in June to August (2016) from 6 Texel rams. Following the collection of samples by artificial vagina the ejaculates were directly evaluated for sperm motility and appearance. The samples appeared thick and creamy (high concentration) with a motility score of 3 or more (Table 2.1). Samples were accepted for the experiment when free from blood and/or urine (free from any contaminants). These measurements are considered very important to evaluate semen quality and volume. Seminal plasma samples (n = 150) were collected (section 2.3) three times per week for three months. These samples were centrifuged at 13000 g for 15 min, model 1–14 centrifuge (Centurion scientific Ltd, UK), the supernatant aspirated and spun for a further 15 min at 13000 g to remove cell debris and all remaining spermatozoa. The SP samples were aliquoted into 500 µl in an Eppendorf tube and stored at -80°C, and were thawed and mixed together for protein fractionation.

3.2.2.2. Fractionation of seminal plasma proteins using cut-off filter

Seminal plasma samples (stored as 500 µl aliquots) were thawed and mixed together to 5.0 ml volume and diluted to 15 ml using phosphate buffer saline (PBS) for each assessment. Samples were fractionated in a sequential manner using centrifugal cut-off filter devices with minor molecular mass limits of 30 and 100kDa (Thermo scientific. Rockford, USA) as in (Figure 3.2). The 15 ml sample was initially loaded into a 20 ml filter device with a molecular mass cut off of 100kDa and centrifuged at 4500 g for 30 minutes at 4°C. The retained volume after centrifugation was stored at -80°C as the >100kDa fraction of seminal plasma protein. The portion of samples that passed through the 100kDa cut-off filter was subsequently loaded into a filter device with a molecular mass cut off of 30kDa and were centrifuged as described above. Once more, the retained volume, after centrifugation, was stored as the 30–100kDa fraction and the remaining filtrate was as the <30kDa fraction of seminal plasma protein stored at -80°C. Thus, the whole process yielded three fractions of SP based on approximate molecular mass >100kDa, 30–100kDa and < 30kDa.

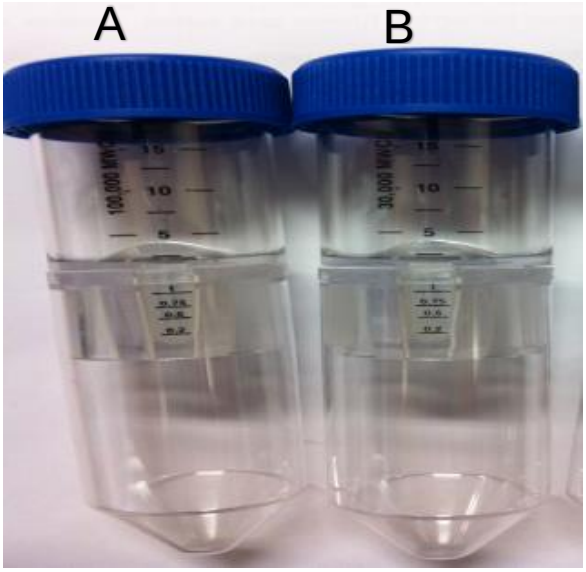


Figure 3. 2. The cut-off filter of seminal plasma (Thermo scientific. Rockford, USA), A) 20 ml of 100kDa filter, B) 20 ml of 30kDa filter.

3.2.2.3. Concentration of seminal plasma protein fractionations

The concentrations of proteins in the seminal plasma were determined according to manufacturers instructions of Pierce BCA protein assay kit No. 23225 (Thermo scientific. Rockford, IL, USA). The fractionated samples of SP (whole SP, >100kDa, 30-100kDa and < 30kDa) were diluted with a phosphate buffer saline (PBS) for four different concentrations 1: 80, 1:40, 1: 20 and 1:10. The samples and the standards (bovine serum albumin (BSA)) provided in the kit were analyzed using a spectrophotometer (Thermo Scientific) to read at 562nm. This method was replicated three times to confirm the concentration of seminal plasma proteins. The results of the samples were compared with the standards to calculate the protein concentration of each sample.

3.2.2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The approximate molecular weight of proteins in the seminal plasma fractions was determined using SDS-PAGE with 10% polyacrylamide gel (Bio-Rad, USA) according to (Laemmli, 1970). Samples of SP fractions of the whole SP, >100, 30-100 and <30 kDa were suspended in 100 µl of PBS. Each sample was mixed with Laemmli buffer 4:1 µl (Sigma Aldrich, USA). Samples were boiled for 5 min and centrifuged at 12000 x g for 30 sec, then loaded (10 µl) onto the gel (Min-Protean TGX precast gels, Bio-Rad Laboratories, Inc., USA). The electrophoresis buffer was Tris /Glycine/ SDS buffer (10x TGS Buffer, Sigma Aldrich, USA), and the final concentration of 1x solution contained 25mM tris,192Mm

Glycine, and SDS 0.1 % w/v) SDS, pH8.3, and the gel was run for 150 min at 30 volts. Afterwards, the electrophoresis gel was stained with Coomassie satin solution (Coomassie brilliant blue R-250 1.0 g, Ethanol 150 ml, Glacial acetic acid 50 ml, deionized distal water 300 ml) for 30 minutes. Following staining, the gel was de-stained with 25% (v/v) methanol, 10% (v/v) acetic acid, and distilled water for 2 hours with shaking. Protein fractions were compared with the precision dual colour standard (Bio-Rad, USA) which contains graduated molecular proteins from 10 to 250kDa to identify the molecular weight of seminal plasma protein samples.

3.2.3. Collection of fresh ejaculates

Semen samples (n=8) were collected during the breeding season from two Texel rams with four ejaculates from each ram using an artificial vagina in the presence of a teaser ewe. Semen samples were assessed directly to measure total motility of sperm. The sperm concentration of the semen samples was determined by hemocytometer. Samples with a thick creamy appearance, with a concentration of spermatozoa of 3 to 5 x 10^9 sperm/ml, and free of blood and urine contaminations were only accepted according to (Evans and Maxwell, 1987; Avdi *et al.,* 2004) (Table 2.1). The total motility of sperm was measured by placing 10 µl of fresh semen on a pre-warmed slide with a coverslip at 37°C using hot stage under a microscope (Olympus CX31, Tokyo. Japan) at magnifying power (100 X) within 3-5 minutes of collection. The samples that had a score of more than 3 (>80 %) were accepted, and less than three were not used in this study Avdi *et al.* (2004) (Table 2.2).

3.2.4. Collection of epididymal spermatozoa

Epididymal spermatozoa samples were collected from testes of Texel rams obtained from a local an abattoir (Euro Quality Lambs Ltd, Craven Arms, Shropshire, UK). After collection testes were covered with gauze moistened with PBS then transported to the lab in a cooling box. Spermatozoa were then extracted from the caudal part of the epididymis. A small incision was made into the cauda epididymis using a sterile scalpel and a 0.1ml syringe filled with 37°C PBS was inserted into the lumen of the epididymis and depressed slowly to flush the epididymis. The luminal fluids of the epididymis were collected in a sterile petri dish at 37°C, then centrifuged at 300xg for 3 minutes to pellet epididymal spermatozoa, and the supernatant decanted and discarded. Epididymal spermatozoa were suspended with 1.0 ml tris citrate glucose (TCG) at 37°C, and the total motility and concentration were assessed as per the procedure for fresh ejaculated samples (see section 3.2.3). Samples which had high motility (>80 %) and concentration 3 to 5 x 10^9 sperm/ml were used.

3.2.5. Treating spermatozoa with SP fractions

After the initial evaluation, fresh samples of ejaculated and epididymal spermatozoa were divided into two equal aliquots. The first aliquot was used for parameters which related to treatments of fresh samples and the second aliquot was used for parameters which related to treatments of cryopreserved samples. The fresh aliquot was centrifuged at 300xg for 3 minutes at 37°C to remove cell debris and seminal plasma. Samples were washed with tris citrate glucose (TCG; 300mM tris, 94.7mM citrate, 27.8mM glucose) at 37°C. Samples of seminal plasma proteins were resuspended in TCG to 3.0 mg/ml for each fraction of proteins. Washed spermatozoa samples were resuspended in TCG as to achieve $50x10^6$ sperm/ml and then mixed with an equal volume of seminal plasma proteins (final concentration 25 x 10^6 sperm /ml, 1.5 mg protein/ml). This created five treatment groups: whole seminal plasma, >100kDa, 30-100kDa, < 30kDa and control without seminal plasma. Control samples were prepared with the same concentration of spermatozoa ($50x10^6$ sperm/ml) and resuspended with an equal volume of TCG. After treatment with SP proteins all the samples were examined under the same environmental conditions at 37^{o}C at 0, 30, 60,180 and 360 minutes for motility, viability, and acrosome integrity. A penetration test was assessed at 0 and 60 minutes on all samples which were incubated at 37°C in a water bath during the period of the assessments.

3.2.6. Preparation of frozen-thawed sperm

Preparation of frozen-thawed sperm was determined according to the method described in section 2.7. Frozen semen samples were thawed and washed using TCG, then diluted to a final concentration of 25 X 10^6 sperm/ml. Each semen sample was treated by SP proteins fractions at post-thaw and assessed as described in section 3.2.5

3.2.7. Penetration of cervical mucus

3.2.7.1. Ewe synchronization and mucus collection

Ewe synchronization and mucus collection were determined according to the method described in section 2.10.1.

3.2.7.2. Penetration test

Penetrability for all types of spermatozoa (fresh and FT ejaculated and epididymal) was determined at 0 and 60 min according to the method described in section 2.10.3. During this experiment natural cervical mucus was used which was collected for the ewes during the period of oestrous.

3.2.8. Proportion of motile spermatozoa

The proportion of motile spermatozoa for all types of spermatozoa (fresh and FT ejaculated and epididymal) were determined according to the method described in section 2.9.1.

3.2.9. Acrosome integrity and viability

Acrosome integrity and viability for all types of spermatozoa were determined according to the method described in section 2.9.3.

3.2.8. Statistical analyses

Whilst the deisgn of this study allows for statistical comparisons between sperm types (ejaculated, epididymal, fresh or frozen-thawed) the aim of the study was to determine the effect of seminal plasma proteins on four defined sperm types. Due to the natural variations in sperm function and integrity that are observed between fresh and frozen-thawed, and ejaculated and epididymal sperm, including sperm type as a variable within the analysis would limit interpretation of the effect of treatment. Consequently each sperm type was analysed independently.

Data were analysed using a repeated measure analysis of variance (ANOVA) with a linear mixed model (REML) Gen Stat (17th edition). To accommodate the experimental design, the random effects were treatment nested within ejaculate nested within ram. The factors used in the fixed model were treatment (SPP fraction) and time (0, 30, 60,180, and 360 min). The fixed model included the treatment*time interaction, and where there was no significant interaction the finxed model was treatment+time. The variables analysed were motility, viability, acrosome integrity, and penetrability through cervical mucus. Post-hoc analyses were performed using the least significant difference (LSD) test. Means were reported with a ± standard error of the mean (SEM) and $P < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Positive and negative control of propidium Iodide (viability)

The results of this technique were a guideline to ensure the accuracy of the results and analysis. Dead spermatozoa were stained by PI stain in red colour as a positive control showed (Figure 3.3). Spermatozoa with non-intact acrosome were stained green with FITC stain (Figure 3.4). These samples were examined under the fluorescence microscope.

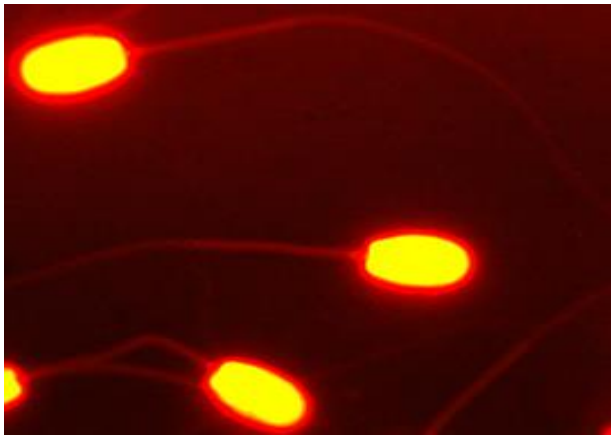


Figure 3. 3. Spermatozoa stained by PI stain as a positive control.

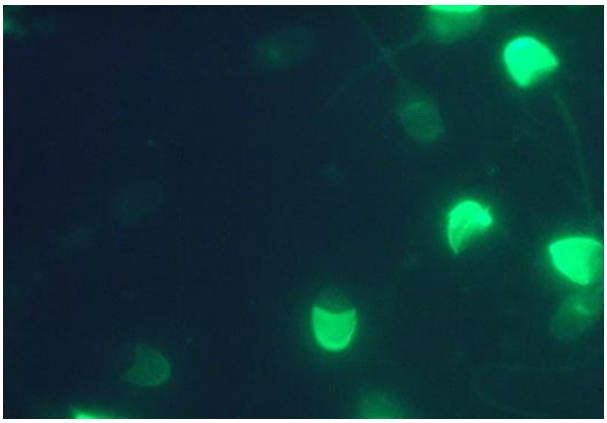


Figure 3. 4. Acrosome integrity of spermatozoa using FITC stain.

3.3.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gel electrophoresis analysis identified the molecular weight of seminal plasma proteins when compared to the standard molecular weight (ladder) (Figure 3.5). The molecular weight of whole seminal plasma proteins ranged from 10 to 250kDa. The proteins bands of >100kDa ranged from approximately 75kDa to 250kDa. The 30-100kDa seminal plasma protein fraction contained proteins of 25 to 100kDa, while proteins within the <30kDa protein fraction ranged from 10 to 30kDa. The slight overlap between fractions indicates

that the protein cut off filters are not highly precise, however the method use was effective at separating out the large, mid, and low molecular weight proteins.

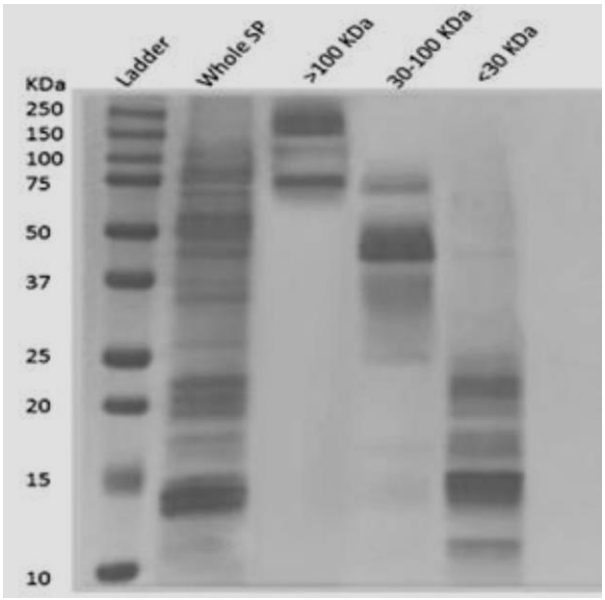


Figure 3. 5. Ram seminal plasma proteins separated by SDS-PAGE. Samples of whole ram seminal plasma proteins bands with different fractions, >100kDa, 30 -100kDa and <30kDa with the ladder as the standard of molecular weight of protein bands stained by Coomassie brilliant blue.

3.3.3. Effect of seminal plasma proteins on freshly ejaculated ram spermatozoa

3.3.3.1 Motility

Motility was affected by SP treatment and time, with a significant interaction ($P < 0.001$). The motility was generally greatest in samples treated with WSP and <30kDa SPP, intermediate in 30-100kDa and >100kDa samples, and lowest in the control sample which did not have any SP although the level of significance differed between time points (Table 3.1). Motility declined over time ($P < 0.001$) in all treatments (Table 3.1).

Table 3. 1. Mean (± SEM) % motility of freshly ejaculated spermatozoa at 0, 30, 60,180 and 360 min incubation without SP (control), with whole seminal plasma (WSP), and with SPP fractions at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Time(min)	Control (8)	WSP (8)	>100kDa (8)	30-100kDa (8)	<30kDa (8)
0	$77.5 \pm 0.945^{b}_{v}$	$83.75 \pm 0.818^{a}_{v}$	$81.25 \pm 1.25^{b}_{v}$	$83.12 \pm 0.915^{a}_{v}$	$84.12 \pm 0.639^{a}_{v}$
30	$70.25 \pm 0.977^{c}_{w}$	$78.37 \pm 1.017^{a}_{w}$	$73.75 \pm 1.25^{bc}_{w}$	$76.62 \pm 1.253^{ab}_{w}$	$78.75 \pm 0.818^{a}_{w}$
60	$54.12 \pm 2.615^{c}_{x}$	$70.62 \pm 1.133^{a}_{x}$	$64.38 \pm 1.475^{b}_{x}$	$70.00 \pm 0.945^{a}_{x}$	$73.12 \pm 0.915^{a}_{x}$
180	$34.00 \pm 2.557^{c}_{y}$	$53.12 \pm 1.716^{a}_{y}$	$45.62 \pm 1.752^{b}_{y}$	$50.00 \pm 1.89^{a}_{y}$	$53.13 \pm 1.875^{a}_{y}$
360	$18.75 \pm 1.25^{d}_{z}$	$38.75 \pm 1.567^{a}_{z}$	$28.12 \pm 0.915^{c}_{z}$	$32.50 \pm 1.336^{b}_{z}$	$37.50 \pm 1.637^{a}_{z}$

a, b, c, and d: values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

v, w, x, y, z: values with different subscripts in the same column are significantly different ($P < 0.001$).

3.3.3.2. Viability

There was no interaction between treatment and time for viability ($P > 0.05$), and therefore data are presented as the effect of treatment across all time points, or effect of time irrespective of treatment. Although viability (mean % ± SEM) tended to be higher in samples treated with WSP and <30kDa SPP, it did not differ significantly between WSP (70.02 ± 2.442), >100kDa (60.45 ± 3.113), 30-100kDa (64.27 ± 2.883), <30kDa (70.65 ± 2.577) and the control (57.42 ± 3.516). Viability (mean % ± SEM) declined significantly over time ($P < 0.001$) being greatest at 0h (81.93± 0.78) and declining at 30 min (77.4 ± 0.92), 1 h (71.25 ± 1.22), 3h (55.75 ± 1.74) and 6h (36.5 ± 2.28).

3.3.3.3. Acrosome integrity

There was no significant interaction between treatment and time ($P > 0.05$), and so these were analysed independently. The acrosome integrity deteriorated over time in all treatment groups ($P < 0.001$), and therefore data are presented as the effect of treatment across all time points, or effect of time irrespective of treatment. The acrosome integrity (mean % ± SEM), did not differ significantly between WSP (75.8 ± 1.70), >100kDa (67.00 ± 2.19), 30-100kDa (69.22 ± 2.00), <30kDa (74.72 ± 1.93) and the control (65.60 ± 2.650). The acrosome integrity (mean % ± SEM) declined significantly over time ($P < 0.001$) being greatest at 0h (83.65 ± 0.66) and declining at 30 min (79.80 ± 0.75), 1 h (74.08 ± 0.87), 3h (63.47 ± 1.36) and 6h (51.35 ± 1.83).

3.3.3.4. Penetrability through cervical mucus

The distance travelled (mean % ± SEM) was greater ($P < 0.001$) in samples treated with WSP and <30kDa SPP at 0 min and 60 min compared to the other treatment groups (Figure 3. 6). Furthermore, the distance travelled at 60 min (4.43 ± 0.11, 4.15 ± 0.12 cm/h) was greater than at 0 min (2.69 ± 0.09, 2.8 ± 0.07 cm/h) in samples treated with WSP or <30kDa SPP respectively ($P < 0.001$).

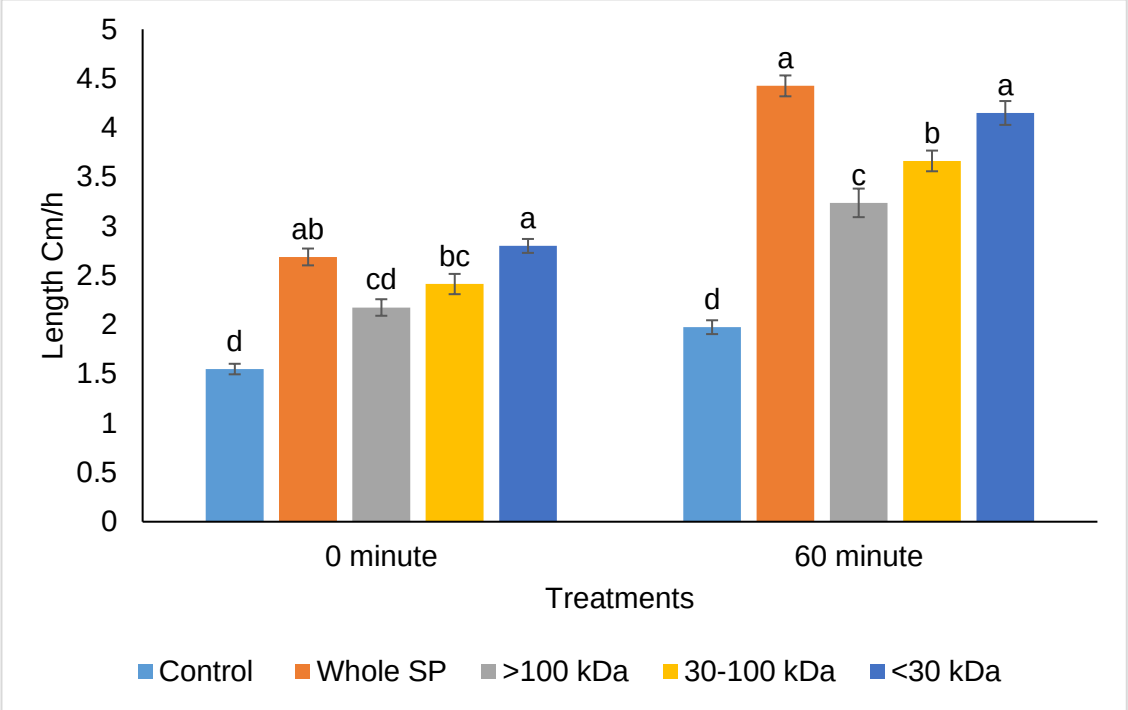


Figure 3. 6. Effect of whole seminal plasma (WSP), different SPP fractions(>100kDa, 30-100kDa,and <30kDa) and without SP (control) on *in vitro* penetration of the cervical mucus by fresh ejaculated spermatozoa at 0 and 60 min incubation at 37°C as measured by centimetre per hour cm/h, (means ± SEM; $P < 0.001$). Within each time point, treatments with different letters (a, b, c, d) differ significantly ($P < 0.001$).

3.3.4 Effect of seminal plasma proteins on frozen-thawed ejaculated ram spermatozoa

3.3.4.1 Motility

The % motility (mean ± SEM) of FT ejaculated spermatozoa was greatest at 0 h (53.30 ± 0.55), declined significantly at 30 min (45.05 ± 0.75), and continued to decline at 1 h (33.80 ± 0.99), 3 h (17.25 ± 0.89) and 6 h (4.87 ± 0.73) ($P < 0.001$). The motility did not differ ($P > 0.05$) between treatments, and therefore data are presented as effect of treatment across all time points, or effect of time irrespective of treatment between ejaculates treated with WSP (34.95 ± 2.77), >100kDa (27.85 ± 2.99), 30 -100kDa (30.97 ± 2.87), <30kDa (34.63 ± 2.83) and no SP (control: 25.87 ± 2.99).

3.3.4.2. Viability

There was a significant interaction between treatment and time for viability ($P < 0.001$). The % viability (mean ± SEM) declined over time in all treatment groups and was significantly higher ($P < 0.001$) in samples treated with WSP and fraction <30kDa SPP, intermediate in 30-100kDa and >100kDa samples, and lowest in the control sample which did not have any SP although the level of significance differed between time points (Table 3.2).

3.3.4.3. Acrosome integrity

There was a significant interaction between treatment and time ($P < 0.001$) for acrosome integrity. The acrosome integrity % (mean ± SEM) of FT ejaculated sperm declined over time in all treatment groups and there was significantly higher proportion of sperms with intact acrosome ($P < 0.001$) in samples treated with WSP and fraction <30kDa SPP compared to the control and other fractions, although the effect of treatment differed between time points (Table 3.3).

3.3.4.4. Penetrability through cervical mucus

There was no interaction between treatment and time, and there was no effect ($P > 0.05$) of WSP, and SPP fractions on penetration values of FT ejaculated spermatozoa. At time 0h the mean (± SEM) distance travelled was 1.2 (± 0.05) cm/h in the control group, 2.04 (± 0.07) in samples treated with WSP, 1.9 (± 0.06) cm/h in <30kDa) 1.76 (± 0.09) in 30-100KDa and 1.58 (± 0.04) in >100KDa. At time 60 min distance travelled range from 1.65 ± 0.06 cm/hr (control) to 2.72 ± 0.086 (WSP) and 2.6 ± 0.07 cm/h (<30kDa). The distance for 30-100 kDa and >100kDa were (2.4 ± 0.07, 2.14 ± 0.05 cm/h) respectively. The overall distance (cm/h ± SEM) travelled at 60min (2.02 ± 0.07) was greater than that at 0 min (1.55 ± 0.05; $P < 0.001$).

Table 3. 2. Mean (± SEM) % viable frozen-thawed (FT) ejaculated spermatozoa at 0, 30, 60,180 and 360 min incubation without SP (control), with whole seminal plasma (WSP), and with SPP fractions at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Time(min)	Control (8)	WSP (8)	>100kDa (8)	30-100kDa (8)	<30kDa (8)
0	$54.50 \pm 1.225^{c}_{v}$	$61.00 \pm 0.845^{ab}_{v}$	$57.38 \pm 1.149^{bc}_{v}$	$59.37 \pm 1.051^{bc}_{v}$	$62.50 \pm 0.982^{a}_{v}$
30	$43.63 \pm 1.017^{d}_{w}$	$55.25 \pm 1.130^{ab}_{w}$	$48.25 \pm 1.065^{c}_{w}$	$51.75 \pm 1.264^{b}_{w}$	$57.13 \pm 1.156^{a}_{w}$
60	$31.00 \pm 1.102^{c}_{x}$	$47.13 \pm 1.274^{a}_{x}$	$36.50 \pm 1.439^{b}_{x}$	$39.87 \pm 1.368^{b}_{x}$	$49.00 \pm 1.592^{a}_{x}$
180	$14.87 \pm 0.833^{d}_{y}$	$33.00 \pm 2.652^{a}_{y}$	$20.38 \pm 1.164^{c}_{y}$	$24.87 \pm 1.008^{b}_{y}$	$34.63 \pm 2.542^{a}_{y}$
360	$7.75 \pm 1.333^{d}_{z}$	$21.00 \pm 2.009^{a}_{z}$	$12.50 \pm 0.627^{c}_{z}$	$15.37 \pm 0.822^{b}_{z}$	$24.75 \pm 1.971^{a}_{z}$

a, b, c and d: values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

v, w, x, y and z: values with different subscripts in the same column are significantly different ($P < 0.001$).

Table 3. 3. Mean (± SEM) % acrosome intact frozen-thawed (FT) ejaculated spermatozoa at 0, 30, 60,180 and 360 min incubation without SP (control), with whole seminal plasma (WSP), and with SPP fractions at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Time(min)	Control (8)	WSP (8)	>100kDa (8)	30-100kDa (8)	<30kDa (8)
0	$56.25 \pm 2.336^{c}_{v}$	$65.00 \pm 1.118^{ab}_{v}$	$59.63 \pm 2.471^{bc}_{v}$	$61.88 \pm 2.207^{ab}_{v}$	$67.13 \pm 1.481^{a}_{v}$
30	$51.00 \pm 2.104^{c}_{w}$	$61.50 \pm 0.824^{ab}_{w}$	$54.13 \pm 2.386^{c}_{w}$	$56.12 \pm 2.074^{bc}_{w}$	$63.13 \pm 1.217^{a}_{w}$
60	$42.50 \pm 2.478^{c}_{x}$	$58.50 \pm 0.802^{a}_{x}$	$47.63 \pm 2.337^{bc}_{x}$	$49.37 \pm 2.146^{b}_{x}$	$58.75 \pm 0.977^{a}_{x}$
180	$31.75 \pm 2.789^{c}_{y}$	$51.25 \pm 1.623^{a}_{y}$	$36.25 \pm 2.396^{bc}_{y}$	$40.00 \pm 2.822^{b}_{y}$	$53.63 \pm 0.962^{a}_{y}$
360	$19.50 \pm 2.171^{c}_{z}$	$38.63 \pm 2.815^{a}_{z}$	$23.00 \pm 1.753^{bc}_{z}$	$26.12 \pm 2.741^{b}_{z}$	$42.88 \pm 1.394^{a}_{z}$

a, b, and c: values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

v, w, x, y, and z: values with different subscripts in the same column are significantly different ($P < 0.001$).

3.3.5. Effect of seminal plasma proteins on fresh epididymal ram spermatozoa

3.3.5.1 Motility

There was a significant interaction between treatment and time ($P < 0.001$). The % motility of fresh epididymal spermatozoa (mean ± SEM) was generally highest in samples treated with WSP and <30kDa SPP, intermediate in 30-100kDa and >100kDa samples, and lowest in the control sample which did not have any SP although the effect of treatment differed between time points ($P < 0.001$). The % motility declined over time in all treatments (Table 3.4).

3.3.5.2 Viability

There was a significant interaction between treatment and time for viability ($P < 0.001$). The % viability (mean ± SEM) declined over time in all treatment groups and tended to be higher ($P < 0.001$) in samples treated with WSP and fraction <30kDa SPP, intermediate in 30-100kDa and >100kDa samples, and lowest in the control sample which did not have any SP although the level of significance differed between time points (Table 3.5).

3.3.5.3 Acrosome integrity

No interaction was observed between treatment and time ($P > 0.05$). The overall % acrosome integrity (mean ± SEM) deteriorated over time being greatest at 0 h (87.92 ± 0.74), and declining at 30 min (83.92 ± 0.72), 1h (78.50 ± 1.04), 3 h (70.05 ± 1.21) and 6 h (57.85 ± 2.13) ($P < 0.001$). The % acrosome integrity (mean ± SEM) did not differ between WSP (81.15 ± 1.36), >100kDa (71.00 ± 2.04), 30-100kDa (74.73 ± 2.17), <30kDa (79.75 ± 2.14) and control (71.43 ± 2.31).

3.3.5.4 Penetrability through cervical mucus

The mean (± SEM) distance travelled by fresh epididymal spermatozoa was greater ($P < 0.001$) in samples treated with WSP, <30kDa SPP and 30-100kDa SPP at 0 min and 60 min post-thaw compared to >100kDa SPP and the control (Figure 3.7). The distance travelled by fresh epididymal spermatozoa was greater ($P < 0.001$) at 60 min (5.11 ± 0.16, 4.34 ± 0.11, 3.38 ± 0.11, 3.01 ± 0.07 cm/h) than 0 min (3.39 ± 0.18, 3.09 ± 0.10, 2.71± 0.10, 2.28 ± 0.10 cm/h) in samples treated with WSP, <30kDa, 30-100kDa and >100kDa, respectively.

Table 3. 4. Mean (± SEM) % motility of fresh epididymal spermatozoa at 0, 30, 60,180 and 360 min incubation without SP (control), with whole seminal plasma (WSP), and with SPP fractions at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Time	Control (8)	WSP (8)	>100kDa (8)	30-100kDa (8)	<30KDa (8)
0	$85.62 \pm 0.625^{a}_{v}$	$88.12 \pm 1.619^{a}_{v}$	$83.75 \pm 0.818^{a}_{v}$	$84.38 \pm 0.625^{a}_{v}$	$85.62 \pm 0.625^{a}_{v}$
30	$76.88 \pm 1.619^{bc}_{w}$	$83.12 \pm 1.619^{a}_{w}$	$73.75 \pm 1.25^{c}_{w}$	$76.25 \pm 1.25^{bc}_{w}$	$79.38 \pm 0.625^{ab}_{w}$
60	$65.00 \pm 2.50^{bc}_{x}$	$76.88 \pm 0.915^{a}_{x}$	$58.75 \pm 1.83^{d}_{x}$	$63.75 \pm 1.567^{c}_{x}$	$69.38 \pm 0.625^{b}_{x}$
180	$40.62 \pm 2.397^{b}_{y}$	$55.62 \pm 3.332^{a}_{y}$	$33.75 \pm 2.059^{c}_{y}$	$33.12 \pm 1.315^{c}_{y}$	$39.38 \pm 1.752^{b}_{y}$
360	$20.00 \pm 2.67^{b}_{z}$	$33.12 \pm 1.619^{a}_{z}$	$7.50 \pm 1.336^{c}_{z}$	$10.62 \pm 0.625^{c}_{z}$	$16.88 \pm 1.619^{b}_{z}$

a, b, c and d: values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

v, w, x, y and z: values with different subscripts in the same column are significantly different ($P < 0.001$).

Table 3. 5. Mean (± SEM) % viability of fresh epididymal spermatozoa at 0, 30, 60,180 and 360 min incubation without SP (control), with whole seminal plasma (WSP), and with SPP fractions at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Time	Control (8)	WSP (8)	>100kDa (8)	30-100kDa (8)	<30kDa (8)
0	$83.50 \pm 1.008^{ab}{}_{v}$	$88.13 \pm 1.008^{a}{}_{v}$	$80.13 \pm 1.394^{b}{}_{v}$	$80.88 \pm 1.663^{b}{}_{v}$	$86.88 \pm 1.432^{a}{}_{v}$
30	$77.38 \pm 1.149^{bc}{}_{w}$	$83.25 \pm 1.206^{a}{}_{w}$	$73.75 \pm 2.218^{b}{}_{w}$	$76.00 \pm 1.680^{b}{}_{w}$	$81.50 \pm 1.165^{ab}{}_{w}$
60	$69.25 \pm 1.849^{b}{}_{x}$	$76.50 \pm 1.150^{a}{}_{x}$	$61.25 \pm 2.128^{c}{}_{x}$	$66.88 \pm 1.641^{b}{}_{x}$	$74.62 \pm 0.905^{a}{}_{x}$
180	$48.63 \pm 1.295^{c}{}_{y}$	$62.63 \pm 1.375^{a}{}_{y}$	$39.50 \pm 1.389^{c}{}_{y}$	$43.63 \pm 1.375^{b}{}_{y}$	$51.25 \pm 2.119^{a}{}_{y}$
360	$29.38 \pm 2.171^{b}{}_{z}$	$42.25 \pm 1.971^{a}{}_{z}$	$17.63 \pm 2.044^{c}{}_{z}$	$20.13 \pm 1.288^{c}{}_{z}$	$29.75 \pm 2.658^{b}{}_{z}$

a, b, and c: values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

v, w, x, y and z: values with different subscripts in the same column are significantly different ($P < 0.001$).

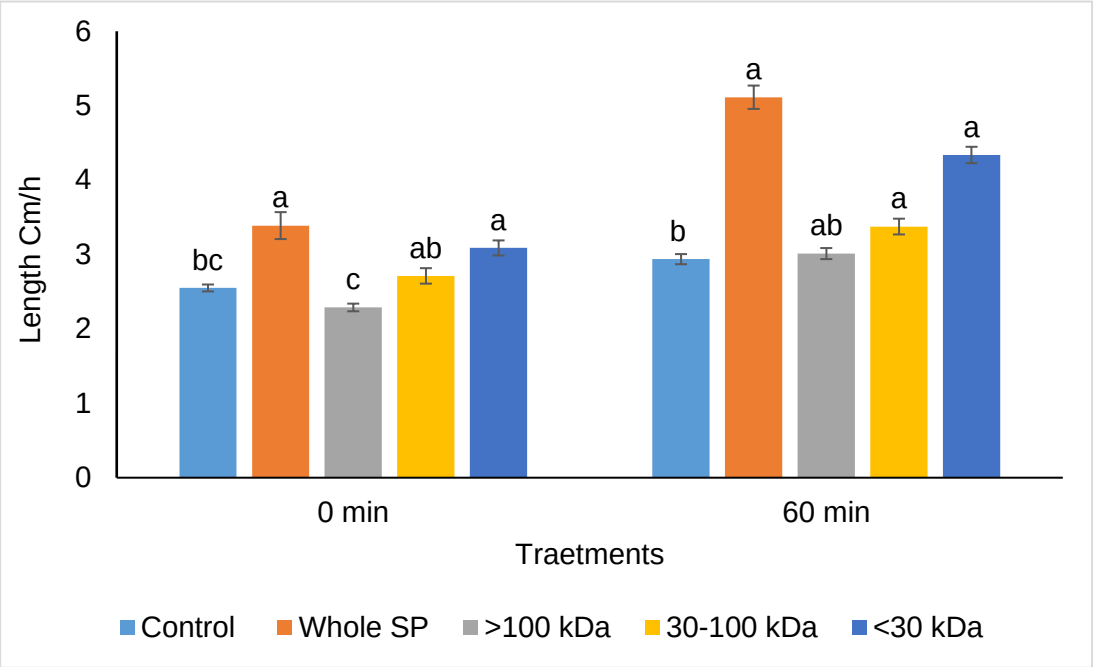


Figure 3. 7. Effect of whole seminal plasma (WSP), different SPP fractions (>100kDa, 30-100kDa,and <30kDa) and without SP (control) on *invitro* penetration of the cervical mucus by fresh epididymal spermatozoa at 0 and 60 min incubation at 37°C as measured by centimetre per hour cm/h, (means ± SEM; $P < 0.001$). Within each time point, treatments with different letters (a, b, c) differ significantly ($P < 0.001$).

3.3.6. Effect of seminal plasma proteins on frozen-thawed epididymal ram spermatozoa

3.3.6.1 Motility

There was a significant ($P < 0.001$) interaction between treatment and time. Generally, % motility in WSP and <30kDa was greater ($P < 0.001$) than the % motility in >100 and 30-100kDa treatments. The % motility of FT epididymal spermatozoa declined significantly over time, although the rate of deterioration was different between treatment groups. Motility was zero at 360 min in all the treatment groups (Table 3.6).

3.3.6.2 Viability

There was no interaction between treatment and time for viability. Moreover, mean (± SEM) % viability did not differ significantly ($P > 0.05$) between the treatment groups and the overall values for WSP, >100kDa, 30-100kDa, <30kDa and the controls were 38.88 ± 2.69, 29.83 ± 2.54, 36.15 ± 2.77, 40.33 ± 2.63 and 30.4 ± 2.49, respectively. Sperm viability deteriorated over time being greatest at 0 h (54.13 ± 1.01) and declining at 30 min (48.00 ± 1.13) 1 h (38.38 ± 1.08), 3 h (24.13 ± 1.00) and 6 h (10.95 ± 0.72) ($P < 0.001$).

3.3.6.3 Acrosome integrity

There was no interaction between treatment and time for the % acrosome integrity (mean ± SEM) of FT epididymal sperm significantly ($P > 0.05$), and therefore data are presented as effect of treatment across all time points, or effect of time irrespective of treatment between samples treated with WSP (56.77± 1.15), >100kDa (50.22 ± 1.39), 30-100kDa (53.48 ± 1.15), <30kDa (55.75 ± 1.25) and control (49.25 ± 1.55). The % acrosome integrity was significantly higher at 0 h (62.13 ± 0.68), and declined significantly at 30 min (58.48 ± 0.64) 1 h (54.15 ± 0.63) 3h (49.30 ± 0.75) and 6 h (41.43 ± 1.07) ($P < 0.001$).

3.3.6.4 Penetrability through cervical mucus

There was no effect ($P > 0.05$) of treatment on the distance travelled through cervical mucus of FT epididymal spermatozoa. At 0 min the mean ± SEM distance travelled ranged from 0.74 ± 0.04 cm/hr (control) to 1.43 ± 0.08 cm/hr (WSP) and 1.36 ± 0.07 cm/h (<30kDa). However, for 30-100kDa and >100kDa were (1.15 ± 0.06, 0.89 ± 0.06) respectively. At 60 min the mean (± SEM) distance travelled ranged from 0.85 ± 0.04 cm/hr (control) to 1.68 ± 0.05 cm/hr (WSP) and 1.53 ± 0.08 (<30kDa). The distance of 30-100kDa and >100kDa were (1.31± 0.06, 1.06 ± 0.07) respectively. There was a significant effect of time on the distance travelled by spermatozoa ($P < 0.001$).The mean (± SEM) distance (cm/h) travelled at 60 min (1.25 ± 0.43) was greater than that at 0 min (0.9 ± 0.07).

Table 3. 6. Mean (± SEM) % motility of frozen-thawed (FT) epididymal spermatozoa at 0, 30, 60,180 and 360 min incubation without SP (control), with whole seminal plasma (WSP), and with SPP fractions at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Time(min)	Control (8)	WSP (8)	>100kDa (8)	30-100kDa (8)	<30kDa (8)
0	$41.25 \pm 0.818^{a}_{w}$	$41.25 \pm 0.818^{a}_{w}$	$40.62 \pm 0.625^{a}_{w}$	$40.62 \pm 0.625^{a}_{w}$	$40.62 \pm 0.625^{a}_{w}$
30	$32.50 \pm 0.945^{cd}_{x}$	$36.25 \pm 0.818^{a}_{x}$	$30.00 \pm 0.945^{d}_{x}$	$33.13 \pm 0.915^{bc}_{x}$	$35.62 \pm 0.625^{ab}_{x}$
60	$22.50 \pm 1.336^{b}_{y}$	$29.38 \pm 1.475^{a}_{y}$	$18.12 \pm 2.1^{c}_{y}$	$24.38 \pm 1.133^{b}_{y}$	$28.75 \pm 0.818^{a}_{y}$
180	$6.00 \pm 1.165^{bc}_{z}$	$13.75 \pm 1.497^{a}_{z}$	$5.00 \pm 0.945^{c}_{z}$	$8.50 \pm 0.802^{b}_{z}$	$12.88 \pm 1.481^{a}_{z}$
360	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

a, b, c and d: values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

w, x, y and z: values with different subscripts in the same column for each treatment are significantly different ($P < 0.001$).

3.4. Discussion

This study aimed to investigate the effect of SPP on four ram spermatozoa populations: fresh ejaculated, FT ejaculated, fresh epididymal and FT epididymal spermatozoaIn general, the results obtained have demonstrated that SPP fractions, when added in the semen extender, improved sperm motility and viability, reduced acrosome damage, and enhanced the ability of spermatozoa to penetrate cervical mucus *in vitro*. The treatments used in this study were either WSP or SP fractions which contain proteins >100kDa, 30-100kDa and <30kDa. Although all SPP treatments improved sperm functions, WSP and <30kDa were more effective than other fractions. Previously the beneficial effect of SPP on fresh and FT spermatozoa has been reported mainly in the ram and the bull (Maxwell *et al.,* 2007; Juyena and Stelletta, 2012; Rickard *et al.,* 2014; Soleilhavoup *et al.,* 2014; Ledesma et al 2016 and 2017), however, the reports on the effect of SP proteins on the epididymal spermatozoa and sperm migration through cervical mucus are limited.

The improved motility of fresh ejaculated, fresh epididymal and FT epididymal spermatozoa with WSP treatment may be explained by SP components including sugars, citric acid, prostaglandins, salts and lipids but mostly, and importantly, proteins that could recover the sperm membrane permeability characteristic of a live cell (Leahy and de Graaf, 2012; Druart *et al.,* 2013). Treatment with SPP (<30kDa) had similar effects to WSP suggesting that the effects of WSP on motility is mitigated by low molecular weight proteins including ram seminal vesicle protein (RSVP) 20 and RSVP 24 that are readily adsorbed by the membrane aiding protection of the sperm membrane and improving sperm motility (Barrios *et al.,* 2005; Xie *et al.,* 2016). In addition, Tris citrate glucose (TCG) was considered to be essential to preserve sperm motility when only interacting SPP fractions were added (Naing *et al.,* 2011). It has been stated that glucose has a high ability to protect cells from the stress by heat and H_2O_2 through providing NADPH by its metabolism via the cycle of pentose phosphate (Lord-Fontaine and Averill-Bates, 2002). This maintained the idea that SP not only has protective proteins, but also play an essential role in maintaining the metabolic support for sperm motility, and this corroborated with the results of Bernardini *et al.* (2011) who used fructose.

Conversely, the motility of FT ejaculated spermatozoa was not affected by treatment, despite the fact that the effect of treatments was better than the control group. Unlike freshly ejaculated sperm, FT sperm have endured the stresses associated with cryopreservation. The low ratio of cholesterol to phospholipids in the ram sperm membrane increases sensitivity to cold shock and osmotic stress (Muino-Blanco *et al.,* 2008; Bernardini *et al.,* 2011). Whilst the addition of

SPP can reduce the impact of cold shock through delayed alterations in the membranes of FT spermatozoa (Elhajj Ghaoui *et al.,* 2007; Domínguez *et al.,* 2008), the results of the present study suggest that the addition of SP or SPP could not mitigate the effect of cryopreservation on motility. Additionally, FT ejaculated spermatozoa were cryopreserved in the presence of SP, then washed at thawing prior to addition of WSP or SPP. It is possible that SPP was already bound to the sperm membrane post-thaw, limiting the effect of SPP fractions on motility. Freezing and thawing process increase osmotic stress on sperm membrane, addition WSP or SPP fractions to ejaculated ram spermatozoa reduced the resistance to hypotonic stress (Tsikis *et al.,* 2018), which could explain the non-significant results in this study. Different breeds of sheep can show significant difference in SPP molecular weight (10 to 334kDa), however, most effective proteins were in the range of 20, 22, 25 and 43 kDa (Carvajal-serna *et al.,* 2018).

Furthermore, it could be related to the decrease of antioxidant level during cryopreservation and thawing which is associated with an increase in lipid peroxidation due to high production of reactive oxygen species (ROS), leading to a decrease in sperm motility (Bansal and Bilaspuri, 2008; Bucak *et al.,* 2008). These results in agreement with Al-Essawe *et al.* (2018) who stated that adding SP on post-thaw stallion sperm did not have an advantageous effect on sperm quality which suggestion inability of SP to reduce the effect of cryopreservation on sperm. The authors also reported that SPP supplementation pre-freeze reduced the activity of mitochondria of bovine sperm (Al-Essawe *et al.,* 2018). It has been stated that phosphorylation of protein at residues of tyrosine rises in numerous species of animal during cryopreservation (Barbonetti *et al.,* 2010), and the sperm tail is highly differentiated which is the primary location of tyrosine phosphorylation, and its role is thoroughly related to the motility of sperm (Naresh and Atreja, 2015). Additionally, bovine SP proteins BSP1, BSP3, and especially BSP5 have an essential role in fertilisation (Manjunath *et al.,* 2009). BSP5 proteins (28-30kDa) are predominately related to bull fertilising ability through their role in the AI industry as fertility associated antigen (FAA) (McCauley *et al.,* 1999; Sutovsky and Kennedy, 2013). Pande *et al.* (2018) stated FAA-positive bulls had been related with enhanced freezability, and improved conception rates (Bellin *et al.,* 1998; Karunakaran and Devanathan, 2017; Singh *et al.,* 2017). It has been stated that mitochondrial proteins can be oxidised through heat stress specifically the proteins with low molecular weight 24 kDa (Mujahid *et al.,* 2007).

Therefore, supplemented sperm cell with these proteins (<30kDa) plays an important role to protect the mitochondria and improve motility, as confirmed previously in bull (Ivanova-Kicheva and Dimov, 2011; Krishnan *et al.,* 2016; Del Valle *et al.,* 2017; Karunakaran and Devanathan, 2017), with stabilising sperm plasma membrane over the acrosome and protection following

freezing-thawing. Furthermore, 28-30kDa proteins are proposed to maintain viability and protect the sperm membrane against LPO and oxidative stress (Karunakaran *et al.,* 2012; Krishnan *et al.,* 2016; Patel *et al.,* 2016). The epididymal spermatozoa do not have the coating with SPP on their membrane, and the cryodiluent could not simply provide protection from the cryodamage. This could explain the death of frozen-thawed epididymal samples at 6h after incubation observed in this study.

The observed lack of effect on the acrosome integrity and viability within fresh ejaculated spermatozoa may be explained on the basis that despite washing, freshly ejaculated spermatozoa are likely to have SPP bound to their membrane (Druart *et al.,* 2013), and the sperm may not lack important proteins associated with acrosome integrity and are therefore unaffected by treatment (Luna *et al.,* 2015). The presence of polyunsaturated fatty acids (PUFAs) in the plasma membrane of mammalian spermatozoa gives the membrane flexibility and fluidity which increase the susceptibility of the sperm membrane to oxidative stress. In addition, the presence of double bonds in these molecules makes the PUFAs susceptible to free radical attack which intiates the lipid peroxidation cascade. This results in the subseqent loss of membrane and morphological integrity, reduced cell functions, and reduced sperm motility and viability (Bansal *et al.,* 2010). Alternatively, this could be due to the response of sperm membrane to interaction with the protein fraction because it is notoriously variable (Muiño-Blanco *et al.,* 2008). Furthermore, the main effect of oxidative damage on sperm membrane was on the unsaturated carbons in PUFA and proteins, which could have a damaging effect on the hydrophobic interaction of lipid-protein in the bilayer (Paul *et al.,* 2017).

The improved acrosome integrity and viability of FT ejaculated samples following treatment by WSP and SPP can be attributed to the rapid interaction and adsorption of the components of these SP fractions particularly <30kDa by the sperm membrane after cryopreservation (Goularte *et al.,* 2014). The effects of WSP and SPP fractions contribute to the protein structure surrounding the spermatozoon in a way similar to that reported previously for fibronectin, stabilizing membrane phospholipids and cytoskeleton for bovine SP proteins (Manjunath and Therien, 2002; Leahy *et al.,* 2010; Xie *et al.,* 2016) and for RSVP14 and RSVP20 (Barrios *et al.,* 2005; Muiño Blanco *et al.,* 2008).

This could explain the protective effect of these proteins in SP against cold-shock injury at cryopreservation to the membranes of ram spermatozoa. The components of SP were diluted during the cryopreservation process which reduced the level and effect of SPP on sperm function. The improved acrosome integrity and viability of spermatozoa when supplemented with SPP fractions may have been due to the lower relative abundance of small molecular

weight factors that also have a direct damaging effect on sperm function post freeze thaw (García-López *et al.*, 1996). Furthermore, low molecular protein such as 30.05kDa was detected at a high level in extracts of liquid stored semen than in fresh semen sample. This could be considered an indicator of the critical role of this protein to reduce the destabilization and any decrease in plasma membrane integrity (Paul *et al.*, 2017).

The lack of effect of treatment on FT epididymal spermatozoa is interesting and may be explained by the slow rate of decline in acrosome integrity over time from 62% post-thaw to 41% at 6h post-thaw compared to 62% post-thaw and 30% 6h post-thaw in FT ejaculated samples. It is possible that epididymal sperm are not subjected to acrosome damage during cryopreservation due to the presence of low molecular proteins (17and 23kDa) (Gatti *et al.,* 2000). The viability of fresh epididymal spermatozoa improved following treatment with WSP and SPP. While these spermatozoa do not suffer from cryodamage and have some binding proteins originating from epididymal secretions (Watson, 2000; Pini *et al.,* 2016), they still lack in some membrane-bound proteins that are derived from the SP (Pini *et al.,* 2016) only. Consequently, treatment with WSP and SPP may aid viability through interaction with proteins and other SP components such as antioxidants. Epididymal spermatozoa are not affected by auto-destructive activity through capacitation (efflux of choline phospholipid and cholesterol from the membrane during ejaculation) (Graham, 1994; Maxwell *et al.,* 1999) which may also contribute to improving the viability of epididymal spermatozoa and this is consistent with the finding of Ehling *et al.* (2006). The viability of FT epididymal samples post-thaw was extremely low, at approximately 35% for all samples, suggesting that cryopreservation was exceptionally detrimental to epididymal sperm which is consistent with previous findings (Thuwanut and Chatdarong, 2009). It is possible that the magnitude of cryodamage to epididymal spermatozoa was too high to be compensated by SPP treatments.

In the present study, SPP treatment improved the passage of fresh ejaculated and fresh epididymal ram spermatozoa through the natural cervical mucus of the ewe. The increased penetration at 60 min compared to 0 min post-thaw could be related to the longer duration of exposure of spermatozoa to SPP fractions thus allowing interaction and adsorption of the SP proteins by the sperm membrane (Maxwell *et al.,* 1999) which improved the ability of spermatozoa to penetrate the cervical mucus. In addition to the effect of exposure time, there could also be some components of SP fractions responsible for the improved ability of sperm to penetrate the cervical mucus. Previous studies have shown that the low molecular weight SP protein fractions (17kDa and 23kDa) can be directly connected with specific domains of the sperm plasma membrane and might have a role in sperm motility (Gatti *et al.,* 2000; El-Hajj Ghaoui *et al.,* 2007; Bernardini *et al.,* 2011; Gwathmey *et al.,* 2018). SP treatment has been

shown to improve the penetrability of cervical mucus by buffalo (Arangasamy *et al.,* 2005) and macaques spermatozoa (Tollner *et al.,* 2008). However, in the present study significant increase in penetration through cervical mucus was observed only for the fresh but not for FT spermatozoa. Although FT semen penetrated mucus with different distance, there was no significant for the effect of SPP supplementation. Addition of SPP has not mitigated the negative effect of cryopreservation on spermatozoa penetration in the cervix.

Our results did not agree with those of some other studies that reported frozen-thawed ram spermatozoa were capable of improving their ability to pass through the cervical mucus when accompanied by SP. However, our results showed an improvement though not statistically significant for penetration of the cervical mucus with SPP fractions. These differences between our and the other studies could be due to either the concentration of SPP fractions or the time of incubation (Maxwell *et al.,* 1999; Barrios *et al.,* 2005). Equally, this might be due to the effect of excessive levels of ROS that reduce the motility and cause damage to the sperm membrane (Agarwal, 2005). SP and SPP fractions improved acrosome integrity and viability but not penetrability of frozen-thawed spermatozoa. These results could be in agreement with other reports which did not relate the penetration of cervical mucus with an increase in motility and/or velocity (Robayo *et al.,* 2008; Rickard *et al.,* 2014).

3.5. Conclusion

WSP and <30kDa SPP fractions were more effective than other fractions in improving the sperm function and integrity although the effect of SPP treatment varied with sperm type and was not consistent for all parameters. This study highlights the factors that need to be considered, such as the source of spermatozoa and the cryopreservation protocol when using SPP to improve sperm function and integrity. Whole SP and <30kDa were equally effective at improving sperm integrity highlighting that SPP <30kDa play an essential role in sperm function. The importance of low MW SPP, such as ram seminal vesicle proteins (RSVP) is further supported by their effect on epididymal spermatozoa, which have not been exposed to SP previously. This experiment could not identify the specific proteins that improve all sperm types and that perhaps trying to identify proteins to aid sperm function is not ideal. The main limitations associated with trying to find one protein instead of antioxidants or other molecules that protect sperm and the proteins on the sperm membrane (i.e. antioxidants). Overall, the results of this study can assist in the identification of novel but critical proteins that may be involved in sperm maturation, function and integrity. To improve the integrity of FT ram spermatozoa need more investigation about the reasons that caused a limitation on sperm functions in the present study. This study highlights the potential importance of low molecular weight and non-protein molecules such as antioxidants in sperm function.

Chapter 4. Effect of antioxidants supplementation pre-freeze and post-thaw on the integrity of cryopreserved ram spermatozoa

4.1. Introduction

As shown in Chapter 3, there was a harmful effect of cryopreservation on ram sperm integrity which could be reduced by the addition of whole seminal plasma and low molecular weight seminal plasma protein fractions which contained 10-30kDa proteins. Ram sperm membrane integrity reduces due to the stress of cooling, freezing and thawing resulting in reduced sperm fertilizing ability. This cold shock and ROS production causes stress on the sperm membrane (Zhu *et al.,* 2017). The effect of ROS damage on the sperm membrane is induced by oxidative damage of polyunsaturated fatty acids which bind to phospholipids in the sperm membrane, consequently causing lipid peroxidation (Irvine, 1996). The high quantity of polyunsaturated fatty acid in the sperm membrane increases the susceptibility to lipid peroxidation by ROS (Osipova *et al.,* 2016; Tvrdá *et al.,* 2016). The damaging effect of lipid peroxidation on the sperm membrane includes loss of intracellular enzymes, damage to sperm DNA, loss of sperm motility (Bansal and Bilaspuri, 2011), and leakage in sperm-oocyte fusion (Aitken *et al.,* 1998). Antioxidants are compounds that prevent the production of ROS or reduce their actions, and protect the sperm from ROS induced damage. Thiol groups also act as a defence against oxidative stress in sperm cells by detoxifying and antioxidising ROS (Bansal and Bilaspuri, 2008). Previous studies have shown that inclusion of antioxidants in cryopreservation extenders increases factors such as motility and membrane integrity after thawing in different species including ram sperm (Bucak *et al.*, 2007). Supplementation with these antioxidants prior to the cryopreservation process may improve cryopreservation methods used in the sheep industry (Bucak *et al.,* 2010).

The antioxidant defence system in mammalian sperm cells is formed of a variety of enzymes including superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase as well as non-enzymatic components like α-tocopherol, methionine, and ascorbic acid (Bucak *et al.,* 2012). The limited ability of sperm to biosynthesise (Aitken, 1995), coupled with the reduction in antioxidant concentrations present in the semen after dilution, reduces the constructive effect of these endogenous antioxidative defences. Therefore, even a small addition of antioxidants to cryopreservation media may have a positive effect on sperm function.

Vitamin C (also termed ascorbic acid and ascorbate) is a water-soluble antioxidant present in seminal plasma and has the ability to reduce ROS (Agarwal and Sekhon, 2010; Asadpour *et*

al., 2011), to H_2O, producing oxidized forms of vitamin C that are moderately stable and do not cause cell destruction (Figueroa *et al.*, 2018). Gangwar *et al.* (2015) reported that the vitamin C concentration in SP is 10 times higher than in blood plasma (360 vs 40 µmol/l), and it has an important role as an antioxidant in extracellular fluid (Padayatty *et al.*, 2003). Through the synergic effect with enzymatic antioxidants it can neutralize the oxidative damage induced by cryopreservation thereby improving sperm motility (Ashamu *et al.*, 2010; Martínez-Páramo *et al.*, 2012). Previous studies have shown that the vitamin C supplementation of semen extender at pre-freezing improves post-thaw sperm integrity. The positive effect of vitamin C is observed in frozen-thawed bull sperm using concentrations of 2.5 mM (Asadpour *et al.,* 2011; Eidan, 2016), 10 mM (Paudel *et al.*, 2008), and 4.5 mg/ml (Hu *et al.,* 2010), in buffalo sperm using 2.5 µM vitamin C (Amini *et al.*, 2015; Sandeep *et al.*, 2015) , and buck sperm using 56.78 µM (Gangwar *et al.*, 2015) and 0.1006 mg/ml (Brito *et al.,* 2017). Research reporting the effect of vitamin C in ram spermatozoa is confounding. Whilst vitamin C has a significant effect on motility, viability, morphology and acrosome integrity in cooled ram semen (Azawi and Hussein, 2013), no effect of vitamin C was observed in cryopreserved ram semen (Sanchez-Partida *et al.*, 1997). Furthermore, at concentrations greater than 50mM, vitamin C had a detrimental effect on sperm function (Sanchez-Partida *et al.,* 1997).

Cysteine is an amino acid considered to be a non-enzymatic antioxidant. It has a low molecular weight and contains a thiol group allowing it to enter sperm cells where it acts as an intracellular glutathione precursor (Çoyan *et al.,* 2011; Topraggaleh *et al.,* 2014). The concentration of cysteine in human seminal plasma is reported at 0.41µM/ml, and this concentration reduced to 0.29 µM/ml in oligozoospermic (Silvestroni *et al.*, 1979). Cysteine protects sperm lipid membranes by indirect radical scavenging through improved intracellular glutathione (GSH) biosynthesis (Bansal and Bilaspuri 2011). It is regularly used in electron transmission reactions and helps in enzymatic catalytic responses (Piste, 2013). It has been stated that cysteine can protect chromatin composition and the integrity of boar sperm membrane during liquid preservation (Szczęśniak-Fabiańczyk *et al.,* 2003). It has also been reported that cysteine has a cryoprotective effect through pre-freeze supplementation to prevent acrosome damage, improving viability, and reducing the total abnormality count as well as improving sperm motility in freeze-thawed goat semen (5mM) (Bucak and Uysal, 2006), bull semen (5mM) (Uysal and Bucak 2007), and after liquid storage of ram semen (5mM) (Bucak and Tekin, 2007; Tonieto *et al.,* 2010).

Taurine is a sulfonic amino acid and non-enzymatic scavenger which is found in oviduct fluid and epididymal fluid. It has been stated that the concentration of taurine in the human sperm seminal plasma is 0.79 µM/ml and this concentration reduced to 0.67µM/ml in oligozoospermic

(Silvestroni *et al.,* 1979). Taurine has a significant role in protecting spermatozoa against ROS and lipid peroxidation when exposed to aerobic conditions and storage (Foote *et al.,* 2002; Bucak and Tekin, 2007). Kirk and Kirk (1994) stated that taurine has an ability to regulate the electrolytes such as magnesium and potassium inside the cell which assists in the movement of calcium, potassium and sodium across the cell plasma membrane, and acts as a capacitation factor, and sperm motility factor (Yang *et al.,* 2010). The addition of taurine results in improved motility of rabbit (Alvarez and Story, 1983), and rat (Sanchez-Partida *et al.,* 1997) spermatozoa. Taurine has been supplemented to cryopreservation media in human (Agarwal and Allamaneni, 2004), ram (Bucak *et al.,* 2008), goat (Bucak and Uysal, 2007), boar (Gutiérrez-Pérez *et al.,* 2009; Hu *et al.,* 2009, Paál *et al.,* 2018), dog (Martins-Bessa *et al.,* 2009), buffalo (Reddy *et al.,* 2010; Chhillar *et al.,* 2012), bull (Uysal and Bucak, 2007), and turkey (Slanina *et al.,* 2018) spermatozoa, with a view to improving particular spermatozoa characteristics after thawing and consequently improve fertilizing ability.

These antioxidants are necessary to maintain sperm integrity and prevent lipid peroxidation of spermatozoa (Gungor *et al.,* 2017). The most vital observation of the previous studies was the effect of pre-freeze supplementation of these antioxidants (vitamin C, cysteine, and taurine) on the integrity of frozen-thawed ram spermatozoa and other species. It has been stated in several species that sperm damage also occurs during thawing such as DNA damage and oxidisation and reducing viability (Pérez-Cerezales *et al.,* 2009). Therefore, it may be beneficial to treat semen samples with antioxidants after thawing to help reverse or prevent damage.

Low concentrations of ROS is one of the main requirements to stimulate sperm capacitation (Aitken, 2017). Consequently excessively high levels of antioxidants can actually be detrimental to sperm function (Salmani *et al.*, 2013).Previous studies did fail to determine the optimal timing and concentrations of each type of antioxidants that can protect sperm from the harmful effects of ROS and improve sperm integrity (Table 1.6).

Therefore, there is a need to determine the potential of these antioxidant in cryopreserved ram semen, and in particular to ascertain the optimal concentration and timing of supplementation. The novelty of this study was to identify the effect of cysteine, taurine and vitamin C on cryopreserved ram semen integrity at different concentrations (0.0, 0.5 and 1.0 mg/ml) either pre-freeze, post-thaw or both pre-freeze and post-thaw. This will optimise the use of these antioxidants and also highlight their importance in preventing sperm damage. Whilst currently there are numerous reports of the potential of these antioxidants, they are still not used commercially in semen extenders, possibly due to a lack of an optimised protocol. Furthermore, there is a requirement for investigation into the mode of action of these antioxidants so that the

full potential can be utilised in semen freezing protocols. A reliable, defined protocol for their use would advance the storage of ram semen and potential improve fertility rates with frozen-thawed semen, thereby advancing the sheep AI industry.

4.2. Materials and methods

4.2.1. Animals and diets

This chapter included three experiments. For each experiment three mature (2 - 4years old), Texel rams with an average body weight of 84 kg and body score condition of 3-4 were used throughout 16 weeks from September to December during breeding season. Rams used in this experiment were obtained from Harper Adams University flock. Rams were housed individually in metal pens bedded with straw during the period of the study. Rams were fed 1 kg/day of concentrate feed specific for rams (Wynnstay Ram Master Coarse Mix 2281) with *ad libitum* access to straw and fresh water.

4.2.2. Sample preparation

In this chapter, three experiments were carried out separately to determine the effects of the antioxidants vitamin C (ascorbic acid), cysteine, or taurine (Sigma Aldrich, UK). Antioxidants were as powder and prepared in the lab with a stock solution in Phosphate-buffered saline PBS (20mg/10ml), and were split into small aliquots with 1.0 ml. Ejaculates were collected during the breeding season from three Texel rams using an artificial vagina. For each experiment nine semen samples were collected (3 ejaculates per ram). All samples were collected in the morning twice weekly. Samples were transported directly to the lab after collection.

Each semen sample for each experiment was diluted 1:4 in Tris-Citrate-Glucose cryodiluent, (300 mM Tris, 94.7 mM citric acid, 27.8 mM D-Glucose, 15% egg yolk, 5% glycerol). Semen samples were analysed in a similar manner for each experiment and the concentration of each sample was determined using a haemocytometer. Sperm concentration was normalized to $50X10^6$ sperm/ml by adding PBS. These were split into three aliquots of volume 1ml in a 2ml Eppendorf tube and chilled for 2h at 4ºC. Each aliquot of semen was treated pre-freeze (before cooling) with one type of antioxidant at one of three final concentrations: 0.0, 0.5, or 1.0 mg/ml. After the treatment by an antioxidant, semen sample was pelleted in dry ice 200µl sperm/ml, and then stored in liquid nitrogen at -196 °C until further assessment. Cryopreserved samples were thawed in a water bath in a clean dry glass test-tube with vigorous shaking for 1min at 37°C. Immediately after thawing, samples were centrifuged at 300xg for 3 min to remove prior residual antioxidant. The supernatant was discarded and the pellet resuspended to same volume of the pellet in tris-citrate glucose buffer (TCG; 300 mM Tris, 94.7 mM citric acid, 27.8 mM D-Glucose). Next resuspended samples were supplemented with the same type of antioxidants generating seven treatments:

1) Control 0.0 mg/ml pre-freeze (PF) was treated post-thaw (PT) by a) 0.0 mg/ml, b) 0.5 mg/ml, c) 1.0 mg/ml.

2) PF 0.5 mg/ml was treated PT by a) 0.0 mg/ml, b) 0.5 mg/ml.

3) PF 1.0 mg/ml was treated PT by a) 0.0 mg/ml, b) 1.0 mg/ml.

Therefore, there were a total seven treatments per antioxidant: 1) Control 0.0 mg/ml, 2) PF 0.5 mg/ml, 3) PF 1.0 mg/ml, 4) PF 0.0 + PT 0.5 mg/ml, 5) PT0.0 + 1.0 mg/ml, 6) PF 0.5 + PT 0.5 mg/ml, and 7) PF 1.0 + PT 1.0 mg/ml. All semen samples were maintained in a water bath at 30ºC for the duration of the experiment. Post-thaw sperm motility, viability, acrosome integrity and ROS production were measured at all time points, and the penetrability was measured just at 60 min (Figure 4.2).

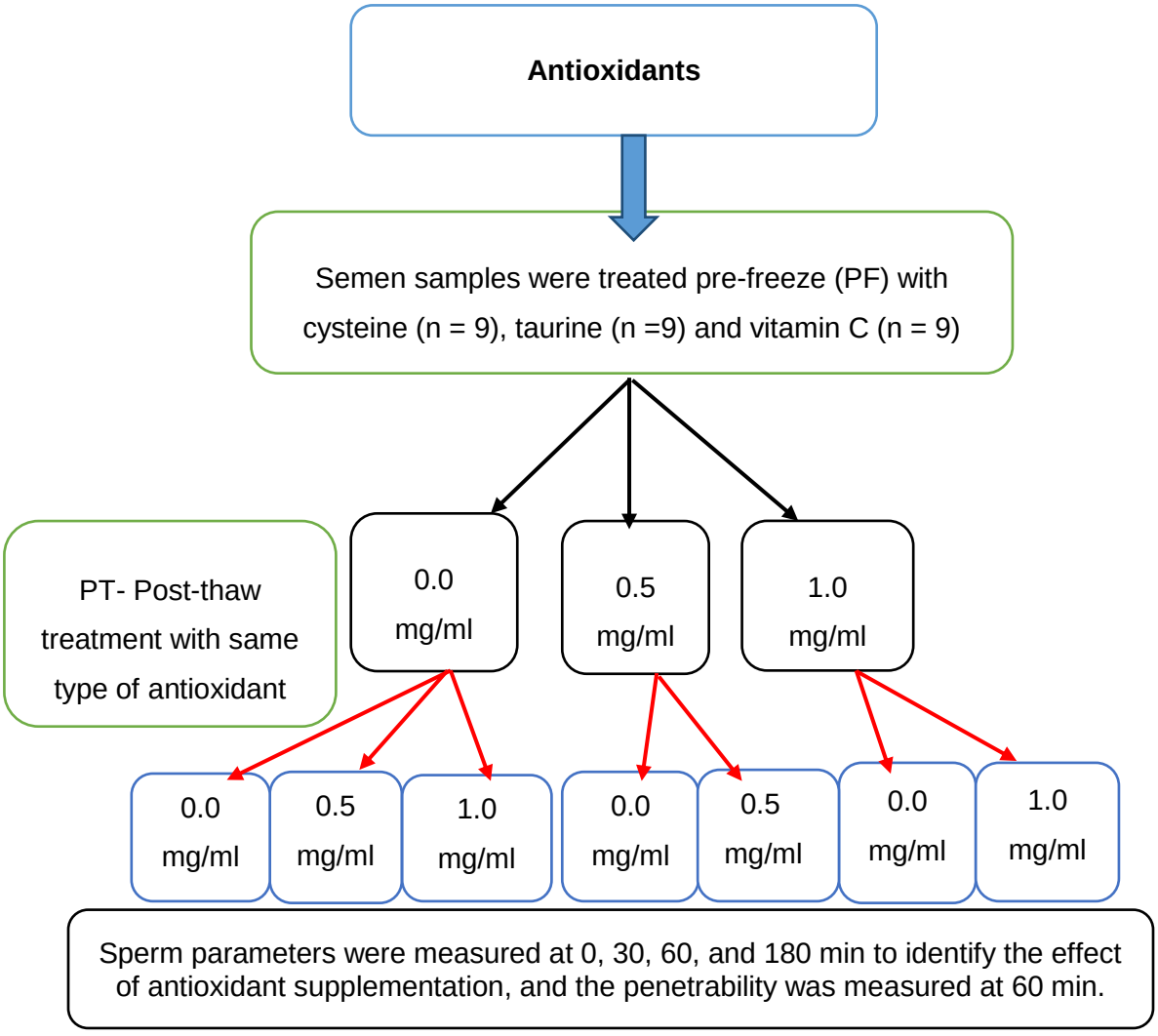


Figure 4. 1. Experimental design of the effect of antioxidants on frozen-thawed ram sperm.

4.2.2.1. Proportion of motile spermatozoa

Proportion of motile spermatozoa was determined according to the method described in section 2.9.1.

4.2.2.2. Acrosome integrity and Viability

Acrosome integrity and viability were determined according to the method described in section 2.9.3.

4. 2.2.3. Penetrability

Sperm penetrability through the artificial mucus was determined according to the method described in section 2.10.2 and 2.10.3. The penetrability was measured in this study just at 60 min.

4.2.2.4 Percentage of ROS production on sperm

Percentage of ROS production on sperm was determined according to the method described in section 2.11.

4.2.4. Statistical Analysis

Whilst this study could have taken a 3 x 3 factorial design, the volume of ejaculates compared to the volume of semen required for treatments and for post-thaw sperm parameter tests was limited. Therefore only seven treatments were used. This study aimed to determine the effect of either 0.5mg/ml or 1.0mg/ml antioxidant (not varying concentrations) pre-freeze, post-thaw or pre-free plus post-thaw, and therefore the experimental design used was with just one concentration of antioxidant in the pre-freeze plus post-thaw treatment groups. Data were analysed using a repeated measure analysis of variance (ANOVA) with a linear mixed model (REML) Gen Stat (17th edition). To accommodate the experimental design, the random effects were concentration nested within ejaculate nested within ram. The factors used in the fixed model were concentration and time (0, 30, 60, and 180 min). The fixed model included the concentration *time interaction, and where there was no significant interaction the fixed model was concentration+time. The variables analysed were motility, viability, acrosome integrity, and ROS proportion. The penetrability through the artificial mucus was measured just at 60 min using one way ANOVA. Post-hoc analyses were performed using the least significant difference (LSD) test. Means were reported with a ± standard error of the mean (SEM) and $P < 0.05$ was considered statistically significant.

Chapter 4. A. The effect of Vitamin C supplementation pre-freeze and post-thaw on cryopreserved ram spermatozoa

4. A.1. Results

4. A.1.1. Motility

There was no significant interaction between treatment and time and so these were analysed independently ($P > 0.05$). There was no significant effect of treatment on % motility (mean ± SEM) although PF+ PT 1.0 mg/ml (33.56 ± 2.85), and PF + PT 0.5 mg/ml (33.08 ± 2.80) tended to have the greatest motility compared to PT 0.5 mg/ml (29.67 ± 2.95), PT 1.0 mg/ml (29.25 ± 2.92), PF 0.5 mg/ml (29.44 ± 2.86), PF 1.0 mg/ml (29.58 ± 2.84) and the control 0.0 mg/ml (28.72 ± 3.01). Motility (mean ± SEM) declined significantly at each time point ($P < 0.001$) being greatest at 0h (47.86 ± 0.21) and declining at 30 min (40.43 ± 0.34), 1h (30.386 ± 0.59), and 3h (3.22 ± 0.33).

4. A.1.2. Viability

There was no interaction between treatment and time for viability ($P > 0.05$). The percentage of viable spermatozoa (mean ± SEM) did not differ significantly between treatments although tended to be greatest in samples treated with 1.0 mg/ml PF + PT (42.25 ± 1.96), and 0.5 mg/ml PF + PT (41.58 ± 1.98), moderate in PF 1.0 mg/ml (38.06 ± 2.06), PF 0.5 mg/ml (37.33 ± 1. 95), PT 1.0 mg/ml (38.03 ± 1.95), and PT 0.5 mg/ml (36.03 ± 2.07), and was lowest in control 0.0 mg/ml (31.03 ± 1.90). Viability (mean % ± SEM) declined significantly over time ($P < 0.001$) being greatest at 0h (49.29 ± 0.60) and declining at 30 min (44.27 ± 0.61), 1h (38.16 ± 0.61), and 3h (19.32 ± 0.44).

4. A.1.3. Acrosome Integrity

There was no significant interaction between treatment and time and so these were analysed independently ($P > 0.05$). Acrosome integrity (mean % ± SEM), did not differ significantly between control 0.0 mg/ml (32.5± 1.747), PF 0.5 mg/ml (41.47 ± 1.749), PF 1.0 mg/ml (41.89 ± 1.77), PT 0.5 mg/ml (38.47 ±1.796), PT 1.0 mg/ml (41.14 ± 1.60), 1.0 mg/ml (46.56 ± 1.62), PF + PT 0.5 mg /ml (44.17 ± 1.59) and PF + PT 1.0 mg/ml (46.56 ± 1.62). Acrosome integrity (mean ± SEM) declined significantly between each time point ($P < 0.001$) being greatest at 0h (51.56 ± 0.70) and declining at 30 min (45.62 ± 0.74), 1h (39.70 ± 0.74), and 3h (26.67 ± 0.74).

4. A.1.4. Penetrability through artificial mucus

There was a significant effect of vitamin C supplementation on the ability of frozen-thawed ram spermatozoa to penetrate artificial mucus. There was interaction between treatment and time at 60 min the distance travelled (mean ± SEM) was greatest ($P < 0.001$) in samples treated with 1.0 mg/ml vitamin C (2.82 ± 0.09), and lowest in the control group 0.0 mg/ml (1.47 ± 0.05). There was a moderate effect on samples that had been treated with 1.0 mg/ml PF or PT (Table 4.1).

Table 4. 1. Mean (± SEM) distance reached by the furthest spermatozoon (vanguard distance) measured in centimetre (cm/h) at 60 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml vitamin C pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment.

Treatment (9)	Distance (cm/h)
Control 0.0 mg/ml	1.47 ± 0.05^{f} cm/h
PF 0.5 mg/ml	1.94 ± 0.04^{e} cm/h
PF 1.0 mg/ml	2.31 ± 0.08^{d} cm/h
PT 0.5 mg/ml	1.64 ± 0.06^{cd} cm/h
PT 1.0 mg/ml	2.12 ± 0.09^{bc} cm/h
PF + PT 0.5 mg/ml	2.50 ± 0.09^{b} cm/h
PF + PT 1.0 mg/ml	2.82 ± 0.09^{a} cm/h

a, b, c, d, and e: values with different superscripts in the same column at 60 min are significantly different ($P < 0.001$).

4. A.1.5. Percentage of ROS production as determined by formazan formation at the sperm head

4. A.1.5.1. Proportion of spermatozoa with 0% formazan formation

There was an interaction between treatment and time for the percentage of sperm that expressed 0% formazan at the sperm head ($P < 0.001$). The proportion of sperm with 0% formazan in the sperm head (mean % ± SEM) declined over time in all treatment groups. The effect of treatment differed between time points but tended to be higher ($P < 0.001$) in samples treated with vitamin C PF + PT 0.5 mg/ml and PF + PT 1.0 mg/ml (5.56 ± 0.58, 6.33 ± 0.58) respectively, compared to the control (0.0 mg/ml) 0.11 ± 0.11) (Table 4.2).

Table 4. 2. Mean (± SEM) proportion of spermatozoa with 0 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml vitamin C pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parantheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$8.78 \pm 0.47^{e}_{w}$	$4.89 \pm 0.48^{e}_{x}$	$1.89 \pm 0.35^{d}_{y}$	$0.11 \pm 0.11^{d}_{z}$
PF 0.5 (9)	$14.78 \pm 0.97^{d}_{w}$	$9.67 \pm 0.80^{d}_{x}$	$5.89 \pm 0.94^{bc}_{y}$	$2.44 \pm 0.48^{cd}_{z}$
PF 1.0 (9)	$17.67 \pm 0.87^{c}_{w}$	$13.33 \pm 0.82^{c}_{x}$	$8.11 \pm 0.89^{b}_{y}$	$3.78 \pm 0.52^{abc}_{z}$
PT 0.5 (9)	$13.00 \pm 0.83^{d}_{w}$	$9.11 \pm 0.89^{d}_{x}$	$5.00 \pm 0.62^{c}_{y}$	$1.44 \pm 0.50^{cd}_{z}$
PT 1.0(9)	$15.78 \pm 1.10^{cd}_{w}$	$11.00 \pm 1.11^{cd}_{x}$	$6.89 \pm 0.86^{bc}_{y}$	$2.78 \pm 0.43^{bc}_{z}$
PF + PT 0.5 (9)	$20.89 \pm 0.92^{bc}_{w}$	$16.33 \pm 0.76^{b}_{x}$	$11.11 \pm 0.59^{a}_{y}$	$5.56 \pm 0.58^{ab}_{z}$
PF + PT 1.0 (9)	$26.22 \pm 1.13^{a}_{w}$	$20.33 \pm 1.17^{a}_{x}$	$13.78 \pm 1.38^{a}_{y}$	$6.33 \pm 0.58^{a}_{z}$

a, b, c, d, and e: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. A.1.5.2. Proportion of spermatozoa with < 50% formazan formation

There was a significant interaction between treatment and time (P <0.001). The proportion of sperm with < 50% formazan in the sperm head (mean % ± SEM) declined over time in all treatment groups. The effect of treatment differed between time points, though tended to be greater in samples treated with vitamin C both pre-freeze and post-thaw (0.5 and 1.0 mg/ml, compared to the control (Table 4.3).

Table 4. 3. Mean (± SEM) proportion of spermatozoa with < 50 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml vitamin C pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$33.00 \pm 1.23^{bc}_{w}$	$27.00 \pm 1.27^{bc}_{x}$	$19.44 \pm 1.33^{bc}_{y}$	$1.11 \pm 0.75^{b}_{z}$
PF 0.5 (9)	$34.33 \pm 1.83^{bc}_{w}$	28.00 ± 1.80^{bc}x	$19.56 \pm 1.72^{bc}_{y}$	$4.89 \pm 0.72^{b}_{w}$
PT 1.0 (9)	$31.67 \pm 1.66^{bc}_{w}$	$24.44 \pm 1.90^{bc}_{x}$	$17.33 \pm 1.59^{c}_{y}$	$4.33 \pm 0.69^{b}_{z}$
PF 0.5 (9)	29.67 ± 1.54^{c}w	$23.00 \pm 1.27^{c}_{x}$	$16.44 \pm 1.04^{c}_{y}$	$4.78 \pm 0.80^{b}_{z}$
PT 1.0 (9)	32.44 ± 2.01b$^{c}_{w}$	$25.67 \pm 2.27^{bc}_{x}$	$17.89 \pm 1.61^{c}_{y}$	$4.78 \pm 0.76^{b}_{z}$
PF + PT 0.5 (9)	$39.89 \pm 1.17^{a}_{w}$	$34.67 \pm 1.13^{a}_{x}$	$26.33 \pm 0.78^{a}_{y}$	$10.33 \pm 0.55^{a}_{z}$
PF + PT 1.0 (9)	$35.22 \pm 1.31^{ab}_{w}$	$29.78 \pm 1.42^{ab}_{x}$	$23.89 \pm 1.59^{ab}_{y}$	$10.33 \pm 1.19^{a}_{z}$

a, b, and c: values with different superscripts in the same column for each incubation time are significantly different (P < 0.001).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different (P < 0.001).

4. A.1.5.3. Proportion of spermatozoa with > 50% formazan formation

There was a significant interaction between treatment and time ($P < 0.001$). The proportion of sperm with >50% formazan in the sperm head (mean % ± SEM) increased over time in all treatment groups. The effect of treatment differed between time points but tended to be lowest in samples treated with vitamin C both pre-freeze and post-thaw (0.5 and 1.0 mg/ml, compared to the control (0.0 mg/ml) and other treatments (Table 4.4).

Table 4. 4. Mean (± SEM) proportion of spermatozoa with > 50 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml vitamin C pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$26.56 \pm 1.11^{a}_{w}$	$31.56 \pm 1.06^{a}_{x}$	$36.33 \pm 0.96^{ab}_{y}$	$44.22 \pm 0.91^{a}_{z}$
PF 0.5 (9)	$26.44 \pm 0.85^{a}_{w}$	$32.33 \pm 0.78^{a}_{x}$	$38.33 \pm 0.82^{a}_{y}$	$46.33 \pm 0.67^{a}_{z}$
PF 1.0 (9)	$28.11 \pm 0.82^{a}_{w}$	$33.67 \pm 0.71^{a}_{x}$	$38.78 \pm 0.55^{a}_{y}$	$45.22 \pm 0.40^{a}_{z}$
PT 0.5 (9)	$29.11 \pm 0.75^{a}_{w}$	$34.89 \pm 0.66^{a}_{x}$	$39.22 \pm 0.70^{a}_{y}$	$46.22 \pm 0.57^{a}_{z}$
PT 1.0 (9)	$26.56 \pm 0.97^{a}_{w}$	$32.78 \pm 1.01^{a}_{x}$	$38.44 \pm 0.67^{a}_{y}$	$45.67 \pm 0.73^{a}_{z}$
PF + PT 0.5 (9)	$21.11 \pm 1.11^{b}_{w}$	$26.00 \pm 1.13^{b}_{x}$	$32.78 \pm 0.78^{c}_{y}$	$43.78 \pm 0.86^{a}_{z}$
PF +PT 1.0 (9)	$21.44 \pm 0.82^{b}_{w}$	$27.44 \pm 0.67^{b}_{x}$	$34.11 \pm 1.05^{bc}_{y}$	$44.22 \pm 0.83^{a}_{z}$

a, b, and c : values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. A.1.5.4. Proportion of spermatozoa with 100% formazan formation

There was no significant interaction between treatment and time, and so these were analysed independently ($P > 0.05$). The proportion (%) of sperm that expressed ROS in 100% of the sperm head increased over time ($P < 0.001$) being lowest at 0h (23.90 ± 0.66) and increasing at 30 min (29.22 ± 0.66), 1 h (35.51 ± 0.66), and 3h (45.90±0.66). The proportion of sperm with 100% formazan at the sperm head (mean % ± SEM) did not differ significantly between PF + PT 1.0 mg/ml (26.72 ± 1.43), PF + PT 0.5 mg/ml (27.92 ± 1.47), PT 1.0 mg/ml (34.83 ± 1.41), PF 0.5 mg/ml (34.28 ± 1.44), PF 1.0 mg/ml (34.36±1.42), control 0.0 mg/ml (41.28 ± 1.51) and PT 0.5 mg/ml (36.06 ± 1.43).

4. A. 2. Discussion

Currently as far as is known, this is the first study that investigates the optimal concentration (0.0, 0.5 or 1.0 mg/ml) and timing (pre-freeze and post-thaw) of vitamin C as an antioxidant in ram spermatozoa cryopreserved in Tris extender. The membrane of ram spermatozoa has high levels of polyunsaturated fatty acids (PUFAs) (Brinsko *et al.,* 2005), which causes the membrane to be sensitive to lipid peroxidation by ROS, such as superoxide, hydroxyl radicals, and hydrogen peroxide (Bansal and Bilaspuri, 2011). The ROS attack subsequently leads to impaired sperm function, including reduced motility, acrosome integrity, viability, loss of intracellular enzymes and destruction of DNA by oxidative stress (Alvarez and Storey, 1989; Bansal and Bilaspuri, 2011). Cryopreservation causes a decline in sperm integrity due to osmotic stress (Watson, 2000; Azevedo, 2006; Maia, 2006; Rodello, 2006; Mahfouz *et al.,* 2010; Wu *et al.,* 2015), and lipid peroxidation due to increase production of ROS (Aitken *et al.,* 1998; Del Olmo *et al.,* 2015).

Vitamin C is a water-soluble, which effectively eliminates hydroxyl (HO), hydrogen peroxide (H_2O_2) and radical's superoxide (O_2-). ROS can remove hydrogen from vitamin C, which then develops into monodehydroascorbate. Then, this antioxidant can scavenge another electron to be dehydroascorbate. In this procedure, ROS are changed to H_2O, and the reacted forms of vitamin C are moderately steady and do not cause cell destruction. Additionally, vitamin C has an essential role in the alteration of the tocopheryl radical (vitamin E-O) into reduced α-tocopherol (vitamin E-OH), having a synergistic effect of the antioxidant action of α-tocopherol (Kefer *et al.,* 2009). Lewis *et al.* (1997) stated that levels of vitamin C detected in sperm at a concentration of 1pmol/10 l° per cell, is required for motility in SP. This could explain the importance of vitamin C concentration on sensitivity of sperm.

The results of the present study determined that whilst supplementation with vitamin C pre-freeze and post-thaw tended to improve sperm integrity, the effect was not significant. These findings are in agreement with previous observations that reported no positive effect of vitamin C supplementation when added prior to cryopreservation (Aurich *et al.,* 1997; Ball and Vo, 2001). Aurich *et al.* (1997) stated that the addition of 0.9 mg/ml vitamin C to stallion semen extender reduced the percentage of progressive sperm motility. Furthermore, the addition of more than 2.0 mg/ml of vitamin C in semen extender decreased ram sperm motility during liquid storage (Sönmez and Demirci, 2004; Sonmez *et al.,* 2005)**.** It is likely that this observed toxic effect of vitamin C is caused by a reduction in pH due to the presence of vitamin C (ascorbic acid) which is strongly acidic and causes a reduction in sperm function (Acott and Carr, 1984; Ménézo *et al.,* 2007; Michael *et al.,* 2007; Cabrita *et al.,* 2011; Lopes *et al.,* 2018). It could be

expected that the concentration of vitamin C used in the present study would not be detrimental to spermatozoa as the mM concentrations 0.5 mg/ml (2.35 mM) and 1.0 mg/ml (5.7 mM) are similar to the natural concentration of vitamin C in seminal plasma which is reported at 3.6 mM (Gangwar *et al.,* 2015).

Vitamin C protects sperm cells from oxidative stress but demonstrates a low capability to protect the cell membrane from osmotic stress during cryopreservation and thus did not improve membrane stability (Martínez-Páramo *et al.,* 2012). Through cryopreservation, osmotic stress is caused by alterations in cell volume subsequent from the association of water and solutes through the sperm plasma membrane (Ball, 2008; Li *et al.,* 2010). Previous studies showed that vitamin C with 2.5 mM in bull and 0.02-0.06 mM in human sperm had a harmful effect on motility of frozen-thawed semen (Foote *et al.,* 2002). Conversely, addition of 0.9 mg/ml vitamin C showed a significant protective effect preserving motility (52.8 ± 4.3) at 5ºC for 12 hours of ram sperm. Moreover, lipid peroxidation was reduced in cryopreserved bull sperm with 5mM of vitamin C (Beconi *et al.,* 1993).

In the current study supplementation with vitamin C improved the passage of frozen-thawed ram spermatozoa through artificial mucus. The greatest effect was observed in samples treated with 0.5 mg/ml and 1.0 mg/ml pre-freeze and post-thaw. It is possible that the increased penetrability is caused by the ability of vitamin C to reduce oxidative stress, as it has been reported that sperm metabolism in humans is protected by vitamin C supplementation (Ahmad *et al.,* 2017).

It is worth noting that whilst vitamin C improved penetrability, it did not cause a significant effect on motility. This is in agreement with previous reports that suggest the ability of sperm to penetrate the cervical mucus is not correlated with sperm motility (Rickard *et al.,* 2014). Furthermore, vitamin C is oxidized into inactive dehydroascorbate when exposed to highly oxidative condition (Linster and Van Schaftingen, 2007). This effect could decrease the beneficial effect of vitamin C on the sperm membrane (Vera *et al.,* 1993).

This instability makes the function of vitamin C hard to maintain as an antioxidant during exposure of spermatozoa to highly oxidative condition for long periods. As Amidi *et al.* (2016) stated transition metal chemical elements have an ability to affect vitamin C making it pro-oxidant increasing effect of free radicals and causing more damage to the sperm.

Nitroblue Tetrazolium (NBT) reacts with superoxide ions (ROS) in the cell to form formazan granules, providing an indication of oxidative stress. In the present study, the percentage of the sperm head which contained formazan granules was assessed as 0, >50 >50 and 100 % as

described previously (Esfandiari *et al.,* 2003). Cryopreserved ram spermatozoa supplemented with 0.5 and 1.0 mg/ml vitamin C both pre-freeze and post-thaw had the least ROS in the sperm head. Vitamin C neutralizes superoxide anions and singlet ROS (Gangwar *et al.,* 2015), reducing oxidative stress, and this may explain the reduced ROS in samples supplemented with vitamin C. The results of the present study highlight the need to use antioxidants both pre-freeze and post-thaw in order to minimize ROS production. Whilst vitamin C supplementation improved sperm penetrability and reduced ROS production, overall there was no other effect on sperm function and integrity.

4. A.3. Conclusion

In conclusion, despite the limited effects on sperm integrity, supplementation with 0.5 or 1.0 mg/ml vitamin C pre-freeze and post-thaw together (PF + PT) reduces ROS production as measured by the formation of formazan granules and improves the penetrability of ram spermatozoa through artificial cervical mucus. The results suggest that it could be difficult to optimise the use of vitamin C as an antioxidant in ram semen cryopreservation as there are limitations such as the limited effect on sperm integrity and the potentially toxic effects if used at concentrations that are too high. The effect of highly oxidative conditions on vitamin C limits its use and therefore utilizing vitamin C in combination with additional antioxidants may help to reduce ROS production. Further, minimizing oxidative stress and improving sperm integrity to enhance the development of cryopreservation protocols in the ram may occur. Therefore, vitamin C is not a reliable and effective antioxidant to protect cryopreserved ram sperm, and has not been used in further investigations within this thesis.

Chapter 4. B. Cysteine supplementation pre-freeze and post-thaw improves integrity and reduces oxidative stress of cryopreserved ram spermatozoa

4. B.1.Results

4. B.1.1. Motility

There was a significant interaction between treatment and time ($P < 0.001$). Motility (mean % ± SEM) was generally greatest ($P < 0.001$) in samples treated with 1.0 mg/ml both PF and PT, intermediate in samples treated PF + PT with 0.5 mg/ml cysteine and lowest in the control sample which did not have any cysteine. As expected, motility declined over time in all treatments (Table 4.5).

4. B.1.2 Viability

There was a significant interaction between treatment and time ($P < 0.001$). The percentage viability (mean ± SEM) declined over time in all treatment groups and tended to be higher ($P < 0.001$) in samples treated with cysteine both before freezing and after thawing (PF + PT 0.5 and 1.0 mg/ml cysteine). There was an intermediate effect when cysteine was supplemented either pre-freezing or post-thaw. However, the lowest percentage viability was found in samples which did not have any cysteine (control 0.0 mg/ml) (Table 4.6).

4. B.1.3 Acrosome Integrity

There was no significant interaction between treatment and time and so these were analysed independently ($P > 0.05$). The percentage acrosome integrity (mean ± SEM) deteriorated over time, being greatest at 0 min (55.54 ± 0.636), and declining at 30 min (50.68 ± 0.748), 1h (45.08 ± 0.827), and 3 h (26.81 ± 0.697) ($P < 0.001$). The percentage acrosome integrity (mean ± SEM), did not differ significantly between PF + PT 1.0 mg/ml (51.47 ± 1.939), PF 1.0 mg/ml (46.31 ± 2.022), PF + PT 0.5 mg/ml (48.28 ± 1.796), PT 0.5 mg/ml (44.25 ± 1.932), PF 1.0 mg/ml (42.83 ± 2.116), PF 0.5 mg/ml (41.42 ± 1.862) and control (37.14 ± 1.935).

Table 4. 5. Mean (± SEM) percentage motility of spermatozoa at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml cysteine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$50.00 \pm 0.00^{d}_{w}$	$29.44 \pm 1.002^{d}_{x}$	$15.78 \pm 1.140^{d}_{y}$	$0.56 \pm 0.556^{d}_{z}$
PF 0.5 (9)	$50.67 \pm 0.333^{bc}_{w}$	$40.44 \pm 0.884^{bc}_{x}$	$31.89 \pm 0.992^{c}_{y}$	$10.78 \pm 0.662^{c}_{z}$
PF 1.0 (9)	$51.67 \pm 0.726^{abc}_{w}$	$43.89 \pm 0.790^{c}_{x}$	$34.89 \pm 1.241^{bc}_{y}$	$16.44 \pm 1.396^{bc}_{z}$
PT 0.5 (9)	$50.22 \pm 0.222^{bc}_{w}$	$40.11 \pm 0.676^{c}_{x}$	$32.22 \pm 1.211^{b}_{y}$	$10.78 \pm 1.310^{b}_{z}$
PT 1.0 (9)	$52.11 \pm 0.772^{b}_{w}$	$42.89 \pm 0.841^{bc}_{x}$	$35.00 \pm 1.312^{bc}_{y}$	$16.78 \pm 0.741^{b}_{z}$
PF + PT 0.5 (9)	$54.78 \pm 0.222^{ab}_{w}$	$46.22 \pm 0.760^{ab}_{x}$	$38.44 \pm 1.625^{ab}_{y}$	$18.00 \pm 0.866^{b}_{z}$
PF + PT 1.0 (9)	$58.00 \pm 0.645^{a}_{w}$	$50.56 \pm 0.7290^{a}_{x}$	$43.89 \pm 0.935^{a}_{y}$	$24.00 \pm 0.553^{a}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

Table 4. 6. Mean (± SEM) percentage viability of spermatozoa at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml cysteine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$50.67 \pm 0.577^{d}_{w}$	$43.89 \pm 0.655^{d}_{x}$	$36.00 \pm 0.667^{d}_{y}$	$22.11 \pm 0.716^{c}_{z}$
PF 0.5 (9)	$55.44 \pm 0.242^{bc}_{w}$	$50.33 \pm 0.645^{c}_{x}$	$44.44 \pm 0.988^{c}_{y}$	$26.78 \pm 0.760^{b}_{z}$
PF 1.0 (9)	$57.22 \pm 0.434^{abc}_{w}$	$51.33 \pm 1.047^{c}_{x}$	$46.22 \pm 1.362^{c}_{y}$	$26.78 \pm 0.778^{b}_{z}$
PT 0.5 (9)	$56.11 \pm 0.351^{bc}_{w}$	$51.89 \pm 0.564^{c}_{x}$	$45.44 \pm 0.868^{c}_{y}$	$28.11 \pm 0.889^{b}_{z}$
PT 1.0 (9)	$57.56 \pm 0.648^{abc}_{w}$	$52.89 \pm 1.207^{bc}_{x}$	$46.89 \pm 1.620^{bc}_{y}$	$27.67 \pm 0.882^{b}_{z}$
PF + PT 0.5 (9)	$59.44 \pm 0.503^{ab}_{w}$	$56.00 \pm 0.373^{ab}_{x}$	$50.33 \pm 0.645^{ab}_{y}$	$35.33 \pm 0.928^{a}_{z}$
PF + PT 1.0 (9)	$60.11 \pm 0.807^{a}_{w}$	$56.44 \pm 0.626^{a}_{x}$	$51.56 \pm 0.852^{a}_{y}$	$35.56 \pm 1.642^{a}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).
w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. B.1.4. Penetrability through artificial mucus

Distance travelled (mean ± SEM) by frozen-thawed ram spermatozoa was greater ($P < 0.001$) in samples treated with cysteine at 60 min compared to the control group. The greatest distance was observed in samples treated with 1.0 mg/ml cysteine. The shortest distance travelled was in the control 0.0 mg/ml group (1.567 ± 0.120 cm/h) Figure 4.2.

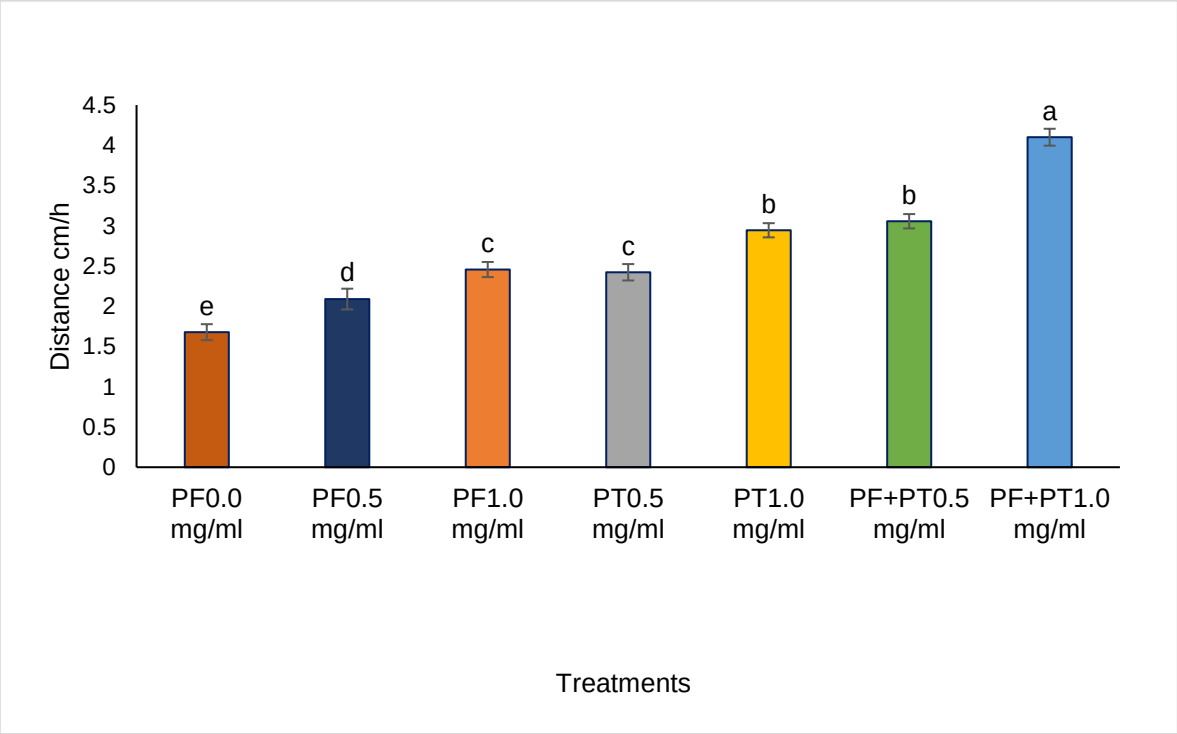


Figure 4. 2. Mean (± SEM) furthest distance reached by the spermatozoon (Vanguard distance) measured in centimetre (cm/h) at 60 min post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml cysteine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT).

4. B.1.5. Percentage of ROS production as determined by formazan formation

4. B.1.5.1. Proportion of spermatozoa with 0% formazan formation

There was an interaction between treatment and time on the proportion of spermatozoa with 0% formazan formation ($P < 0.001$). The proportion of sperm with 0% formazan within the sperm head (mean % ± SEM) declined over time in all treatment groups but tended to be higher ($P < 0.001$) in samples treated with cysteine pre-freeze and post-thaw (PF + PT 0.5 and PF + PT 1.0 mg/ml). (Table 4.7).

4. B.1.5.2. Proportion of spermatozoa with < 50% formazan formation

There was an interaction between treatment and time on the proportion of sperm with <50% formazan within the sperm head ($P < 0.001$). The proportion of sperm with <50% formazan within the sperm head (mean % ± SEM) declined over time in all treatment groups but tended to be higher ($P < 0.001$) in samples treated with cysteine pre-freeze and post-thaw (PF + PT 0.5 mg/ml and PF + PT 1.0 mg/ml). The proportion of spermatozoa with < 50% formazan formation was intermediate in other treatments. The proportion of sperm with <50% formazan within the sperm head (mean % ± SEM) tended to be lowest in the control group which was not treated with cysteine (control 0.0 mg/ml) Table 4.8.

Table 4. 7. Mean (± SEM) proportion of spermatozoa with 0% ROS formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml cysteine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$10.33 \pm 1.027^{d}_{w}$	$6.22 \pm 0.954^{d}_{x}$	$1.56 \pm 0.530^{d}_{y}$	$0.0 \pm 0.0^{b}_{z}$
PF 0.5 (9)	$16.67 \pm 0.5^{c}_{w}$	$11.44 \pm 0.648^{c}_{x}$	$6.67 \pm 0.471^{c}_{y}$	$0.0 \pm 0.0^{b}_{z}$
PF 1.0 (9)	$19.44 \pm 0.818^{b}_{w}$	$13.78 \pm 0.687^{b}_{x}$	$6.89 \pm 0.807^{b}_{y}$	$0.333 \pm 0.333^{b}_{z}$
PT 0.5 (9)	$18.22 \pm 0.954^{b}_{w}$	$12.67 \pm 0.726^{b}_{x}$	$5.89 \pm 0.790^{b}_{y}$	$0.0 \pm 0.0.0^{b}_{z}$
PT 1.0 (9)	$19.44 \pm 0.729^{b}_{w}$	$13.56 \pm 0.972^{b}_{x}$	$6.89 \pm 0.807^{b}_{y}$	$0.0 \pm 0.0^{b}_{z}$
PF + PT 0.5 (9)	$25.78 \pm 0.521^{ab}_{w}$	$19.78 \pm 0.547^{ab}_{x}$	$12.11 \pm 0.611^{ab}_{y}$	$4.56 \pm 0.475^{a}_{z}$
PF + PT 1.0 (9)	$28.78 \pm 0.745^{a}_{w}$	$23.11 \pm 0.889^{a}_{x}$	15.00 ± 0.928^{ay}	$5.78 \pm 0.619^{a}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).
w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

Table 4. 8. Mean (± SEM) proportion of spermatozoa with ROS < 50 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0, 0.5 or 1.0 mg/ml cysteine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$42.78 \pm 1.245^{ab}{}_{w}$	$36.11 \pm 1.207^{bc}{}_{x}$	$26.44 \pm 1.591^{bc}{}_{y}$	$3.78 \pm 0.619^{c}{}_{z}$
PF 0.5 (9)	$35.11 \pm 1.495^{c}{}_{w}$	$30.56 \pm 2.115^{c}{}_{x}$	$24.89 \pm 2.118^{bc}{}_{y}$	$6.78 \pm 0.778^{bc}{}_{z}$
PF 1.0 (9)	$40.33 \pm 1.143^{abc}{}_{w}$	$34.56 \pm 1.292^{bc}{}_{x}$	$28.89 \pm 1.550^{bc}{}_{y}$	$7.56 \pm 0.709^{b}{}_{z}$
PT 0.5 (9)	$39.67 \pm 1.77^{abc}{}_{w}$	$33.11 \pm 1.822^{ab}{}_{x}$	$24.33 \pm 1.491^{c}{}_{y}$	$6.67 \pm 0.527^{b}{}_{z}$
PT 1.0 (9)	$37.78 \pm 0.846^{bc}{}_{w}$	$32.56 \pm 1.056^{ab}{}_{x}$	$26.11 \pm 1.662^{ab}{}_{y}$	$8.11 \pm 0.735^{b}{}_{z}$
PF + PT 0.5 (9)	$43.78 \pm 1.164^{a}{}_{w}$	$38.89 \pm 1.317^{a}{}_{x}$	$33.11 \pm 1.419^{a}{}_{y}$	$15.56 \pm 0.818^{a}{}_{z}$
PF + PT 1.0 (9)	$41.00 \pm 0.726^{ab}{}_{w}$	$36.89 \pm 1.184^{a}{}_{x}$	$31.67 \pm 1.572^{a}{}_{y}$	$15.11 \pm 1.184^{a}{}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$). w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. B.1.5.3. Proportion of spermatozoa with > 50 % formazan formation

There was an interaction between treatment and time on the proportion of sperm with >50% formazan within the sperm head ($P < 0.001$). The proportion of sperm with >50% formazan within the sperm head (mean % ± SEM) increased over time in all treatment groups but tended to be lowest ($P < 0.001$) in samples treated with cysteine pre-freeze and post-thaw (PF + PT 0.5 and PF + PT 1.0 mg/ml). The proportion of sperm with >50% formazan within the sperm head (mean % ± SEM) increased over the incubation time and tended to be intermediate in the other treatments (PF 0.5, PF 1.0, PT 0.5 and PF 1.0 mg/ml), and was lowest in the control group that did not have any treatment (control 0.0 mg/ml) at the end of the incubation time (Table 4.9). The low percentage of spermatozoa with >50% ROS at the sperm head in the control group is caused by a very high proportion of sperm within the control group expressing 100% formazan within the sperm head.

4. B.1.5.4. Proportion of spermatozoa with 100% formazan formation

There was an interaction between treatment and time on the proportion of sperm with 100% formazan within the sperm head ($P < 0.001$).The proportion of sperm with 100% formazan within the sperm head (mean % ± SEM) increased over time in all treatment groups but tended to be lowest ($P < 0.001$) in samples treated with cysteine pre-freeze and post-thaw (PF + PT 0.5 and PF + PT 1.0). The proportion of spermatozoa with 100% ROS was intermediate in other treatment groups, and the highest percentage of ROS over the incubation time was in the control group which was not treated with cysteine (Table 4.10).

Table 4. 9. Mean (± SEM) proportion of spermatozoa with ROS > 50 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml cysteine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$20.67 \pm 0.882^{b}{}_{w}$	$24.56 \pm 0.801^{b}{}_{x}$	$28.67 \pm 1.0.54^{a}{}_{y}$	$35.22 \pm 0.813^{a}{}_{z}$
PF 0.5 (9)	24.22 ± 1.064^{cw}	$29.54 \pm 1.492^{c}{}_{x}$	$34.89 \pm 1.338^{b}{}_{y}$	$47.00 \pm 0.957^{c}{}_{z}$
PF 1.0 (9)	$20.56 \pm 0.835^{bc}{}_{w}$	$26.33 \pm 0.726^{b}{}_{x}$	$33.56 \pm 1.226^{b}{}_{y}$	$49.56 \pm 0.603^{c}{}_{z}$
PT 0.5 (9)	$21.44 \pm 1.015^{bc}{}_{w}$	$26.67 \pm 1.130^{bc}{}_{x}$	$34.22 \pm 1.451^{b}{}_{y}$	$47.44 \pm 1.365^{bc}{}_{z}$
PT 1.0 (9)	$22.44 \pm 0.709^{bc}{}_{w}$	$28.44 \pm 0.853^{bc}{}_{x}$	$35.33 \pm 1.213^{b}{}_{y}$	$49.11 \pm 0.588^{c}{}_{z}$
PF + PT 0.5 (9)	$15.11 \pm 0.949^{a}{}_{w}$	$21.00 \pm 1.130^{a}{}_{x}$	$28.89 \pm 1.695^{a}{}_{y}$	$45.78 \pm 0.969^{b}{}_{z}$
PF + PT 1.0 (9)	$16.89 \pm 0.512^{a}{}_{w}$	$22.44 \pm 1.180^{a}{}_{x}$	$29.11 \pm 1.940^{a}{}_{y}$	$45.89 \pm 1.073^{b}{}_{z}$

a, b, and c: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).
w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

Table 4. 10. Mean (± SEM) proportion of spermatozoa with 100% formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml cysteine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$26.22 \pm 0.846^{e}{}_{w}$	$33.11 \pm 0.889^{c}{}_{x}$	$43.33 \pm 1.333^{d}{}_{y}$	$61.00 \pm 0.957^{d}{}_{z}$
PF 0.5 (9)	$24.00 \pm 0.764^{de}{}_{w}$	$28.56 \pm 0.899^{b}{}_{x}$	$33.33 \pm 1.258^{bc}{}_{y}$	$46.22 \pm 0.572^{c}{}_{z}$
PF 1.0 (9)	$19.67 \pm 1.080^{ab}{}_{w}$	$25.33 \pm 1.027^{b}{}_{x}$	$30.67 \pm 1.247^{b}{}_{y}$	$42.56 \pm 0.747^{b}{}_{z}$
PT 0.5 (9)	$20.67 \pm 0.866^{cd}{}_{w}$	$27.56 \pm 0.729^{b}{}_{x}$	$35.56 \pm 1.556^{c}{}_{y}$	$45.89 \pm 978^{bc}{}_{z}$
PT 1.0 (9)	$20.33 \pm 0.816^{cd}{}_{w}$	$25.44 \pm 1.056^{b}{}_{x}$	$31.67 \pm 1.213^{bc}{}_{y}$	$42.78 \pm 0.862^{bc}{}_{z}$
PF + PT 0.5 (9)	$15.33 \pm 0.441^{ab}{}_{w}$	$20.33 \pm 0.687^{a}{}_{x}$	$25.89 \pm 0.539^{a}{}_{y}$	$34.11 \pm 1.073^{a}{}_{z}$
PF + PT 1.0 (9)	$13.33 \pm 0.645^{a}{}_{w}$	$17.56 \pm 0.709^{a}{}_{x}$	$24.22 \pm 1.234^{a}{}_{y}$	$33.22 \pm 1.470^{a}{}_{z}$

a, b, c, d and e: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).
w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. B.2. Discussion

Several studies have shown that semen extender supplemented with antioxidants has a beneficial effect by reducing the production of ROS in numerous farm animal species (Bucak and Tekin, 2007; Tonieto *et al.,* 2010). However, to our knowledge, there are no studies describing the effect of cysteine supplementation pre-freeze and post-thaw and its influence during the freezing and thawing process in ram spermatozoa. Furthermore there are no studies that have optimised the use of cysteine or thoroughly investigated the optimal timing and concentration.

ROS have a beneficial effect on sperm membrane permeability and fluidity under normal circumstances (Agarwal *et al.*, 2014), but excessive ROS production leads to damage to the sperm functions such as motility, viability, membrane integrity, and the activity of mitochondria and consequently results in lower fertility (Agarwal *et al.,* 2008; Bansal and Bilaspuri, 2011).

Due to the a greater sensitivity of the ram sperm membrane to cryopreservation, the rates of fertility do not reach the desired levels (10-30%) through TCAI (Salamon and Maxwell, 2000). The low concentration of antioxidants in sperm and seminal plasma of frozen-thawed semen coupled with the decrease the level intracellular antioxidant ability in sperm following the freeze-thawing procedure (Trinchero *et al.,* 1990), results in increased oxidative stress (Lahnsteiner *et al.,* 2011; Osipova *et al.,* 2016).

Cysteine supplementation to the semen extender protects sperm motility by control of lipid peroxidation caused by ROS in frozen-thawed bull semen (Bilodeau *et al.*, 2000).In the present study, the addition of cysteine to the ram semen extender (pre-freeze and post-thaw) improved sperm motility, viability, penetrability and reduced the proportion of sperm with a large percentage of ROS in the sperm head.These findings are in agreement with previous studies that report the addition of cysteine improved sperm parameters on ram semen (Uysal and Bucak, 2007), canine (Michael *et al.,* 2007), cat (Thuwanut *et al.,* 2008), bull (Sariözkan *et al.,* 2009), boar (Funahashi and Sano, 2005; Kaeoket *et al.,* 2010) and chicken (Partyka *et al.,* 2013) semen. A pre-freeze supplementation of 10 mM cysteine was found to be more effective in case post-thaw motility (59.0 ± 4.35) of ram sperm (Uysal and Bucak, 2007). Memon *et al.* (2014) stated the supplementation of cysteine improved the quality of frozen-thawed goat semen such as motility through a reduction in the vulnerability of the plasma membrane to cryo-damage during the process of freeze-thawing. Interestingly these positive effects were more pronounced with 5 mM cysteine than with 10 mM.

However, in previous study (Memon *et al*., 2014) post-thaw motility was checked immediately after thawing (0 min) which is comparable to our results (motility%; 58.00 ±

0.645) at 0 min. In our study sperm motility was 24.00 ± 0.55% with the (PF + PT) 1.0 mg/ml compared to 0.56 ± 0.5% for the controls at 180 min post-thaw. The improved motility in the present study which was maintained to 3 hours post-thaw highlights the importance of the post-thaw cysteine supplementation to enhance frozen-thawed ram sperm integrity. The post-thaw supplementation could be considered a support source of cysteine to maintain the activity and the role of antioxidants. This is likely through their activity to scavenge the free radicals and protect the sperm membrane and decrease DNA fragmentation thereby improving the post-thaw motility and viability of ram spermatozoa (Bucak *et al.,* 2013; Sharafi *et al.,* 2015).

Although DNA fragmentation was not assessed in the current study, sperm chromatin is tightly packed using cysteine residues (Vilfan *et al.,* 2004), and cysteine has the ability to produce intra- or interprotamine cross-linking (Vilfan *et al*., 2004). Furthermore cysteine residues can increased spermatozoa DNA stability (Gosalvez *et al*., 2011). This suggests that in the present study, cysteine supplementation may protect spermatozoa DNA against oxidative damage.

It has been stated that cysteine addition improved the viability of ram sperm (4 mM, Çoyan *et al.,* 2011; 4 mM, Gungor *et al.,* 2017), buffalo (7.5 mM, Topraggaleh *et al.,* 2014), buck (10 mM, Mondal, 2014; 5 mM, Fayyaz *et al.,* 2018), and boar (5 mM, Funahashi and Sano, 2005). However, there was high stress in the present study by cryopreservation and incubation time, the viability of post-thaw ram sperm improved by cysteine supplementation, and that could be related to the effect of cysteine on protect sperm form the damaging effect by ROS, and also could be due to the technique (PF + PT) which used, which confirm the importance of this technique.

In the current study, the addition of cysteine did not significantly improve acrosome integrity. This result was in agreement with previous studies that also did not show the role of cysteine for protecting acrosome integrity from the damaging effect of ROS (Michael *et al.,* 2007; Bucak *et al.,* 2008; Tuncer *et al.,* 2010). It has been stated that the acrosome reaction is more sensitive to oxidative damage than other parameters of sperm integrity such as motility (Whittington and Ford, 1998). Peris *et al.* (2008) reported that high concentrations of ROS during cryopreservation induced capacitation-like changes which increases the level of intracellular calcium and membrane permeability. As capacitation is a pre-requisite to the acrosome reaction, this could induce damage in the acrosome region, or stimulate the acrosome reaction which cannot be recovered by antioxidant supplementation. This damage to the plasma membrane observed in the anterior part of sperm included swelling and breaking, causing loss of acrosome integrity and activity (Aisen *et al.,* 2005). This could be the main reason for the low ratio of acrosome-intact to non-intact in treated samples compared to control samples. However, a few studies that added cysteine to the semen

extender improved the acrosome integrity in bull (Sariözkan *et al.*, 2009), and buffalo semen (Ansari *et al.*, 2011). These differences could either be related to different experimental methodologies or species.

Cysteine supplementation in the present study showed improvement in the penetrability of frozen- thawed ram sperm on artificial mucus, this could be because cysteine protects the sperm membrane and mitochondria from the oxidative effect of ROS (Bucak *et al.,* 2008; Sharafi *et al.,* 2015). There are limited data on the effect of antioxidants in sperm penetrability of mucus. In the present study post-thaw supplementation was more beneficial than pre-freeze supplementation of cysteine in the penetrability of post-thawed ram spermatozoa in the artificial mucus.

Coyan *et al.* (2011) stated that pre-freeze supplementation with 4 mM cysteine improved post-thaw ram sperm integrity, however the results were not statistically significant. These results could support our theory that determine the optimal timing and concentration of antioxidants supplementation contribute in maintain the activity of these antioxidants and improve others elements in sperm cell, that could lead to increase fertility rates. Specifically, the concentration used in the present study was 8.25 mM/ml compared to natural concentration of cysteine in SP that could the main reason for protecting and maintaining sperm activity. Cysteine has the ability to increase the level of glutathione in sperm cell, and there is a significant relationship between the level of glutathione and the ability of human sperm to penetrate bovine cervical mucus (Ochsendorf *et al.,* 1998). These findings for improving motility, viability and penetrability which have been related with the effect of cysteine to protect sperm membrane from the effect of cryopreservation, and that could be associated with prevent the extensive production of ROS (Allai *et al.,* 2018).

Conversely, the results in the present study did not agree with some previous studies, which stated there was not any beneficial effect of pre-freeze cysteine supplementation on post-thawing ram semen parameters such as motility and viability (5 mM and 10 mM, Yildiz *et al.*, 2015) could be associated with the type of extender used or could be due to different concentration of cysteine, although the concentrations were not dissimilar to those used in the present study. Specifically, the technique of semen preservation used in their studies differed by using straws as opposed to pellets in the present study. The species studied was cyprinus carpio. The lack of effect observed with only pre-freeze supplementation in other studies, help to support the importance of using antioxidants both pre-freeze and post-thaw as in the present study.

In the present study the level of ROS was determined using nitroblue tetrazolium (NBT) which reacts with superoxide ions (ROS) in the sperm head to form formazan granules, providing different level of ROS depending on the level of oxidative stress. The proportion

of sperm with formazan within the sperm head was higher in control samples compared to the lowest proportion of sperm with formazan within the sperm head was found in treatment samples specifically with 1.0 mg/ml (PF + PT). The increase the proportion of sperm with formazan within the sperm head in the present study could be related with incubation time. Cysteine is a precursor of glutathione and works as a glutathione peroxidase cofactor to destruct hydrogen peroxide (Meister, 1994), through the ability of glutathione to control hydrogen molecules and repair damaged DNA. Thus, glutathione (GSH) and the other thiol (-SH) compounds such as cysteine may be an essential component to protect cells from DNA damage. It has been reported that cysteine has the ability to protect the sperm membrane through chelation of transition metal ions such as Fe^{+2} -driven free radical reactions which have a damaging effect on the sperm cell membrane (Hughes *et al.*, 1998). The presence of a thiol group as well as a low molecular weight that makes it easy for cysteine to penetrate into the sperm membranes and protect the spermatozoa from the effect of ROS (Topraggaleh *et al.,* 2014).This demonstrates the need to identify the optimal timing and concentration of antioxidants supplementation which is a vital step to reduce the effect of ROS and protect sperm integrity.

The previous studies reported that the content of sperm glutathione reduced during the process of freezing and thawing in semen extender of bulls (Stradaioli and Monaci, 2007), boars (Gadea *et al.,* 2004), and goats (Atessahin *et al.,* 2008). Pre-freeze supplementation of cysteine improves glutathione content and activated glutathione peroxidase during cryopreservation (Coyan *et al.,* 2011; Topraggaleh *et al.,* 2014; Zhu *et al.,* 2017). The post-thaw supplementation of cysteine could recovered and provided some support to keep the stability of sperm glutathione level, which found that the post-thaw glutathione supplementation improved the integrity and fertilizing ability of frozen-thawed boar spermatozoa (Gadea, *et al.,* 2005), and goat spermatozoa (Gadea *et al.,* 2013). In the current study, was found a low level of cysteine (0.5 mg/ml) reduced the product of ROS. This effect may be attributed to the ability of cysteine to reduce the level of ROS and lipid peroxidation on sperm functions. The present study demonstrated that integrity of ram semen can be significantly improved with the addition an antioxidant. The mechanism underpinning this was the reduction of the concentration/quantity of ROS. However the effectiveness depends on type of antioxidant, concentration of antioxidant, method used to add to the semen *in vitro*.Other factors include differences in species of animal and breed and if the treatment is applied to the semen *in vitro* or directly to the animal eg oral supplement.

4. B.3.Conclusion

These findings showed that supplementation of 0.5 mg/ml or 1.0 mg/ml of cysteine both pre-freeze and post-thaw in ram semen extender, did not improve acrosome integrity, but lead to greater motility, viability and penetrability while reducing the percentage of ROS production compared to control samples, which supports spermatozoa to reduce ROS effect on the sperm plasma membrane and mitochondria. The greatest effect was observed in samples treated with 1.0mg/ml cysteine pre-freeze and post-thaw (PF+PT), and whilst other studies have focussed on adding antioxidants to the semen extender before freezing, this study highlights the importance of treating samples post-thaw to maintain sperm integrity and longevity. Therefore, the use of cysteine supplementation through cryopreservation and post-thaw incubation may be suggested to assist and improve the semen preservation approach in the sheep industry.

Chapter 4.C. Taurine supplementation pre-freeze and post-thaw improves integrity and reduces oxidative stress of cryopreserved ram spermatozoa

4. C.1.Results

4. C.1.1.Motility

There was an interaction between treatment and time for motility percentage ($P < 0.001$). Motility (mean % ± SEM) was generally greatest ($P < 0.001$) in samples treated with 1.0 mg/ml taurine pre-freeze and post-thaw. There was an intermediate effect in samples treated with different concentrations of taurine PF or PT, and the lowest motility was observed in the control samples which were not supplemented with taurine. As expected motility declined over time in all treatments (Table 4.11)

Table 4. 11. Mean (± SEM) percentage motility of spermatozoa at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$48.67 \pm 0.441^{c}_{w}$	$27.22 \pm 1.470^{e}_{x}$	$13.00 \pm 1.167^{e}_{y}$	$0.0 \pm 0.0^{f}_{z}$
PF 0.5 (9)	$49.78 \pm 0.222^{bc}_{w}$	$33.89 \pm 1.645^{d}_{x}$	$24.71 \pm 1.550^{d}_{y}$	$7.22 \pm 0.878^{d}_{z}$
PF 1.0 (9)	$51.31 \pm 0.882^{ab}_{w}$	$39.00 \pm 1.787^{c}_{x}$	$29.44 \pm 1.749^{bcd}_{y}$	$12.11 \pm 0.735^{bc}_{z}$
PT 0.5 (9)	$50.11 \pm 0.423^{a}_{w}$	$36.55 \pm 1.547^{bc}_{x}$	$27.67 \pm 1.414^{bc}_{y}$	$5.55 \pm 1.002^{e}_{z}$
PT 1.0 (9)	$52.11 \pm 0.772^{a}_{w}$	$44.22 \pm 0.894^{b}_{x}$	$33.56 \pm 1.082^{b}_{y}$	$10.56 \pm 0.556^{c}_{z}$
PF + P 0.5 (9)	$56.00 \pm 0.471^{a}_{w}$	$39.67 \pm 1.179^{c}_{x}$	$30.00 \pm 1.269^{bc}_{y}$	$17.44 \pm 0.835^{b}_{z}$
PF + PT 1.0 (9)	$58.56 \pm 0.729^{a}_{w}$	$49.33 \pm 0.957^{a}_{x}$	$38.11 \pm 0.889^{a}_{y}$	$22.44 \pm 0.899^{a}_{z}$

a, b, c, d, e, and f: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. C.1.2. The proportion of sperm viability

There was an interaction between treatment and time for viability ($P < 0.001$). Viability (mean % ± SEM) declined over time in all treatment groups but tended to be higher in samples treated with taurine compared to the control (Table 4.12). The highest results for viability were observed with taurine supplementation PF + PT (0.5 and 1.0 mg/ml), compared to the control group. There was an intermediate effect of taurine supplementation PF or PT treatments compared to the control (0.0 mg/ml) that did not have any treatment.

Table 4. 12. Mean (± SEM) percentage viability of spermatozoa at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$53.22 \pm 0.741^{c}_{w}$	$41.33 \pm 1.167^{e}_{x}$	$30.11 \pm 1.620^{e}_{y}$	$8.11 \pm 1.099^{f}_{z}$
PF 0.5 (9)	$56.44 \pm 0.580^{c}_{w}$	$50.89 \pm 1.348^{d}_{x}$	$44.12 \pm 1.645^{d}_{y}$	$17.11 \pm 0.754^{de}_{z}$
PF 1.0 (9)	$58.89 \pm 0.588^{c}_{w}$	$52.78 \pm 1.289^{c}_{x}$	$47.78 \pm 1.816^{bcd}_{y}$	$20.00 \pm 0.553^{c}_{z}$
PT 0.5 (9)	$58.00 \pm 0.667^{c}_{w}$	$52.67 \pm 1.202^{cd}_{x}$	$45.89 \pm 1.567^{cd}_{y}$	$18.33 \pm 0.866^{e}_{z}$
PT 1.0 (9)	$60.44 \pm 0.709^{bc}_{w}$	$55.78 \pm 0.878^{b}_{x}$	$50.56 \pm 1.192^{b}_{y}$	$22.22 \pm 1.051^{cd}_{z}$
PF + PT 0.5(9)	$61.44 \pm 0.835^{ab}_{w}$	$57.00 \pm 0.972^{c}_{x}$	$51.89 \pm 1.092^{bc}_{y}$	$34.22 \pm 0.846^{b}_{z}$
PF + PT 1.0(9)	$62.22 \pm 0.703^{a}_{w}$	$57.78 \pm 0.830^{a}_{x}$	$53.44 \pm 0.959^{a}_{y}$	$36.78 \pm 0.795^{a}_{z}$

a, b, c,d, e and f: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. C.1.3. Acrosome Integrity

There was an interaction between treatment and time for acrosome integrity (P <0.001). Acrosome integrity (mean % ± SEM) declined over time in all treatment groups but tended to be higher in samples treated with taurine compared to the control (Table 4.13). Acrosome integrity was greatest in samples supplemented with taurine PF + PT compared to the control group at all-time points. In addition, there was an intermediate effect of taurine supplementation PF or PT treatments compared to the control (0.0 mg/ml) that did not have any treatment.

Table 4. 13. Mean (± SEM) percentage of acrosome-intact spermatozoa at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$56.56 \pm 0.801^{d}_{w}$	$45.67 \pm 0.866^{d}_{x}$	$33.56 \pm 1.501^{d}_{y}$	$10.44 \pm 0.475^{d}_{z}$
PF 0.5 (9)	$60.33 \pm 0.624^{cd}_{w}$	$54.33 \pm 1.258^{c}_{x}$	$47.67 \pm 1.700^{c}_{y}$	$21.11 \pm 0.992^{c}_{z}$
PF 1.0 (9)	$62.78 \pm 0.521^{c}_{w}$	$56.67 \pm 1.155^{bc}_{x}$	$50.89 \pm 1.645^{bc}_{y}$	$22.56 \pm 0.818b^{c}_{z}$
PT 0.5 (9)	$62.00 \pm 0.601^{c}_{w}$	$56.22 \pm 1.211^{bc}_{x}$	$49.78 \pm 1.341^{c}_{y}$	$21.67 \pm 1.364^{c}_{z}$
PT 1.0 (9)	$64.44 \pm 0.689^{bc}_{w}$	$59.56 \pm 0.973^{ab}_{x}$	$54.00 \pm 1.014^{ab}_{y}$	$26.33 \pm 1.856^{b}_{z}$
PF + PT 0.5(9)	$64.78 \pm 0.760^{ab}_{w}$	$59.78 \pm 1.140^{ab}_{x}$	$55.33 \pm 1.667^{a}_{y}$	$36.67 \pm 0.816^{a}_{z}$
PF + PT 1.0(9)	$66.11 \pm 0.935^{a}_{w}$	$62.00 \pm 0.928^{a}_{x}$	$56.78 \pm 1.211^{a}_{y}$	$39.22 \pm 1.140^{a}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different (P < 0.001).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different (P < 0.001).

4. C.1.4. Penetrability through artificial mucus

The distance travelled through artificial mucus (mean ± SEM) was greater ($P < 0.001$) in samples treated with taurine at 60 min compared to the control group (Figure 4.3). Furthermore, the distance travelled at 60 min was greater within groups PF + PT (0.5 and 1.0 mg/ml, 4.0 ± 0.076 cm/h, and 2.989 ± 0.111cm/h, respectively). The results showed an intermediate effect of taurine supplementation at different concentrations PF or PT. The lowest distance travelled by frozen-thawed ram spermatozoa during the incubation time was in the control group which had no taurine supplementation (1.433 ± 0.066 cm/h).

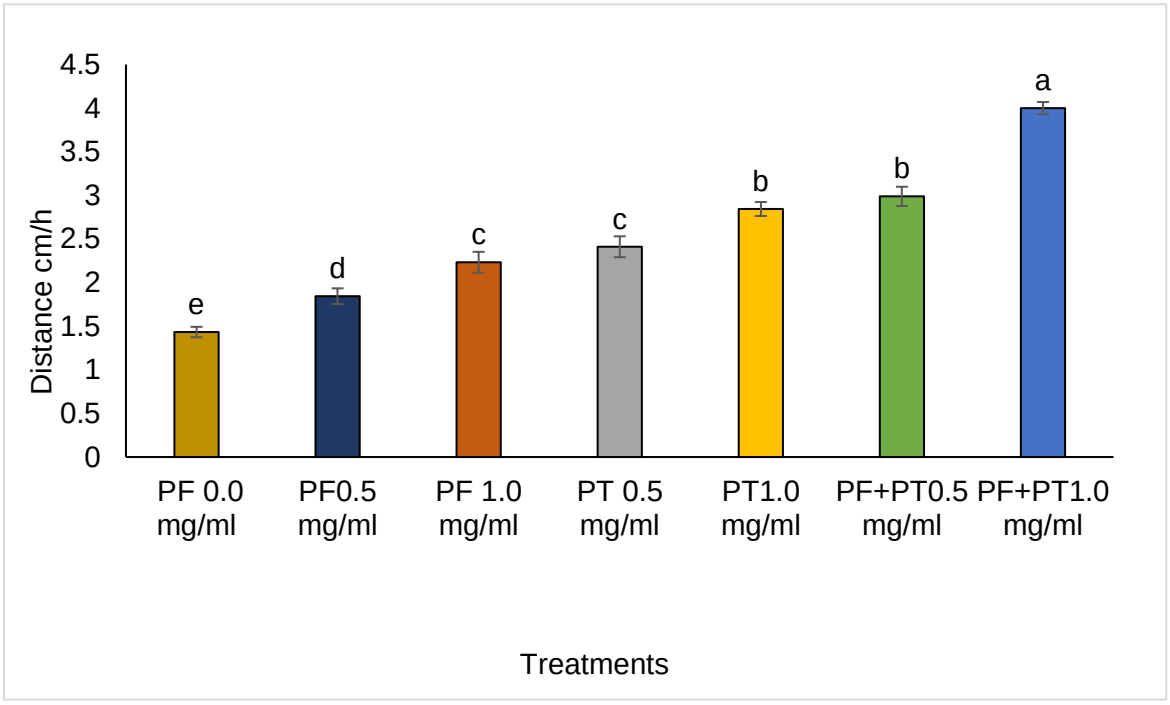


Figure 4. 3. Mean ± SEM of distance travelled of spermatozoa at 60 minutes post-thaw when supplemented with 0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT).

4. C.3.5. Proportion of spermatozoa with formazan formation (ROS production)

4. C.3.5.1. Proportion of spermatozoa with 0 % formazan formation

There was an interaction between treatment and time for the proportion of sperm with 0% formazan present in the sperm head ($P < 0.001$). The proportion of sperm with 0% formazan formation within the sperm head (mean % ± SEM) declined over time in all treatment groups. The effect of treatment differed between time points but tended to be higher in samples treated with taurine pre-freeze and post-thaw (PF + PT 0.5 mg/ml and PF + PT 1.0 mg/ml, compared to the control (0.0 mg/ml) and other treatments (Table 4.14).

Table 4. 14. Mean (± SEM) proportion of spermatozoa with 0 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$10.44 \pm 0.377^{d}_{w}$	$7.11 \pm 0.484^{d}_{x}$	$3.11 \pm 0.309^{d}_{y}$	$0 \pm 0^{c}_{z}$
PF 0.5 (9)	$19.78 \pm 0.222^{c}_{w}$	$14.89 \pm 0.484^{c}_{x}$	$9.11 \pm 0.754^{c}_{y}$	$0 \pm 0^{c}_{z}$
PF 1.0 (9)	$21.22 \pm 0.222^{c}_{w}$	$16.11 \pm 0.716^{c}_{x}$	$9.67 \pm 0.928^{c}_{y}$	$0 \pm 0^{c}_{z}$
PT 0.5 (9)	$20.22 \pm 0.434^{c}_{w}$	$14.67 \pm 0.782^{c}_{x}$	$9.11 \pm 1.047^{c}_{y}$	$0 \pm 0^{c}_{z}$
PT 1.0 (9)	$22.00 \pm 0.333^{c}_{w}$	$16.11 \pm 0.754^{c}_{x}$	$10.00 \pm 0.866^{c}_{y}$	$0.33 \pm 0.333^{c}_{z}$
PF + PT 0.5 (9)	$30.11 \pm 0.423^{b}_{w}$	$24.44 \pm 0.754^{b}_{x}$	$18.00 \pm 0.882^{b}_{y}$	$5.22 \pm 0.324^{b}_{z}$
PF + PT 1.0 (9)	$33.22 \pm 0.572^{a}_{w}$	$26.44 \pm 0.899^{a}_{x}$	$20.22 \pm 1.024^{a}_{y}$	$6.11 \pm 0.455^{a}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. C.1.5.2. Proportion of spermatozoa with < 50 % formazan formation

There was an interaction between treatment and time for the proportion of sperm with < 50% formazan present in the sperm head ($P < 0.001$). The proportion of sperm with < 50 % formazan in the sperm head (mean % ± SEM) declined over time in all treatment groups but tended to be highest ($P < 0.001$) in samples treated with taurine pre-freeze and post-thaw (PF + PT 0.5 and PF + PT 1.0 mg/ml), compared to the control (0.0 mg/ml) which had the lowest proportion.(Table 4.15).

Table 4. 15. Mean (± SEM) proportion of spermatozoa with < 50 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parantheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0(9)	$44.11 \pm 1.086^{c}_{w}$	$37.56 \pm 1.029^{bcd}_{x}$	$30.33 \pm 1.434^{b}_{y}$	$2.22 \pm 0.619^{c}_{z}$
PF 0.5 (9)	$34.89 \pm 0.841^{a}_{w}$	$30.67 \pm 1.130^{a}_{x}$	$24.89 \pm 1.218^{a}_{y}$	$4.11 \pm 0.611^{ab}_{z}$
PF 1.0 (9)	$40.67 \pm 0.882^{bc}_{w}$	$35.44 \pm 0.835^{bc}_{x}$	$31.00 \pm 0.928^{bc}_{y}$	$8.22 \pm 0.940^{b}_{z}$
PT 0.5 (9)	$40.67 \pm 1.537^{bc}_{w}$	$35.11 \pm 1.711^{abc}_{x}$	$26.44 \pm 1.659^{ab}_{y}$	$6.78 \pm 0.866^{ab}_{z}$
PT 1.0 (9)	$39.22 \pm 1.402^{ab}_{w}$	$34.44 \pm 1.425^{ab}_{x}$	$30.56 \pm 1.659^{b}_{y}$	$7.56 \pm 1.271^{b}_{z}$
PF + PT 0.5 (9)	$43.89 \pm 0.683^{c}_{w}$	$40.78 \pm 0.852^{d}_{x}$	$36.78 \pm 1.364^{d}_{y}$	$16.56 \pm 1.271^{c}_{z}$
PF + PT 1.0 (9)	$42.78 \pm 0.795^{bc}_{w}$	$39.44 \pm 0.689^{cd}_{x}$	$35.22 \pm 0.983^{cd}_{y}$	$18.22 \pm 1.115^{c}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. A.1.5.3. Proportion of spermatozoa with > 50 % formazan formation

There was an interaction between treatment and time for the proportion of sperm with >50% formazan present in the sperm head ($P < 0.001$). The proportion of sperm with >50 % formazan in the sperm head (mean % ± SEM) increased over time in all treatment groups but tended to be lowest ($P < 0.001$) in samples treated with taurine pre-freeze and post-thaw and highest in the control (0.0 mg/ml) samples. Supplementation with different concentrations of taurine PF or PT showed an intermediate effect in reducing the proportion of sperm with >50% formazan. AT 180 min incubation, the proportion of sperm with >50% formazan at the sperm head was lowest in the control samples, this is due to the high proportion of untreated sperm (control) at 180 min that had 100% formazan in the sperm head (table 4.17).

Table 4. 16. Mean (± SEM) proportion of spermatozoa with >50 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$23.67 \pm 0.408^{b}_{w}$	$28.67 \pm 0.764^{c}_{x}$	$32.22 \pm 1.064^{c}_{y}$	$38.00 \pm 0.882^{d}_{z}$
PF 0.5 (9)	$24.00 \pm 0.333^{b}_{w}$	$29.11 \pm 0.735^{c}_{x}$	$34.45 \pm 1.285^{bc}_{y}$	$48.89 \pm 0.389^{bc}_{z}$
PF 1.0 (9)	$20.33 \pm 0.471^{b}_{w}$	$25.89 \pm 0.857^{c}_{x}$	$31.67 \pm 0.816^{bc}_{y}$	$50.11 \pm 0.873^{a}_{z}$
PT 0.5 (9)	$20.78 \pm 0.778^{b}_{w}$	$26.00 \pm 1.067^{c}_{x}$	$32.67 \pm 1.111^{bc}_{y}$	$48.56 \pm 0.377^{b}_{z}$
PT 1.0 (9)	$21.00 \pm 0.782^{b}_{w}$	$26.44 \pm 1.064^{c}_{x}$	$31.00 \pm 1.002^{b}_{y}$	$49.22 \pm 0.465^{ab}_{z}$
PF + PT 0.5(9)	$14.78 \pm 0.521^{a}_{w}$	$18.89 \pm 0.818^{a}_{x}$	$25.00 \pm 1.538^{a}_{y}$	$45.89 \pm 0.807^{c}_{z}$
PF + PT 1.0(9)	$15.33 \pm 0.889^{a}_{w}$	$20.56 \pm 1.152^{b}_{x}$	$26.11 \pm 1.620^{a}_{y}$	$43.67 \pm 1.280^{cd}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. C.1.5.4. Proportion of spermatozoa with 100 % formazan formation

There was an interaction between treatment and time for the proportion of sperm with 100% formazan present in the sperm head ($P < 0.001$). The proportion of sperm with 100% formazan in the sperm head (mean % ± SEM) increased over time in all treatment groups was significantly lower ($P < 0.001$) in samples treated with taurine pre-freeze and post-thaw (PF + PT 0.5 and PF + PT 1.0 mg/ml), compared to all other treatments at all-time points. Taurine supplementation at different concentrations either PF or PT only differed from control samples at 60 and 180 min incubation (Table 4.1).

Table 4. 17. Mean (± SEM) proportion of spermatozoa with 100 % formazan in the sperm head at 0, 30, 60, and 180 minutes post-thaw when supplemented with 0.0, 0.5 or 1.0 mg/ml taurine pre-freeze (PF), post-thaw (PT) or pre-freeze and post-thaw (PF + PT). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Treatment	0 min	30 min	60 min	180 min
Control 0.0 (9)	$21.78 \pm 0.899^{b}_{w}$	$26.67 \pm 1.258^{c}_{x}$	$34.33 \pm 1.708^{d}_{y}$	$59.78 \pm 1.090^{d}_{z}$
PF 0.5 (9)	$21.33 \pm 0.500^{b}_{w}$	$25.33 \pm 0.527^{bc}_{x}$	$31.46 \pm 0.754^{bc}_{y}$	$47.00 \pm 0.624^{c}_{z}$
PF 1.0 (9)	$17.78 \pm 0.324^{b}_{w}$	$22.56 \pm 0.580^{b}_{x}$	$27.67 \pm 0.726^{bc}_{y}$	$41.67 \pm 0.687^{b}_{z}$
PT 0.5 (9)	$18.33 \pm 0.745^{b}_{w}$	$24.22 \pm 0.940^{bc}_{x}$	$31.56 \pm 1.453^{cd}_{y}$	$44.44 \pm 0.915^{bc}_{z}$
PT 1.0 (9)	$17.78 \pm 0.741^{b}_{w}$	$22.67 \pm 0.816^{b}_{x}$	$28.00 \pm 1.106^{b}_{y}$	$42.89 \pm 0.904^{b}_{z}$
PF + PT 0.5 (9)	$11.33 \pm 0.373^{a}_{w}$	$15.67 \pm 0.667^{a}_{x}$	$20.56 \pm 0.6488^{a}_{y}$	$32.11 \pm 0.920^{a}_{z}$
PF + PT 1.0 (9)	$9.11 \pm 0.351^{a}_{w}$	$13.33 \pm 0.441^{a}_{x}$	$18.44 \pm 0.530^{a}_{y}$	$32.00 \pm 1.344^{a}_{z}$

a, b, c and d: values with different superscripts in the same column for each incubation time are significantly different ($P < 0.001$).

w, x, y, and z: values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

4. C.2. Discussion

Taurine is considered one of the main scavengers that improves the defence of sperm against ROS and reduce lipid peroxidation (Sharma and Agarwal, 1996; Saleh and Agarwal, 2002). Taurine has a variety of physiological roles including viability, cell propagation, osmoregulation, inhibition of oxidative stress in the cell (Buff *et al.,* 2001); (Chatiza *et al.,* 2018), transport and binding of calcium (Lazarewicz *et al.,* 1985), and the biological stabilisation of membranes (Balkan *et al.,* 2002). It has been stated that taurine has an important role as an antioxidant in cell defence mechanisms (Green *et al.,* 1991).

This study was aimed to determine the optimal timing and concentration of taurine supplementation of the semen extender that can decrease oxidative stress and enhance the integrity of cryopreserved ram spermatozoa. The percentage of motility, acrosome integrity, viability, penetrability, and ROS production were assessed as parameters to rectify of oxidative damage to the sperm membrane and the resulting effect on sperm function during cryopreservation. The addition of different concentrations of taurine to ram semen extender before freezing and after thawing was thought to reduce this damage by promoting the stabilization of the sperm membrane. The results obtained were in agreement with previous studies which showed that supplementation with antioxidants such as taurine improved and maintained the motility and viability of cryopreserved spermatozoa of various species such as the bull (Foote *et al.,* 2002; Chhillar *et al.,* 2012), buffalo (Shiva Shankar Reddy *et al.*, 2010), goat (Salvador *et al.,* 2006), turkey (Donoghue and Donoghue, 1997; Slanina *et al.,* 2018), rabbit (Alvarez, and Storey, 1983), rat (Yang *et al.,* 2010) donkey (Dorado *et al.,* 2014; Bottrel *et al.,* 2018), and horse semen (Ijaz and Ducharme, 1995). Chatiza *et al.* (2018) stated that 75 mM taurine supplementation during liquid storage of boar semen have the greatest effect at 17ºC for 4h, compared with other types of antioxidants such as cysteine and vitamin E. Taurine has the ability as an antioxidant to protect and maintain the integrity of the acrosome (Sariözkan *et al.,* 2009a), stabilizes and protects cytoplasmic membrane and improves the motility of mammalian spermatozoa (Slanina *et al.,* 2018).

Moreover, some studies have been conducted on ram sperm using different concentration of taurine Previous studies (Bucak *et al.,* 2007; Rather *et al.,* 2016; Banday *et al.,* 2017) have demonstrated the effect of taurine on spermatozoa using concentrations of25-50 mM taurine compared to 8 mM in the present study during pre-freeze supplementation on the semen of different species of animals including rams. These high concentrations of taurine improved ram sperm integrity and reduced the level of ROS and oxidative stress in most species. It is possible that at these high concentrations used pre-freeze, there is a reduced need for the addition of taurine to spermatozoa post-thaw. However, in the present study,

supplementation of 1.0 mg/ml of taurine (8 mM) PF and PT improved sperm integrity and reduced the level of ROS. Therefore, this method of using taurine pre-freezing and post-thawing (PF + PT) seemed to be more effective in protecting sperm function as well as being more economical through detecting the optimal time and concentration of taurine in ram semen extender compared with previous studies.

The significant effect of taurine on sperm parameters on the present study could be due to the fact that taurine acts as a sulfonated amino acid which is an essential defender for cells against the production of ROS when exposed during aerobic circumstances (Alvarez and Story, 1983; Holmes *et al.,* 1992). Furthermore, taurine has the ability to penetrate cryoprotectants and reacts as an antioxidant to induce the rearrangement of proteins and lipids within the sperm membrane, which improves membrane fluidity (Yang *et al.,* 2010), and regulates the energy metabolism in the cell (Agarwal and Allamaneni *et al.,* 2004). This effect could minimize the effect of low temperatures to which the sperm are exposed during cryopreservation and subsequently improve the capability of spermatozoa to resist the harmful effects of freezing and thawing (Sanchez-Partida *et al.,* 1993; Maxwell and Watson, 1996). In addition, taurine supplementation of 0.5 and 1.0 mg/ml (PF + PT) improved sperm motility post cryopreservation, and displayed as antioxidative role. This could be due to the ability of taurine to improve the level of antioxidant enzymes in sperm cell through reducing the damaging effect of superoxide anion, which has high activity and cannot distribute easily through the cell membrane, and dismute to generate hydrogen peroxide (H_2O_2) either naturally or by superoxide dismutase (SOD) enzyme (Slimen *et al.,* 2014).

Taurine has the ability to protect and enhance the function of other antioxidant enzymes including catalase (Perumal *et al.,* 2013), superoxide dismutase (Higuchi *et al.*, 2012), glutathione peroxidase (Nonaka *et al.,* 2001), and thioredoxin reductase (Yildirim *et al.,* 2007). The protective feature of taurine on the antioxidant system of spermatozoa improved sperm ability to resist the harmful effect of ROS (Holmes *et al.,* 1992; Bucak *et al.,* 2007; Paal *et al.,* 2018). Additionally, this synergistic effect protects sperm integrity (Slanina *et al.,* 2018). This system can assist to inhibit cholesterol efflux from the cell membrane, reduce production of malondialdehyde, and decrease the damage of cholesterol from cell membranes which is essential for acrosome integrity (Perumal *et al.,* 2013). Moreover, it has been reported that taurine has the ability to transport across the mitochondrial plasma membrane (Suzuki *et al.,* 2002), and supports stabilisation of the chain of electron transportation (Jong *et al.,* 2012). This could explain the role of taurine in the improvement of sperm integrity such as motility, acrosome integrity, viability and penetrability in the present study.

In studies that have been conducted to assess the ability of taurine to protect sperm integrity at 5°C such as in turkey (2.5-7.5 mg/ml, Slanina *et al.,* 2018), and in bull (50 mM, Perumal

et al., 2013), high concentrations of taurine were beneficial to sperm function. However, liquid storage/colled semen is not suitable for long term storage of semen and also the sperm experienced high stress as in cryopreservation (Salamon and Maxwell, 2000). Conversely, 75 mM taurine supplementation did not improve boar sperm integrity in liquid storage at 17 °C (Chatiza *et al.,* 2018), highlighting the beneficial technique in the present study for the optimized time and concentrations of taurine supplementation in cryopreserved semen.

Contrariwise, Partyka *et al.* (2017) found that pre-freeze supplementation of chicken sperm by 10 mM of taurine improved sperm parameters such as motility, mitochondrial activity and viability, and decreased apoptosis and DNA fragmentation of sperm, however they did not test the post-thaw treatment. In the present study, the penetrability of frozen-thawed ram sperm through the artificial mucus was greatest in samples treated with 1.0 mg/ml taurine PF + PT 1.0 mg/ml This effect could be related to the beneficial effect of taurine on endogenous antioxidant capability to improve catalase level in the presence of ROS (Bucak *et al.,* 2007; Sariözkan *et al.,* 2009). Additionally, taurine can maintain sperm integrity at post thaw through stabilization of the mitochondrial membrane and electron passage chain inhibiting it from high production of superoxide anion (Jong *et al.,* 2012). Lopes *et al.* (2018) reported that taurine supplementation of tambaqui sperm extender improved the regulation and transport of Ca^{2+}, which is required to generate sperm activity. This positive effect of taurine on the sperm membrane could also explain their ability to maintain the integrity and improve sperm penetrability.

The direct effect of taurine as an antioxidant could be through its ability to detoxify and reduce some volatile intermediates such as hypochlorous acid generated by myeloperoxidase, and the indirect effect through taurine penetrates into the cell membrane and stabilizing it (Schaalan *et al.*, 2018). In the current study, taurine reduced ROS thus inhibiting the harmful effect of ROS and providing stability to the sperm membrane specifically in the post-thaw phase. Moreover, the presence of amine group in taurine plays an important role through its relationship with nucleic acids and can consequently reduce the ROS production and the damage to DNA (Sokól *et al.*, 2009).

Taurine has the ability to improve either the primary post-thaw motility of sperm or the longevity of motility of frozen–thawed ram sperm (Sanchez-Partida *et al.,* 1997). Consequently in the present study, the use of taurine both pre-freeze and post-thaw could be particularly beneficial.The pre-freeze supplementation allowed taurine to function as an osmoregulater and protect the sperm membrane from the effect of cold shock damage (Sturman and Hayes, 1980) whilst post-thaw supplementation may enable taurine to

support the systemic enzymes of the sperm to reduce the effect of ROS and oxidative stress (Das *et al.,* 2009; Yang *et al.,* 2010). In addition, the sudden variation in temperature through thawing of frozen spermatozoa from (-196 ºC to 37 ºC), that leads to a fast phase change from solid to liquid and severe ROS production by switching on the O^2 creation or a release of free radicals produced along the stages of cryopreservation (Chatterjee and Gagnon, 2001). Therefore, these findings in the current study could determine the effect of optimal timing and /or concentration of taurine supplementation on the frozen-thawed ram semen.

4. C.3.Conclusion

This study has shown that taurine supplementation 0.5 and 1.0 mg/ml PF + PT decreased ROS production and protected parameters of ram spermatozoa such as motility, acrosome integrity, viability and improved penetrability after cryopreservation. The best effects of taurine supplementation were obtained when supplementation occurred pre-freeze and post thaw and the optimal concentration at 1.0 mg/ml. These findings were obtained through using the novel technique for timing and concentration of taurine supplementation may assist the improvement of cryopreservation protocols to increase ram sperm integrity.

4. D. General Conclusion of Antioxidants Supplementation

All antioxidants which have been investigated in the present study, determined that 1.0 mg/ml both pre-freeze and post-thaw was most beneficial. This highlights the importance of protecting sperm during cryopreservation, but also after thawing as the sperm are still exposed to oxidative stress during this time. Through this study the protocol of antioxidants supplementation was developed that could be utilized. The results of vitamin C, cysteine and taurine, suggest that there are limited beneficial effects of using vitamin C in cryopreserved ram semen. Cysteine and taurine showed a high effect to protect and maintain the functions of ram sperm post-thaw, and it is therefore, recommended to utilize it using the optimized protocol developed in the present study. Cysteine and taurine have shown a diversity of physiological functions on sperm cell including motility, viability, osmoregulation and inhibition of oxidative stress in cell which is assumed to be regulated by their ability to penetrate the sperm membrane to regulate and maintain the activity of sperm.

Whilst cysteine and taurine improve sperm function most likely through reducing oxidative stress, the effect of ROS and lipid peroxidation on all sperm components has not been elucidated. The sperm membrane contains many types of proteins including heat shock proteins (HSPs) which have been implicated in sperm function and integrity. Determining the impact of oxidative stress on HSP protein expression and investigating the role of cysteine and taurine in protecting the activity of HSPs in ram sperm could aid development of semen cryopreservation protocols.

Chapter 5. Effects of H_2O_2 exposure on ram sperm motility, reactive oxygen species levels and heat shock proteins 70 and 90 expression

5.1. Introduction

Results of the previous study (Chapter 4) have shown the detrimental effect of ROS on sperm functions and effect of antioxidants supplementation to reduce this effect. However, the defence mechanism of sperm membrane and the role of heat shock proteins (HSP) such as HSP70 and HSP90 to reduce the effect of ROS is not fully elucidated. Cysteine and taurine were successful in reducing ROS and improving sperm integrity. The mode of action of ROS on sperm proteins, DNA and membrane stability has been investigated, however there are a range of proteins that appear to have a critical role on sperm function, that have not been investigated in response to ROS. Heat shock proteins are molecular chaperones which have a critical role in male reproduction such as sperm maturation and functions (Redgrove *et al.,* 2012).

There are numerous ROS which affect sperm such as superoxide (O_2) hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and nitric oxide (NO). Exogenous addition of H_2O_2 increases intracellular ROS which are important intracellular signalling molecules that are necessary at physiological levels to assist in sperm motility and fertilization (Mahfouz *et al.,* 2008; Plessis *et al.,* 2010). It has been suggested that H_2O_2 plays an essential physiological role during the capacitation process, probably by influencing reorganisation of the membrane to aid the fusion through exocytosis of the acrosomal contents (Bize *et al.,* 1991; Griveau and Renard, 1994).H_2O_2 is a critical oxidant and central ROS which causes chemical damage in DNA of the cell and has a high ability to penetrate the cell membrane (Halliwell and Aruoma,1991). Furthermore, H_2O_2 can react with Fe^{2+} or Ca^{2+} to produce the hydroxyl radical (OH•) (e.g., $H_2O_2+Fe^{2+}\rightarrow OH\bullet+OH^{-}+Fe^{3+}$), which results in heavy oxidation. The excessive production of ROS, with limited antioxidants defence, inhibit sperm functions (Agarwal *et al.,* 2006). The high concentrations of ROS have been correlated with reduced motility and subsequently adverse effects on human fertility (Saleh and Agarwal, 2002; Aziz *et al.,* 2004).

Heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70) are proteins that can protect spermatozoa from oxidative stress (Beere and Green, 2001). These proteins can be synthesized by cells in stressful circumstances to reduce aggregation and denaturation. HSP90 has a significant role in acrosome reaction, hyperactivation, and phosphorylation during capacitation in human and boar sperm (Ecroyd *et al.,* 2003; Li *et al.,* 2014; Sagare-Patil *et al.,* 2017). Furthermore, HSP90 has a critical role in preserving sperm motility during cooling or after cryopreservation (Cao *et al.,* 2003; Martin *et al.,* 2007). HSP70 also has the ability to maintain the stability of sperm membrane protein and is involved in the movement

of proteins across intracellular membranes (Zhang *et al.,* 2015). HSP70 was detected in sperm proteins isolated from boar (Yang and Leela, 2000), and bull (Kamaruddin *et al.,* 2004), and may play an essential role in fertilization (Spinaci *et al.,* 2006). A positive correlation has been identified between the motility of frozen-thawed bull sperm and expression level with coefficients ranging from 0.327 to 0.785 for HSP70 (Zhang *et al.,* 2015), 0.364 to 0.953 for HSP90 (Wang *et al.,* 2014).

Whilst HSP 90 and HSP70 can protect against oxidative stress, the impact of oxidative stress on the expression HSP70 and HSP90 on ram sperm has not been investigated. As ROS are detrimental and HSPs are protective, this study aimed to determine the effect of H_2O_2 on the protein expression of HSP90 and HSP70 in ram spermatozoa. In another study by Zhang *et al.* (2018) on the effect of H_2O_2 on the expression level of HSP90 by *E.coli* stated by producing this protective protein, they have the ability to inhibit phospholipid oxidation against oxidative damage by ROS. The findings from this study will aid the understanding of the role heat shock proteins and impact of ROS on protective mechanisms in ram spermatozoa.

5.2. Materials and methods

5.2.1. Animal and semen collection

Semen samples from three mature Texel rams (3 years) with weight 85-87 Kg were used in this study. The animals were housed at Harper Adams University. The rams were fed 1 kg of concentrate daily, and good quality hay and water were supplied *ad libitum*. A total number of nine ejaculates were collected from the rams (3 ejaculates from each ram) twice a week during the breeding season (October) using the artificial vagina. Only ejaculates of 0.75-2.0 ml, a minimum concentration of 3×10^9 sperm/ml and minimum motility of 80% were used in the experiment.

5.2.2. Semen processing

Immediately after collection, the ejaculates were assessed for quality as described above, then immersed in a warm water bath at 37°C. Ejaculates (n = 9) were diluted individually in tris-citrate-glucose (300 mM Tris base, 94.7 mM citric acid, 27.8 mM D-Glucose, 0.0058g penicillin, 0.005g Streptomycin (Sigma Aldrich, UK), and each ejaculate was divided into four aliquots. Each aliquot was further diluted in TCG to a concentration of $25x10^6$ sperm/ml. The first aliquot was considered as a pre-incubated sample without the addition of H_2O_2 or incubation, and the three remaining aliquots received one of three treatments: 1) 15 mM H_2O_2, 2) 5 mM of H_2O_2, and 3) control without any treatment with H_2O_2 (Sigma-Aldrich, UK) then were incubated at 37ºC for 60 min (Figure 5.1). The pre-incubated sample was assessed directly after preparation, and the other three aliquots were examined after incubation for motility, the proportion of formazan at the sperm head, lipid peroxidation of sperm and seminal plasma, and the protein expression of HSP90 and HSP70 protein at different sperm regions.

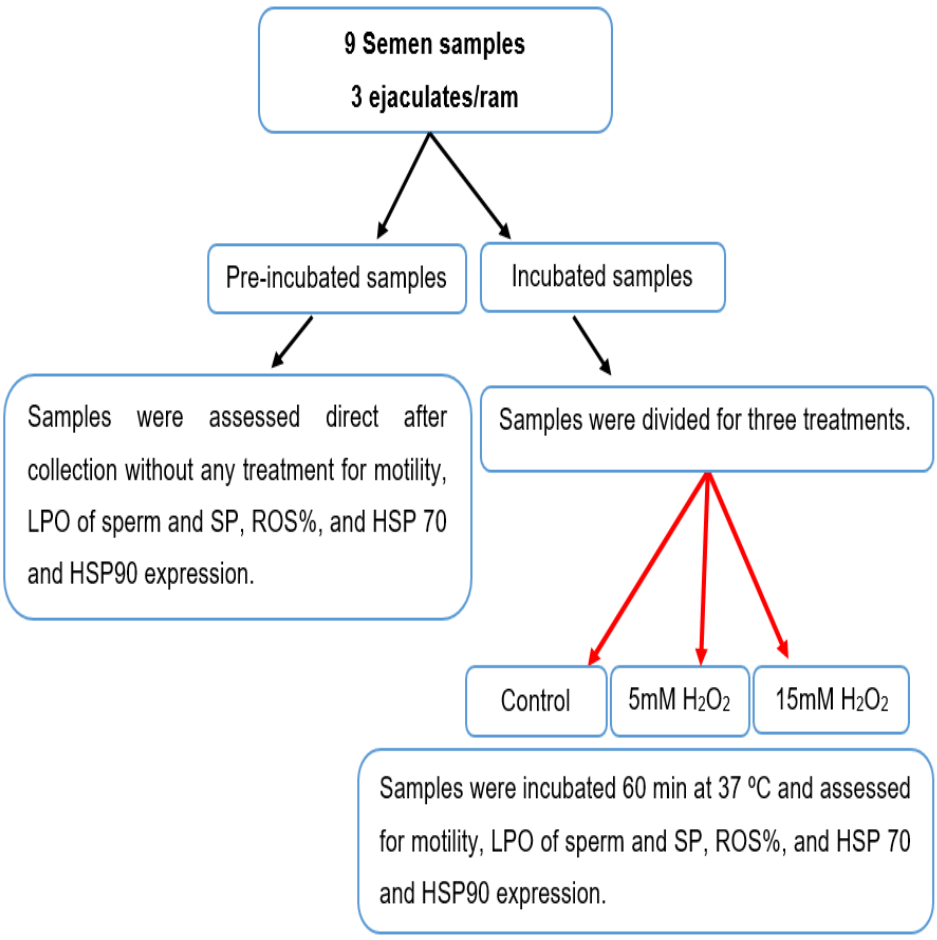


Figure 5.1. Experimental design of the effect of hydrogen peroxide (H_2O_2) on fresh ram sperm.

5.2.3. Assessment of ram spermatozoa following H_2O_2 supplementation

5.2.3.1. The proportion of motile spermatozoa

The proportion of motile spermatozoa was determined according to the method described in section 2.9.1.

5.2.3.2. Proportion of ROS production in sperm

Proportion of spermatozoa with <50% and >50% formazan formation in sperm head was determined according to the method described in section 2.11.

5.2.3.3. Measurement of malondialdehyde (MDA)

Lipid peroxidation of seminal plasma (SP) and sperm membranes was determined according to the method described in section 2.12.1 and 2.12.2 respectively

5.2.3.4. HSP70 and HSP90 expression

HSP70 and HSP90 protein by immunofluorescence was determined according to the method described in section 2.13. The expression of HSP70 and HSP90 was assessed for each sperm region of each sample for pre-incubated and incubated samples as described in section 2.13.

5.2.5. Statistical analyses

The data were analysed using one way (ANOVA) Gen stat (17th edition) with the treatment being H_2O_2 concentration. Ram was used as a block. Means were reported with a ± standard error of the mean (SEM) and $P < 0.05$ was considered statistically significant. Post-hoc analyses were performed using the least significant difference (LSD) test. The variables were motility, proportion of spermatozoa >50 and <50% formazan formation, level of LPO of sperm and SP, and expression level of HSP. During statistical analyses of HSP70 and HSP90 expression, each region of the sperm was considered separately to compare the effect of treatment.

5.3. Results

5.3.1. Motility

Motility (mean % ± SEM) was greatest ($P < 0.001$) in pre-incubated samples (78.480 ±1.263) which did not have any treatment of H_2O_2 or incubation. The percentage motility of incubated samples at 60min was greatest in the control sample, intermediate in samples treated with 5 mM H_2O_2, and lowest in samples treated with 15 mM of H_2O_2 (Table 5.1).

5.3.2. Proportion of ROS > 50% and < 50%

There was a significant effect of treatment in proportion of ROS > 50% and ROS < 50% production ($P < 0.001$). The proportion of sperm with >50% ROS at the sperm head (mean % ± SEM) tended to be lowest ($P < 0.001$) in pre-incubated samples which did not have any treatment or incubation. In the treatment samples, ROS > 50% was lowest in the control group, moderate in the group receiving treatment of 5mM of H_2O_2, and highest in the group receiving treatment of 15mM of H_2O_2 (Table 5.1). The proportion of spermatozoa with <50% formazan formation was highest ($P < 0.001$) in pre-incubated samples and reduced significantly between control (no H_2O_2), 5 mM and 15 mM of H_2O_2 (Table 5.1).

5.3.3. Level of LPO in the seminal plasma and sperm

The value of LPO of SP and sperm (mean % ± SEM) was lowest in pre-incubated samples ($P < 0.001$). The level of LPO of SP and sperm for samples at 60 min incubation was highest in the group treated with 15 mM, and moderate with samples treated with 5 mM of H_2O_2. However, the lowest value of LPO in sperm and SP was in the control group that has no treatment with H_2O_2 (Table 5.1).

Table 5. 1. Means (± SEM) motility %, proportion ROS < 50 and ROS > 50, LPO of SP and sperm values of fresh ejaculated spermatozoa pre-incubation and supplemented without H_2O_2 as control and with 5 mM and 15 mM for 60 minutes incubation at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment.

Parameters	0 min pre-incubation(9)	60 min Control (9)	60 min 5 mM(9)	60 min 15 mM(9)
Motility	78.480 ±1.263[a]	49.44 ± 2.416[b]	33.22 ± 2.350[c]	18.22 ± 2.356[d]
ROS<50	79.00 ± 1.236[a]	67.89 ± 0.920[b]	56.89 ± 0.889[c]	32.22 ± 1.869[d]
ROS>50	21.00 ± 1.236[a]	32.11 ± 0.920[b]	43.11 ± 0.889[c]	67.78 ± 1.869[d]
LPO SP	0.82 ± 0.030[a]	1.71 ± 0.095[b]	3.28 ± 0.197[c]	5.68 ± 0.182[d]
LPO sperm	0.74 ± 0.044[a]	1.31 ± 0.064[b]	3.06 ± 0.133[c]	5.20 ± 0.168[d]

a, b, c, and d values with different superscripts in the same row for each treatment are significantly different ($P < 0.001$).

5.3.4. Level of HSP70 expression

There was high expression of HSP70 in pre-incubated samples on all sperm regions such as the acrosome, post-acrosome, full head, middle piece and tail ($P < 0.001$). At all regions, the protein expression of HSP70 (mean % ± SEM) was highest ($P < 0.001$) in pre-incubated samples which did not have any treatment. HSP70 protein expression decreased after incubation (control) and was further decreased by treatment with H_2O_2 at 5 mM. The protein expression of HSP70 was significantly lower in samples treatment with 15 mM H_2O_2 that all other treatments at all regions of the spermatozoa (Figure 5.2).

5.3.5. Level of HSP90 expression

At all regions, the protein expression of HSP90 (mean % ± SEM) was highest ($P < 0.001$) in pre-incubated samples which did not have any treatment. HSP90 protein expression decreased after incubation (control) and was further decreased by treatment with H_2O_2 at 5mM. The protein expression of HSP90 was significantly lower in samples treatment with 15mM H_2O_2 than all other treatments at all regions of the spermatozoa. (Figure 5.3).

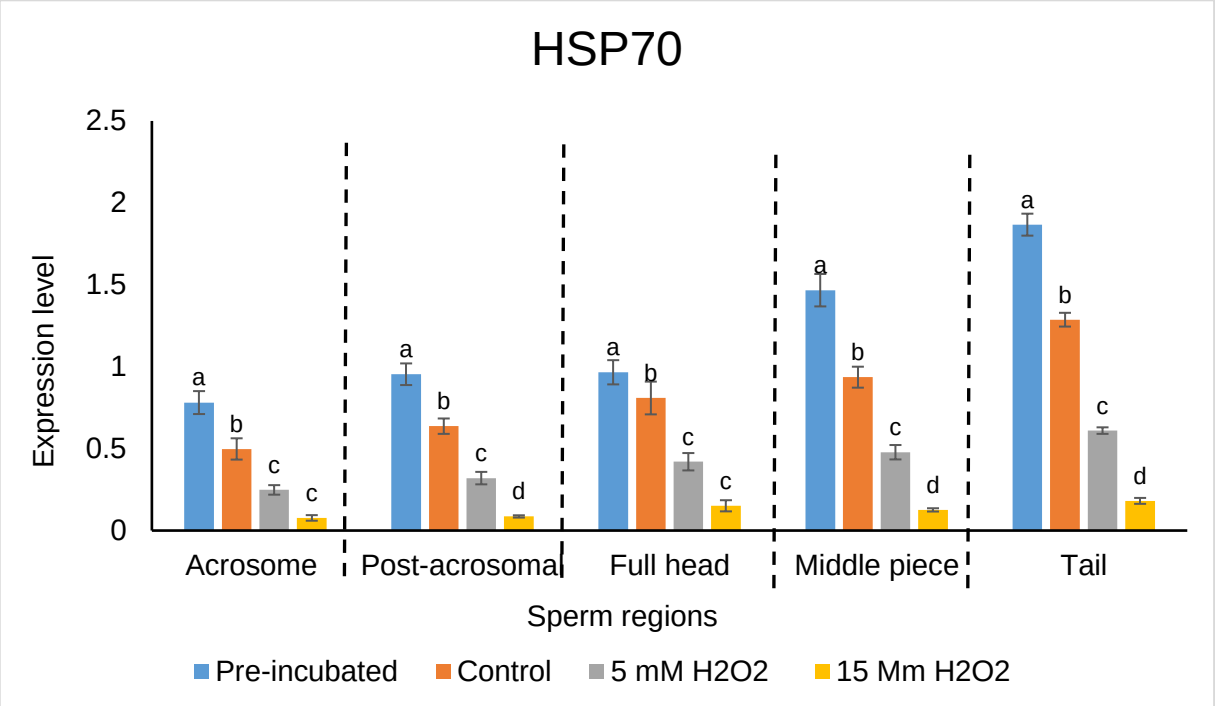


Figure 5. 1. Mean (± SEM) HSP70 expression index in the acrosome, post-acrosome, full head, middle piece and tail of sperm of ram fresh ejaculated spermatozoa pre-incubation and incubated without (control) and with 5 mM and 15 mM H_2O_2 for 60 min at 37°C.

a, b, c, and d values with different superscripts in the same region of the sperm are significantly different ($P < 0.001$).

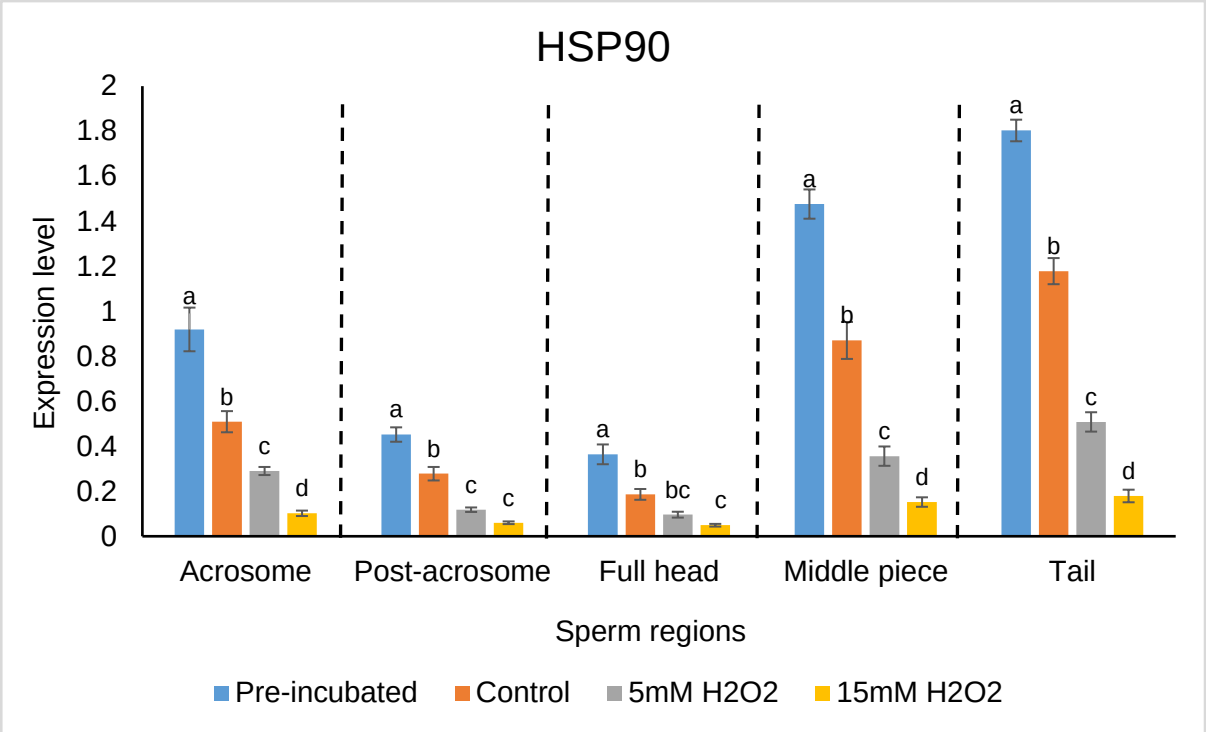


Figure 5. 2. Mean (± SEM) HSP90 expression index in the acrosome, post-acrosome, full head, middle piece and tail of sperm of ram fresh ejaculated spermatozoa pre-incubation and incubated without (control) and with 5 mM and 15 mM H_2O_2 for 60 min at 37°C.

a, b, c, and d values with different superscripts in the same region of the sperm are significantly different ($P < 0.001$).

5.4. Discussion

The antibodies used in the present study were suitable for use in immunofluorescence, cross-reacted with sheep (Abcam, Cambridge, UK: http://www.abcam.com/hsp90-antibodyac88-ab13492-references.html),and have been used in spermatozoa previously (Varela *et al*., 2008). However, it is worth considering that the changes in HSP70 and HSP90 expression observed in the present study may be a consequence of destruction or alteration of the HSP antibody binding site due to H_2O_2 rather than decreased expression or degredation of the HSP protein.

The HSP90 antibody used in the present study has previously been used with success in cryopreserved human semen (Li *et al*., 2014). In addition, it has been reported that HSP90 has the ability to protect cells from the effect of ROS (Fukuda *et al*. 1996), and also suggested that the ATP level is reduced post cold shock and that could demonstrate that HSP90 is involved in ATP metabolism (Prodromou *et al*., 1997).

Cao *et al*. (2003) suggested that a reduction in HSP90 expression in sperm cell after treatment with H_2O_2 was caused by protein degradation. Reactive oxygen species formed during oxidative stress cleaves HSP90 between amino acid residues 126-127 and 131-132 (Beck *et al*., 2012), disrupting its function. As the antibody used in the present study binds to amino acid residues 604-697 it is probable that the antibody is still able to bind despite cleavage, providing the protein is still present. Consequently it is likely that the reduction in HSP90 expression observed with high concentrations of H_2O_2 is caused by protein degradation.

It has been demostrated that HSP70 has the ability to act in specific cells as reactivation system against oxidative damage and reduces the effect of H_2O_2 (Kaur and Bansal, 2003). The HSP70 antibody clone (N27F3-4; ab47454), used in the present study is suitable for use in immunofluorescence and in frozen sections for immunohistochemistry. The antibody shows cross-reactivity with a range of species including sheep (Abcam, Cambridge, UK: https://www.abcam.com/hsp70-antibody-n27f3-4-ab47454-references html). It has been used successfully in mouse sperm (Winklhofer *et al*., 2001) and is therefore an appropriate antibody to detect expression of HSP70 in ram spermatozoa by immunofluorescence. It has also been used in cryopreserved buffalo spermatozoa, which successfully demonstrated that the reduction of HSP70 expression may be the main reason of reduced post-thaw fertility of sperm in this species (Varghese *et al*., 2016).

With this evidence it is likely that the reduced expression of HSP70 and HSP90 in the present study is caused by degradation or loss of the HSP protein and not destruction of the HSP 70 or HSP90 antibody binding region.

To the best of our knowledge, this is the first report of the effect of ROS on HSP70 and HSP90 protein expression in spermatozoa. Sperm motility is generated by the energy from the mitochondria which is the source of oxidative phosphorylation in the middle piece and increases the concentration of free radicals inside and outside of the sperm cells (Martin *et al.,* 2004; Guthrie and Welch, 2006). High concentrations of ROS cause damage to the sperm membrane and decrease sperm motility (Salamon and Maxwell, 1995), consequently leading to decreasing ability of sperm to pass the cervical mucus and penetrate the oocyte (Gillan *et al.*,1997).

Artificial reproduction techniques require the minimizing of ROS production during the preparation and processing of sperm. Hence, it is essential to maintain a balance between the ROS production and total antioxidant ability in seminal plasma and sperm, which reduces oxidative stress and protects sperm integrity (Sharma *et al.,* 2000; Agarwal *et al.,* 2006). Additionally, the decrease in primary protective mechanisms (antioxidant systems) in the sperm cell can lead to decreased sperm integrity. It has been reported that external stress was shown to decrease the activity of the enzymatic system of cells including the inhibition of SOD activity (Lushchak and Bagnyukova, 2006).

Inhibition of antioxidants including SOD during cryopreservation causes increased ROS damage to the chain production of free radicals, enzyme mechanisms including catalase, glutathione peroxidase (GSH-PX), and superoxide dismutase (SOD), aim to prevent ROS-induced injuries (Mennella and Jones, 1980; Sanocka *et al.*, 1997). Initially the superoxide ion (O_2 -) is inactivated by superoxide dismutase (SOD), transforming it into hydrogen peroxide (H_2O_2). The latter is then rapidly catabolised by catalase and peroxidases into dioxygen (O_2) and water (H_2O). It has been confirmed that the production of H_2O_2 under the action of SOD is the triggering component in the natural antioxidant defence mechanisms (Zeitoun and Al-Damegh, 2015).

H_2O_2 is the most harmful of the peroxides when generated in large quantities and can move rapidly within the cells and attack molecules. H_2O_2 is considered the primary ROS responsible for sperm damage (Aitken *et al.*, 1993). The effect of H_2O_2 on the ram sperm parameters in the present study was assumed to be that H_2O_2 has the ability to adversely impact sperm motility and other parameters. These parameters include the level of HSP90 and HSP70 expression. The results showed that H_2O_2 increased ROS proportion demonstrating that the concentrations and methods of H_2O_2 used in the study were sufficient to induce oxidative stress, and thereby determine the effect of oxidative stress on HSP70 and HSP90 expression. The effects observed in the current study was in agreement with previous studies of the effect of H_2O_2 on sperm parameters (Mahfouz *et al.,* 2008; Garg *et al.,* 2009; Plessis *et al.,* 2010) , however, these studies did not determine the effect of H_2O_2 on the protein expression level of HSP70 and HSP90 in spermatozoa.

Whilst controlled ROS concentrations are essential for cell signaling and some sperm functions, the increased concentration of intracellular ROS caused a reduction in the motility of the ram sperm (in present study), and this effect has been shown previously in human sperm motility (Plessis *et al.,* 2010). These authors found a highly significant negative correlation between sperm motility and the concentration of ROS proportion (r^2 = 0.965) using 15 mM H_2O_2. The addition of H_2O_2 to the sperm medium increases lipid peroxidation and the level of MDA (Garg *et al.,* 2009).

In the current study, the results showed a low level of LPO on SP and sperm in the pre-incubated samples compared with high level of LPO in the treatment samples. This effect of ROS was confirmed by Garg *et al.* (2009) who reported that there was a significant effect of H_2O_2 (10, 25, and 50mM) at 37ºC on fresh and frozen-thawed buffalo sperm, and the level of ROS in frozen-thawed sperm was more than fresh samples. They also found in fresh samples that the motility, viability %, and the level of MDA were lower in the control group (42.5 ± 3.2, 77.8 ± 0.6, and 398.1 ± 15.0 respectively) compared with samples treated with 50 mM H_2O_2 after 60 min incubation at 37 ºC (15.0 ± 2.0, 29.5 ± 1.0, 961.6 ± 12.7).

Mahfouz *et al.* (2009) reported increased production of ROS causes lipid peroxidation of the sperm plasma membrane, with a subsequent loss of the structure, fluidity, and function of sperm membrane, and fragmentation of DNA, with excessive effect on the production of NADPH, and reduction of catalase activity (Ollero *et al.,* 1996). This effect was apparent in the results of our study in terms of reduced sperm motility and the increased proportion of ROS > 50 with increased levels of LPO in SP and sperm. This effect was due to the presence of high concentrations of ROS (15 mM H_2O_2), which increased the damage in the spermatozoa plasma membrane and mitochondria as shown in previous studies in bull (Chatterjee *et al.,* 2001), stallion (Baumber *et al.,* 2003), and human (Aitken and Krausz, 2001; Plessis *et al.,* 2010; Sharma, 2015).

The mechanism of action of H_2O_2 on sperm function, could be through the ability of H_2O_2 to penetrate the cytoplasm through the cell membrane and prevent the activity of enzymes such as glucose-6-phosphate dehydrogenase (G6PD). This enzyme regulates the fluidity of the glucose hexose monophosphate shunt and sustains the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). This molecule is considered as a basis of electrons for spermatozoa to generate ROS by NADPH oxidase (Griveau *et al.,* 1995). Therefore, the decline of G6PD lead to a reduction in the availability of NADPH and decreased glutathione (Chatiza *et al.,* 2018), which is the primary type of antioxidants that is believed to reduce the adverse effects of lipid peroxidation and enhances motility, viability and maintain membrane permeability (Satorre *et al.*, 2007).

The technique which was used in the present study to quantify the proportion of ROS, assessed the formazan formation in the sperm head. The increase in ROS following treatment with H_2O_2 has been reported previously in human spermatozoa, although ROS was assessed using 2, 7-dichlorofluorescein diacetate (DCFH %) Plessis *et al. (*2010).

The results in the present study showed a positive relationship between increasing H_2O_2 level and decreasing sperm integrity associated with decreasing in HSP90 and HSP70 protein expression. This could be due to the damage of mitochondria which is the primary site of ROS production and oxidative stress in sperm (Koppers *et al.,* 2008). Furthermore, previous studies reported that HSPs were synthesized in the meiotic phase of spermatogenesis (Allen *et al.,* 1988; Feng *et al.*, 2001). Ren *et al.*, (2017) reported that there is no gene expression in the headpiece as the genes are condensed into chromosomes, and the gene expression in the mitochondria is limited to energy production for motility. Therefore, the quantity and quality of HSPs are limited on the sperm membrane under stress, and that could explain the low expression level of HSPs on the head sperm region. In general, addition of H_2O_2 is detrimental to translation and inhibits protein expression. It is therefore possible that in the present study, HSP90 expression decreases due to degradation of the protein under the influence of H_2O_2, and the protein expression can not be recovered because there is no protein translation of HSPs after spermatogenesis.

The result obtained in the current study that exogenous H_2O_2 can adversely affect sperm functions as reported previously (Garg *et al.,* 2009; Bromfield *et al.,* 2015). The high expression level of HSP70 and HSP90 in the pre-incubated sample and lowest expression that was observed in the treatment samples by H_2O_2, suggests that the harmful effect of H_2O_2 on the sperm membrane integrity would have been through the degradation of these proteins induced by ROS. Calle-Guisado *et al.* (2017) stated that HSP90 in boar spermatozoa is significant to sustain sperm motility and mitochondrial function under stress. They also reported that the high drop in the mitochondrial membrane potential could be related to decreased motility in spermatozoa. Moreover, it has been reported that mitochondrial oxidative phosphorylation is essential for the conservation of boar sperm motility (Guo *et al.,* 2017). HSP90 can maintain the function of mitochondria through its interaction with numerous proteins (Altieri, 2013), comprising protein kinases and phosphatases (Taipale *et al.,* 2010). In a study on the ram epididymis, it has been found that the functions of mitochondria decreased under stress with an increase in the concentration of ROS (Hamilton *et al.*, 2016). On the other hand, Kaur and Bansal, (2003) reported that HSP70 has the ability to play as a systemic reactivation on sperm cell against oxidative stress.

The high protein expression of HSP70 and HSP90 at specific sperm regions may suggest that the primary site of HSPs are localized in the middle piece and tail regions of sperm (Mitchell *et al.*, 2007; Li *et al.,* 2014). In addition, in the present study, the significantly higher expression of HSPs in pre-incubated samples could be related to the low level of stress that allowed and aided to continue the activity of HSPs, compared with other samples that had a higher stress by H_2O_2 and incubation time, which caused protein degradation and reduced the activity of HSPs (Cao *et al.,* 2003). These findings were in agreement with previous research that confirmed that the level of expression of HSP90 or HSP70 was related to sperm integrity (Wang *et al.,* 2014; Zhang *et al.,* 2015).

The critical role of HSPs is to reduce oxidative stress and work as inherent antioxidants (Zhang *et al.,* 2018). This could explain the low levels of HSP90 and HSP70 in the sperm regions of H_2O_2 treated samples. The neutralization of ROS by these proteins would protect sperm at the expense of reduced HSP70 and HSP90 levels. The level of expression decreased in treated samples. This could suggest the high level of ROS and incubation period played a significant role to degradation and exhaust the HSPs on the sperm membrane, and because ejaculated spermatozoa are thought not to synthesize proteins the expression of HSP70 and HSP90 declines.

5.5. Conclusion

The *in-vitro* procedure carried out in the current study is simple to execute and showed the effect of exogenous H_2O_2 on sperm function and HSP70 and HSP90 protein expression. The results of the present study have demonstrated that H_2O_2 which is one of the main reactive oxygen species, reduces the expression of HSP70 and HSP90 protein and this was associated with deteriorated sperm function, especially when used at a 15 mM concentration. These findings could help in reducing the harmful effect of ROS on sperm and improve fertility rates using artificial insemination in sheep, through providing protection of the sperm membrane and maintenance the sperm functions.

Chapter 6. The effect of taurine and cysteine supplementation on HSP90 and HSP70 protein expression in frozen-thawed ram spermatozoa.

6.1. Introduction

As shown in chapter 4, taurine and cysteine when used pre-freezing and post-thaw at 1.0 mg/ml reduce sperm damage and improve sperm integrity and function through a reduction in ROS and lipid peroxidation. ROS (particularly H_2O_2) reduce the expression of HSP70 and HSP90 proteins in ram spermatozoa (chapter 5), and as heat shock proteins protect against cell damage (Beere and Green, 2001), it is possible that decreased expression of these proteins exposes the cell to greater stress. As antioxidants can mitigate the effects of ROS (chapter 4) they may also affect the expression of HSPs through indirect action. The effect of antioxidants on HSP70 and HSP90 expression in cryopreserved ram semen has not been elucidated.

Cysteine has the ability to enter the membrane of mammalian cells easily and improve intracellular glutathione biosynthesis both *in vivo* and *in vitro* (Mazor *et al.,* 1996). It has been reported that addition of cysteine to semen extender enhanced chromatin structure, membrane integrity and viability post-thawed (Jeong and Xiangzhong, 2001; Szczęśniak-Fabiańczyk *et al.,* 2003; Funahashi and Sano, 2005). Cysteine supplementation increases the concentration of catalyse in spermatozoa (Coyan *et al.,* 2011) and one molecule of catalase has been reported to degrade two million molecules of H_2O_2 within one minute and reduce the activity of NADPH oxidase which decreases the free radicals (Agrawal *et al.,* 2005; Abdulkareem and Alzaidi, 2018).

Taurine is a sulfonic amino acid and non-enzymatic scavenger that has a significant effect in protecting spermatozoa against ROS under the aerobic conditions and preservation (Foote *et al.,* 2002; Bucak and Tekin, 2007). Taurine has been added to the semen extender of rams (Bucak *et al.,* 2007), dogs (Martins-Bessa *et al.,* 2009), boars (Gutiérrez-Pérez *et al.,* 2009) and buffalo (Reddy *et al.,* 2010; Kumar *et al.,* 2013) to improve membrane sperm integrity and fertility by preventing lipid peroxidation and defending cells against production of ROS (Partyka *et al.,* 2017).

Heat shock proteins (HSPs) protect cells from oxidative stress and any damage (Beere and Green, 2001). HSP90 has been identified in semen extracts from human and boar ejaculates and have been linked to the sperm acrosome reaction and hyperactivation in humans (Li *et al.,* 2014; Sagare-Patil *et al.,* 2017). HSP90 has been reported to play an essential role in sperm motility which is reduced during cooling or after cryopreservation (Cao *et al.,* 2003; Martin *et al.,* 2007). Zhang *et al.* (2018) observed the presence of cysteine in semen extender have a positive role on the activity of HSP90 to reduce the harmful effect

of ROS such as H_2O_2. HSP70 has the ability to maintain protein structure and is involved in the movement of proteins across intracellular membranes (Zhang *et al.*, 2015). HSP70 seems to play an essential role in sperm function post ejaculation, and it was detected in proteins isolated from boar (Yang and Leela, 2000) and bull sperm (Kamaruddin *et al.,* 2004). It may also play an essential role in fertilisation (Spinaci *et al.,* 2006). Taking into account that cryopreservation is one of the significant stresses that damage ram spermatozoa, resulting in severe economic losses, more research needs to be performed to understand the defence mechanisms that could be activated. Nevertheless, currently, there are no studies that determine the influence of antioxidant supplementation on the protein expression of HSP90 and HSP70 at different regions of the sperm. Therefore, the objective of this study was to determine the effect of supplementing 1.0 mg/ml of taurine or cysteine on the expression of HSP70 and HSP90 protein in ram spermatozoa, and to define the relationship between HSP expression and the integrity of post-thaw ram spermatozoa.

6.2. Materials and methods

6.2.1. Animals and diets

Three mature (3 years old), Texel rams with an average body weight of 87kg and body condition score of 3-4 were used throughout 16 weeks. Rams used in this experiment were obtained from the Harper Adams University flock. Rams were housed individually in metal pens bedded with straw during the period of the study. Rams were fed 1 kg/day concentrate feed specifically for rams (Wynnstay Ram Master Coarse Mix 2281) with *ad libitum* access to straw and fresh water.

6.2.2. Laboratory analysis

Fifteen semen samples were collected by artificial vagina during the breeding season from three Texel rams (5 ejaculates/ram) to identify the effect of each type of antioxidant on frozen-thawed ram semen. The effect of taurine and cysteine concentrations (1.0 mg/ml) was identified for all semen samples. All samples were collected in the morning for 2-3 times weekly and only ejaculates of 0.75-2ml, a minimum concentration of 3×10^9 sperm/ml and minimum motility of 80% were used in the experiment.

6.2.3. Preparation of frozen-thawed sperm

Preparation of frozen-thawed sperm was performed according to the method described in section 2.7.

6.2.4. Sample preparation

Ejaculates (n = 15) were diluted dropwise 1:4 (semen: cryodiluent, v:v) in tris-citrate-glucose cryodiluent (300 mM Tris, 94.7 mM citric acid, 27.8 mM D-Glucose, 15% egg yolk, 5% glycerol), then divided into three equal aliquots and supplemented with 0.0 (control), 0.1 mg/ml (taurine), and 1.0 mg/ml (cysteine) (Sigma-Aldrich, UK) pre-freeze (PF). Next, samples were chilled to 4 °C in a water jacket over 2.5 hours, pelleted on dry ice and stored in liquid nitrogen until further assessment. Cryopreserved samples were thawed in a water bath in a clean dry glass test-tube with vigorous shaking for 1 min at 37°C. Immediately after thawing, samples were centrifuged at 300 x g for 3 min, the supernatant discarded and the pellet resuspended in tris-citrate glucose buffer (TCG; 300 mM Tris, 94.7 mM citric acid, 27.8 mM D-Glucose). The sample was then centrifuged again, and the pellet resuspended in TCG to 50 $x10^6$ sperm/ml. The resuspended samples were then treated as they had been pre-freeze with 0.0 (control), 1.0 mg/ml (taurine), or 1.0mg/ml (cysteine) post-thaw (PT) to generate three treatments as follows: 1) control 0.0 mg/ml, 2) PF+PT 1.0 mg/ml taurine and 3) PF + PT 1.0 mg/ml cysteine.

Sperm parameters motility, viability acrosome integrity, and proportion of ROS were assessed for all samples at 0, 30, 60, 180 min. Penetrability was assessed at 60 min, LPO for sperm and SP and expression of HSP 70 and HSP were assessed at 0 min and 60 min post-thaw at 37°C (Figure 6.1).

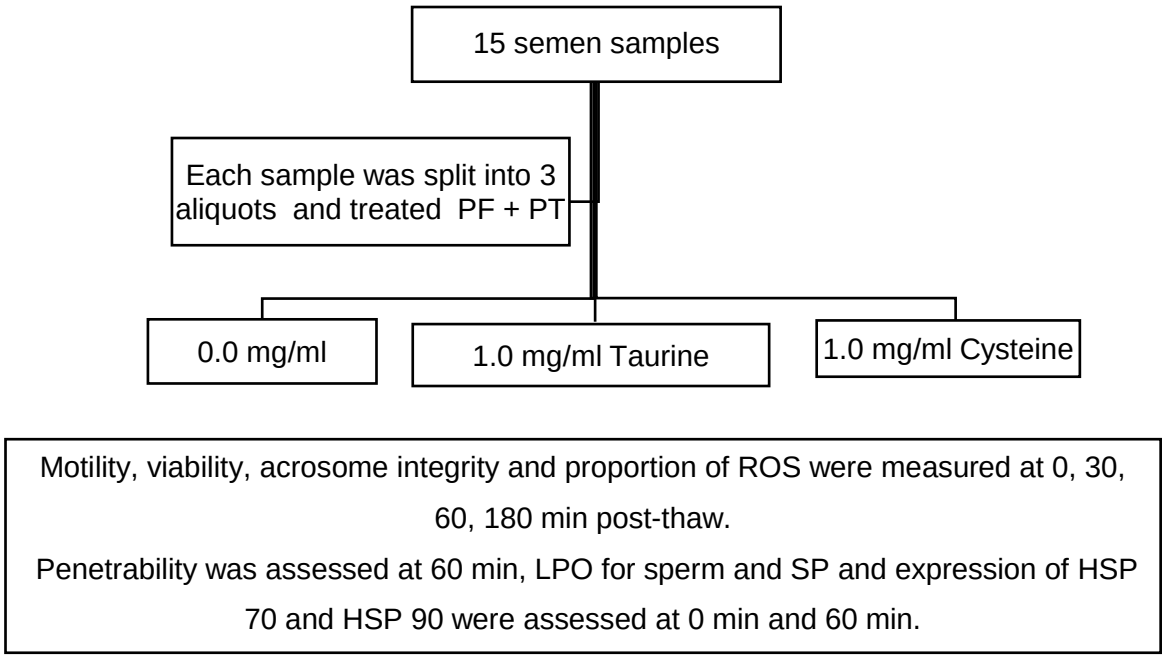


Figure 6. 1. Experimental design to determine the effect of antioxidants on the expression of HSP90, HSP70 and sperm function in cryopreserved ram spermatozoa.

6.2.4. Sperm parameters

6.2.4.1. Proportion of motile spermatozoa

The proportion of motile spermatozoa was determined at post-thaw according to the method described in section 2.9.1.

6.2.4.2. Acrosome integrity and Viability

Acrosome integrity and viability were determined post-thaw according to the method described in section 2.9.3.

6.2.4.3. Penetrability

Penetration test was determined at 60 minutes post-thaw according to the method described in section 2.10.2., and 2.10.3

6.2.4.4. Percentage of ROS production on sperm

Percentage of ROS (< 50% and >50%) through the formazan production on sperm head was determined at post-thaw according to the method was described in section 2.11.

6.2.5. Measurement of lipid peroxidation (LPO)

6.2.5.1. Lipid peroxidation of seminal plasma (SP)

Lipid peroxidation of seminal plasma (SP) for fifteen semen sample were determined at 0, 60 minutes post-thaw according to the method described in section 2.12.1.

6.2.5.2. Lipid peroxidation of sperm

Lipid peroxidation of sperm for each sample was determined at 0, 60 minutes post-thaw according to the method described in section 2.12.2.

6.2.6. Staining of immunofluorescence HSP90 and HSP70

The expression of HSP90 and HSP70 of each semen sample for each treatment (taurine, cysteine, and control) was determined at 0 min and 60 min according to the method described in section 2.13.

6.2.7. Statistical analyses

The data were analysed using a repeated measure analysis of variance (ANOVA) with a linear mixed model (REML) Gen Stat (17th edition). To accommodate the experimental design, the random effects were treatment nested within ejaculate nested within ram. Means were reported with the ± standard error of the mean (SEM) and $P < 0.05$ was considered statistically significant. Post-hoc analyses were performed using the least significant difference (LSD) test. The effect of treatments on the expression level of HSP70 and HSP90 was compared within each sperm region within each time.

The simple linear regression was used to measure the strength of a linear association between treatments and the expression of HSP90 and HSP70 on different regions of sperm, where the value r^2 was used to indicate correlation as follows: no correlation 0-0.1, weak 0.2-0.4, intermediate 0.4-0.6, high 0.6-0.8, and very high 0.8-1.0. The penetrability of spermatozoa through artificial mucus was measured at 60 min only, and was analyzed using one way (ANOVA) Gen stat (17th edition). Comparison of treatments within each of the sperm regions was carried out using one way (ANOVA), and post-hoc analyses were performed using the least significant difference (LSD) test to determine significance. The expression level of HSP70 and HSP90 between the zero and 60min incubation times within each sperm region and within each treatment was calculated using Student's T-test assuming the data followed a normal distribution.

6.3. Results

6.3.1. Motility

There was an interaction of treatment and time for (mean % ± SEM) motility ($P < 0.001$), in samples treated with 1.0 mg/ml taurine, cysteine and control. The results showed that the highest value was with cysteine treatment, and was intermediate in samples treated with taurine, and the lowest values were in the negative control samples which did not have any cysteine or taurine. As expected percentage of motility declined over time in all treatments (Table 6.1).

6.3.2. Acrosome integrity

There was an interaction of treatment and time for (mean % ± SEM), acrosome integrity ($P < 0.001$), in samples treated with 1.0 mg/ml taurine, cysteine and control. The results showed that the highest value was with cysteine treatment, and was intermediate in samples treated with taurine, and the lowest values were in the negative control samples which did not have any treatment. As expected percentage of acrosome integrity declined over time in all treatments (Table 6.1).

6.3.3. Viability

There was an interaction of treatment and time for (mean % ± SEM), viability ($P = 0.024$) in samples treated with 1.0 mg/ml taurine, cysteine and control. The results showed that the highest value was with cysteine treatment, and was intermediate in samples treated with taurine, and the lowest values were in the negative control samples which did not have any cysteine or taurine. As expected percentage of viability declined over time in all treatments (Table 6.1).

Table 6. 1. Means (± SEM) percentage motility, acrosome integrity and viability of frozen-thawed (FT) ejaculated spermatozoa supplemented without antioxidants as control and with 1.0 mg/ml of taurine or cysteine at 0, 30, 60 and 180 minutes incubation at 37°C ($P < 0.001$). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

	Time	Control (15)	1.0 mg/ml Taurine (15)	1.0 mg/ml Cysteine (15)	P value
Motility	0	$45.67 \pm 0.43^{b}_{w}$	$50.13 \pm 0.51^{a}_{w}$	$51.67 \pm 0.59^{a}_{w}$	*P < 0.001*
	30	$31.53 \pm 1.15^{b}_{x}$	$39.80 \pm 1.19^{a}_{x}$	$40.07 \pm 1.21^{a}_{x}$	
	60	$17.40 \pm 0.99^{c}_{y}$	$28.53 \pm 1.70^{ab}_{y}$	$29.87 \pm 1.35^{a}_{y}$	
	180	3.40 ± 0.31^{bz}	$8.00 \pm 0.68^{a}_{z}$	$10.33 \pm 0.71^{a}_{z}$	
Acrosome intact	0	$46.47 \pm 0.31^{c}_{w}$	$54.00 \pm 0.67^{b}_{w}$	$57.20 \pm 0.45^{a}_{w}$	*P* < 0.001
	30	$41.47 \pm 0.31^{c}_{x}$	$46.27 \pm 1.05^{b}_{x}$	$50.27 \pm 0.60^{a}_{x}$	
	60	$33.00 \pm 0.82^{b}_{y}$	$35.60 \pm 1.38^{b}_{y}$	$40.13 \pm 0.98^{a}_{y}$	
	180	$8.40 \pm 0.58^{c}_{z}$	$12.27 \pm 0.81^{b}_{z}$	$15.60 \pm 0.72^{a}_{z}$	
Viability	0	$47.53 \pm 0.47^{b}_{w}$	$53.13 \pm 0.55^{a}_{w}$	$55.53 \pm 0.70^{a}_{w}$	*P* = 0.024
	30	$40.47 \pm 0.83^{b}_{x}$	$44.87 \pm 0.62^{a}_{x}$	$47.27 \pm 0.86^{a}_{x}$	
	60	$31.93 \pm 1.21^{b}_{y}$	$33.13 \pm 0.89^{b}_{y}$	$37.73 \pm 1.03^{a}_{y}$	
	180	$6.20 \pm 0.41^{c}_{z}$	$9.53 \pm 0.52^{b}_{z}$	$13.73 \pm 0.48^{a}_{z}$	

a, b and c values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

w, x, y and z: For each assessment parameter with different subscripts in the same column are significantly different ($P < 0.001$)

6.3.2. Penetrability through artificial mucus

Distance travelled (mean % ± SEM) was greater ($P < 0.001$) in samples treated with cysteine (3.91 ± 0.176 cm/h), and taurine (3.28 ± 0.101 cm/h) at 60 minutes compared to the control (2.11± 0.085 cm/h). In addition, cysteine and taurine were significantly different from each other.

6.3.3. Proportion of ROS > 50 and < 50

There was an interaction between treatment and time on the proportion of spermatozoa with >50% ROS at the sperm head ($P < 0.001$). Proportion of ROS > 50 (mean % ± SEM) increased over time in all treatment groups but tended to be lowest ($P < 0.001$) in samples treated with cysteine, intermediate in those treated with taurine and highest in the negative control group (Table 6.2). There was an interaction between treatment and time of proportion ROS < 50 ($P < 0.001$). Proportion of ROS < 50 (mean % ± SEM) declined over time in all treatment groups but tended to be higher ($P < 0.001$) in samples treated with cysteine intermediate in those treated with taurine and lowest in the control group (Table 6.2).

Table 6. 2. Mean (± SEM) proportion of ROS > 50 and ROS <50 percentage values of frozen-thawed (FT) spermatozoa supplemented without antioxidants as control and with 1.0 mg/ml of taurine or cysteine at 0, 30, 60 and 180 minutes incubation at 37°C. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

ROS%	Time (min)	Control (15)	1.0 mg/ml Taurine (15)	1.0 mg/ml Cysteine(15)
ROS > 50%	0	$55.87 \pm 1.028^{c}_{w}$	$46.2 \pm 1.360^{b}_{w}$	$41.13 \pm 1.028^{a}_{w}$
	30	$63.07 \pm 1.140^{c}_{x}$	$53.13 \pm 1.420^{b}_{x}$	$48.87 \pm 0.951^{a}_{x}$
	60	$71.80 \pm 1.043^{c}_{y}$	$62.93 \pm 1.548^{b}_{y}$	$57.02 \pm 1.010^{a}_{y}$
	180	$86.47 \pm 0.856^{c}_{z}$	$74.20 \pm 1.541^{b}_{z}$	$68.53 \pm 1.486^{a}_{z}$
ROS < 50%	0	$44.13 \pm 1.460^{c}_{w}$	$53.80 \pm 1.360^{b}_{w}$	$58.87 \pm 1.028^{a}_{w}$
	30	$36.93 \pm 1.140^{c}_{x}$	$46.87 \pm 1.420^{b}_{x}$	$51.13 \pm 0.951^{a}_{x}$
	60	$28.2 \pm 1.043^{c}_{y}$	$37.07 \pm 1.548^{b}_{y}$	$42.80 \pm 1.010^{a}_{y}$
	180	$13.53 \pm 0.856^{c}_{z}$	$25.80 \pm 1.541^{b}_{z}$	$31.47 \pm 1.486^{a}_{z}$

a, b, and c, values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$).

w, x, y and z: For each assessment with different subscripts in the same column are significantly different ($P < 0.001$).

6.3.4. Lipid peroxidation of seminal plasma and sperm

There was an interaction between treatment and time of LPO of SP ($P < 0.001$) and sperm ($P < 0.001$). LPO of SP and sperm (mean % ± SEM) increased over time (after 60 min) in all treatment groups and was lowest ($P < 0.001$) in samples treated with cysteine, intermediate in samples treated with taurine and highest in the control group (Table 6.3).

Table 6. 3. Mean (± SEM) lipid peroxidation of sperm (nmol/25x10^6) and SP (nmol/ml) of frozen-thawed (FT) ejaculated spermatozoa supplemented without antioxidants as control and with 1.0 mg/ml of taurine or cysteine at 0 and 60 minutes incubation at 37°C ($P < 0.001$). Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

LPO	Time	Control (15)	1.0mg/ml Taurine (15)	1.0mg/mlCysteine (15)
SP	**0**	$2.44 \pm 0.0665^{c}_{y}$	$2.11 \pm 0.0642^{b}_{y}$	$1.79 \pm 0.0548^{a}_{y}$
	60	$3.23 \pm 0.0710^{c}_{z}$	$2.79 \pm 0.0731^{b}_{z}$	$2.27 \pm 0.0590^{a}_{z}$
Sperm	0	$3.66 \pm 0.078^{c}_{y}$	$3.37 \pm 0.096^{ab}_{y}$	$3.15 \pm 0.048^{a}_{y}$
	60	$6.65 \pm 0.246^{c}_{z}$	$5.13 \pm 0.128^{b}_{z}$	$4.61 \pm 0.103^{a}_{z}$

a, b, and c: values with different superscripts in the same row for each incubation time are significantly different ($P < 0.001$). Numbers in parantheses represent the number of ejaculates within each treatment at each time point.

y and z: for each assessment parameter LPO value with different subscripts in the same column are significantly different ($P < 0.001$).

6.3.5. Level of HSP70 expression

There was an interaction between treatment and time for the protein expression of HSP70 on each parts of sperm such as acrosome, post-acrosome, full head, middle piece and tail ($P < 0.001$). The expression of HSP70 (mean % ± SEM), declined overtime (after 60 min) in all treatment groups tended to be highest ($P < 0.001$) in samples treated with cysteine compared to the moderate level with taurine and lowest with control group. There was a high expression level on the middle piece and tail compared with other sperm regions (Table 6.4, and Figure 6.3).

Table 6. 4. Mean (± SEM) protein expression score of HSP70 at the acrosome, post-acrosome, full head, middle piece and tail regions of cryopreserved ram spermatozoa at 0 and 60 minutes post-thaw. Samples were supplemented without (control) and with 1.0 mg/ml taurine or cysteine before freezing and after thawing. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Sperm Regions	Control (15)		Taurine (15)		Cysteine (15)	
	0 min	60 min	0 min	60 min	0 min	60 min
Acrosome	0.501 ± 0.027^{cp}	0.256 ± 0.035^{cx}	0.755 ± 0.065^{bq}	0.402 ± 0.039^{byz}	1.016 ± 0.072^{br}	0.531 ± 0.047^{bz}
Post- acrosomal	0.504 ± 0.044^{cp}	0.261 ± 0.032^{cx}	0.908 ± 0.057^{bq}	0.524 ± 0.049^{by}	1.130 ± 0.053^{br}	0.699 ± 0.039^{bz}
Head	0.545 ± 0.025^{cp}	0.263 ± 0.34^{cx}	0.817 ± 0.074^{bq}	0.439 ± 0.038^{by}	1.250 ± 0.046^{br}	0.747 ± 0.059^{bz}
Middle piece	0.918 ± 0.076^{bp}	0.554 ± 0.060^{bx}	1.473 ± 0.089^{aq}	0.672 ± 0.074^{bx}	1.981 ± 0.115^{ar}	1.078 ± 0.093^{ay}
Tail	1.220 ± 0.056^{ap}	0.773 ± 0.053^{ax}	1.66 ± 0.107^{aqr}	0.853 ± 0.071^{axy}	2.117 ± 0.103^{ar}	1.061 ± 0.091^{by}

a, b and c: values with different superscripts in the same column for each incubation time are significantly ($P < 0.001$).

p, q and r: values with different superscripts within sperm region and 0 incubation time in the same row are significantly different ($P < 0.05$)

x, y and z: values with different superscripts within sperm region and 60 incubation time in the same row are significantly different ($P < 0.05$).

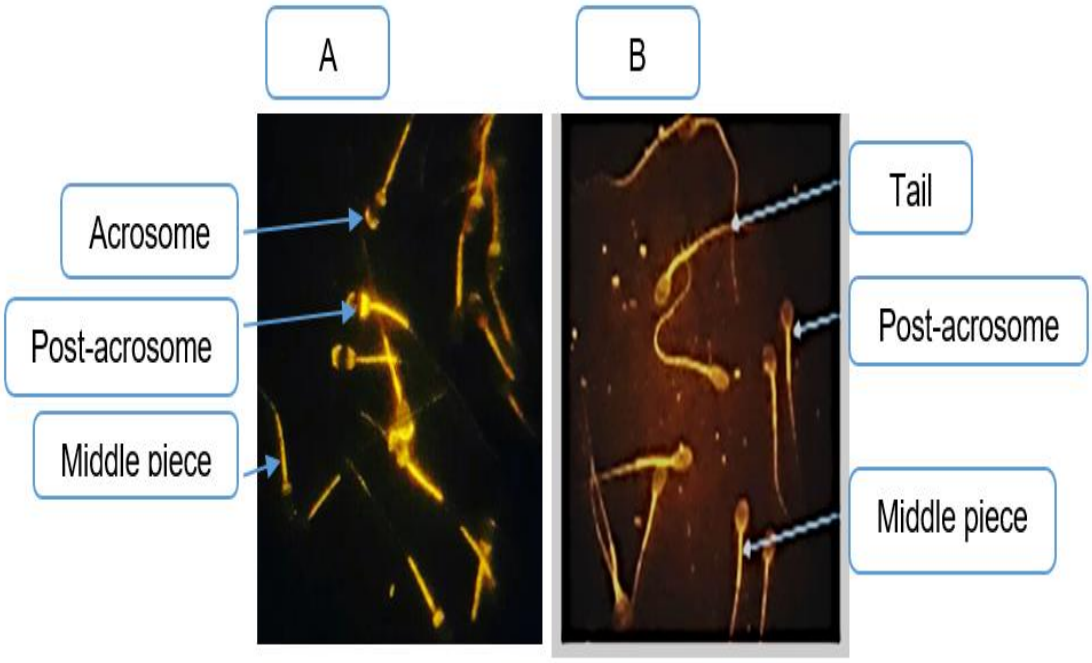


Figure 6. 2. Expression HSP70 on different regions of frozen-thawed ram sperm (acrosome, post-acrosomal, full head, middle piece, and tail) at 0 min (A) and (B) at 60 min which had treated 1.0 mg/ml PF + PT of cysteine.

6.3.6. Level of HSP 90 expression

There was an interaction between treatment and time for the protein expression of HSP90 at the acrosome region, full head and tail ($P < 0.001$). The intensity of HSP90 (mean % ± SEM) declined over time (after 60 min) in all treatment groups but tended to be highest ($P < 0.001$) in samples treated with cysteine compared to the intermediate level with taurine and lowest in the control group (Table 6.4) Figure 6.4. However, there was no interaction between treatment and time of expression of HSP90 on the post-acrosomal region and middle piece (P >0.005). Values obtained for the post-acrosomal region and middle piece were: control (0.129 ± 0.016, 0.591 ± 0.037), taurine (0.196 ± 0.021, 1.029 ± 0.068) and cysteine (0.236 ± 0.023, 1.892 ± 0.071) respectively (Table 6.5).

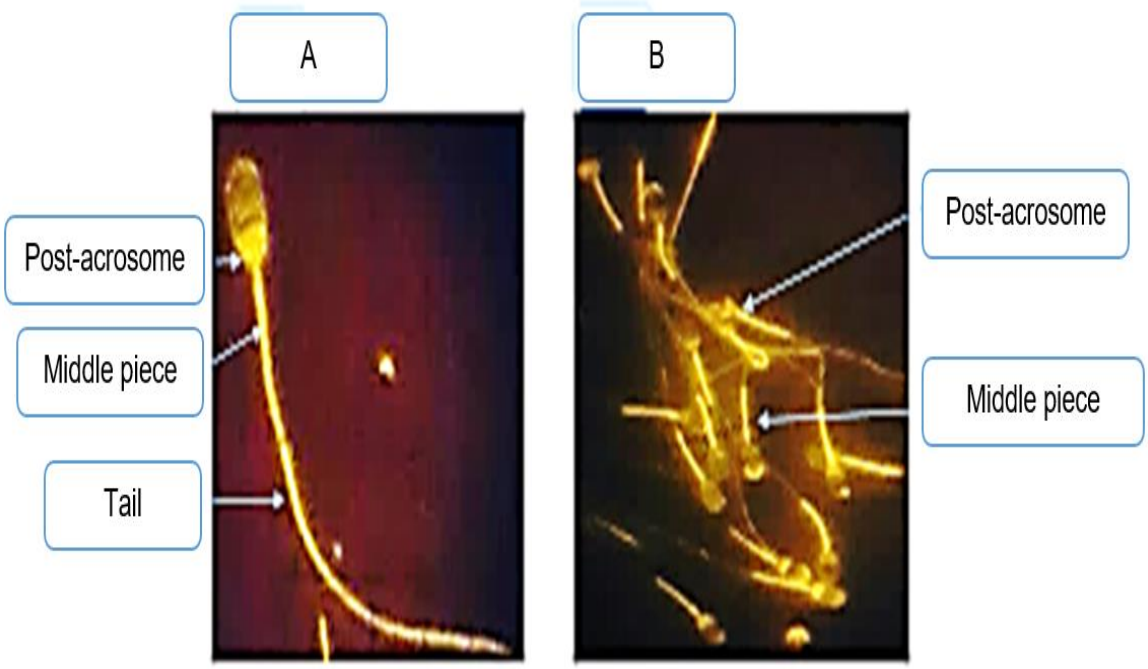


Figure 6.3. Expression HSP90 on different regions of frozen-thawed ram sperm (acrosome, post-acrosome, full head, middle piece, and tail) at 0 min (A) and (B) at 60min which had treated 1.0 mg/ml of cysteine.

Table 6. 5. Mean (± SEM) protein expression score of HSP90 at the acrosome, post-acrosome, full head, middle piece and tail regions of cryopreserved ram spermatozoa at 0 and 60 minutes post-thaw. Samples were supplemented without (control) and with 1.0 mg/ml taurine or cysteine before freezing and after thawing. Numbers in parentheses represent the number of ejaculates within each treatment at each time point.

Sperm Regions	Control (15)		Taurine (15)		Cysteine (5)	
	0 min	60 min	0 min	60 min	0 min	60 min
Acrosome	0.203 ± 0.013^{bp}	0.083 ± 0.009^{bx}	0.271 ± 0.013^{cq}	0.121 ± 0.013^{bxy}	0.345 ± 0.021^{cr}	0.149 ± 0.019^{by}
Post-acrosomal	0.199 ± 0.018^{bp}	0.060 ± 0.004^{bx}	0.277 ± 0.027^{cpq}	0.115 ± 0.013^{by}	0.334 ± 0.026^{cq}	0.138 ± 0.012^{byz}
Head	0.182 ± 0.010^{bp}	0.048 ± 0.003^{bx}	0.215 ± 0.023^{cp}	0.075 ± 0.005^{cy}	0.301 ± 0.026^{cq}	0.103 ± 0.007^{bz}
Middle piece	1.135 ± 0.067^{ap}	0.591 ± 0.037^{bx}	1.560 ± 0.073^{bq}	1.029 ± 0.067^{ay}	1.892 ± 0.071^{br}	1.299 ± 0.071^{az}
Tail	1.724 ± 0.046^{ap}	1.151 ± 0.058^{ax}	2.164 ± 0.086^{aq}	1.409 ± 0.082^{axy}	2.528 ± 0.056^{ar}	1.544 ± 0.008^{ay}

a, b and c: values with different superscripts in the same column are significantly ($P < 0.001$).

p, q and r: values with different superscripts within sperm region and 0 incubation time in the same row are significantly different ($P < 0.05$)

x, y and z: values with different superscripts within sperm region and 60 incubation time in the same row are significantly different ($P < 0.05$).

6.3.7. Correlation of HSP70 and HSP90 expression with sperm parameters

The correlation between the expression of HSP90 on sperm parts and sperm parameters was classified into five scores depending on the value of r^2 , no correlation 0-0.1, weak 0.2-0.4, intermediate 0.4-0.6, high 0.6-0.8, very high 0.8-1.0).

6.3.7.1. Correlation of HSP70 on sperm regions

The results showed some differences in correlations between the expressions of HSP70 and sperm parameters. A very weak correlation (r^2 = 0.388) was found between the acrosome region and motility, and an intermediate correlation (r^2 = 0.455) was found with LPO of SP and post-acrosomal region. HSP70 expression in the sperm head showed high correlation (r^2 = 0.612) with proportion of ROS. A high correlation (r^2 = 0.603) was found between HSP70 expression in the post-acrosomal region and LPO of sperm. Middle piece and tail regions have an intermediate correlation (r^2 = 0.552) with proportion of ROS. The results of HSP70 expression were found to be significant (P < 0.001) Table 6.6.

6.3.7.2 Correlation of HSP90 on sperm regions

The results showed there was a high correlation between motility and HSP90 expression in the acrosome (r^2 = 0.607), and full head region (r^2 = 0.603). However, moderate correlations (r^2 = 0.579; r^2 = 0.472) were detected between middle piece and LPO of sperm and seminal plasma. The middle piece and tail regions have high correlations (r^2 = 0.624; r^2 = 0.637) with proportion of ROS and intermediate correlations (r^2 = 0.549) with the motility. The results of HSP90 expression were found to be significant (P < 0.001) Table 6.

Table 6. 6. The correlation coefficients of HSP90 and HSP70 expression with motility, ROS < 50, LPO SP and LPO of sperm in different sperm regions.

Sperm Regions	Motility		ROS < 50		LPO SP		LPO Sperm	
	HSP90	HSP70	HSP90	HSP70	HSP90	HSP70	HSP90	HSP70
Acrosome	0.607***	0.388*	0.539**	0.451**	0.473***	0.364**	0.404**	0.422***
Post-acrosomal	0.564***	0.426**	0.574 **	0.473**	0.504***	0.455**	0.452*	0.603***
Full head	0.603 ***	0.464**	0.493**	0.612**	0.518***	0.369*	0.482*	0.446**
Middle piece	0.530**	0.409*	0.624***	0.542 **	0.466**	0.385*	0.579**	0.428*
Tail	0.549 ***	0.464*	0.637***	0.552**	0.496**	0.459*	0.472**	0.418**

Significance: (* $P < 0.05$) (** $P < 0.01$) (*** $P < 0.001$)

6.4. Discussion

Cryopreservation is considered the most dependable method to store ram semen for long periods and has an active role in the development of sheep reproduction. Previous studies showed that cryopreservation has an adverse effect on sperm integrity (Salamon and Maxwell, 2000). Ram sperm parameters such as motility, acrosome integrity, viability, penetrability, and proportion of ROS < 50 were affected due to the effect of cold shock (Hu *et al.,* 2010). Sperm integrity parameters in this study were significantly higher than the control group when supplemented with antioxidants such as taurine or cysteine. The present findings were in agreement with previous studies that represented sperm parameters were affected during cryopreservation (Gutiérrez-Pérez *et al.,* 2009; Hu *et al.,* 2010), and also in agreement with the results in chapter four of the effect of antioxidants (cysteine or taurine) on post-thaw ram sperm. The studies about the effect of antioxidants on the activity of HSPs on the integrity of ram sperm membrane are limited.

It has been stated that antioxidant supplementation conserves the motility and viability of cryopreserved ram spermatozoa by protecting and stabilizing the membrane against the oxidative damage during storage of ram semen (Maxwell and Stojanov, 1996), and also for other species, e.g. bull (Foote *et al.,* 2002) goat (Salvador *et al.,* 2006), and turkey (Donoghue and Donoghue, 1997). Antioxidants such as taurine protects frozen-thawed sperm against losses of sperm motility (Uysal *et al.,* 2005), and regulates the electrolytes such as magnesium and potassium inside the cell plasma membrane (Kirk and Kirk, 1994). Cysteine is an antioxidant as an amino acid comprising a thiol group and has a low molecular weight which can be easily combined into sperm membranes. Cysteine is considered as an intracellular glutathione precursor (Gungor *et al.,* 2017), and has the ability to protect the spermatozoa from lipid oxidation by inhibiting the generation of ROS (Çoyan *et al.*, 2011). Meister (1994) stated that the presence of glutathione in cysteine can protect sperm from the harmful effect of H_2O_2 through denoting hydrogen atoms to repair the damage on DNA on spermatozoa (as described in chapter 5).

HSP synthesis increases in response to a sudden rise in temperature and other stresses such as chemicals and physical conditions, to protect essential cell proteins from aggregation and denaturation. Sperm-specific antigens have been identified as objects of interest for immunomediated human infertility medicine and the identification of alternative methods for numerous mammalian species. Soren *et al.* (2018) detected the higher expression of HSP70 and HSP90 in bull spermatozoa under stress designated their significant role in preserving the quality of semen.

In the current study, the results showed that the expression level of HSP70 and HSP90 was high in samples treated with 1.0 mg/ml pre-freeze and post-thaw of taurine or cysteine. This high expression was combined with improving sperm parameters and low level of ROS and LPO in sperm and SP. HSPs have a wide protective effect on sperm functions such as regulating sperm motility through its ability to activate nitric oxide synthase (NOS), which is useful to sperm motility (Huang *et al.,* 2000).

The changes in HSP expression observed in the present study are expected to be indicate of protein degradation or decreased expression rather than inhibition of antibody to the HSP antibody binding region. If cryopreservation induced destruction of the antibody binding region, we would expect to see a similar rate of decline in HSP expression against all treatment groups. In the present study, differences were observed between samples treated with different antioxidants, and there was no expression in the negative control samples, suggesting that the antibody was able to bind to its specific binding region.

Despite, HSPs having a protective effect, the harmful effect of cryopreservation in the present study decreased the role of HSPs gradually over incubation time. This effect could be due to the degrading the HSPs during cryopreservation, as observed on cryopreserved human spermatozoa (Cao *et al.,* 2003), and also due to the production of ROS (H_2O_2) as shown in Chapter 5. It has been reported that HSP70 has the ability to regulate the activity of superoxide dismutase which is an enzymatic antioxidant in the sperm cells (Zhang *et al.,* 2015). The high level of HSP70 in cells reserves the activity of protein kinase and prevents cell death with support the resistance of cell (Kennedy *et al.*, 2014). Huszar *et al.* (2000) stated that there is a biological function for the role level expression of HSP70 on sperm-related with function, maturity and fertility. Alvarez-Rodriguez *et al.* (2013) reported that HSPA8 (HSP70) has the ability to protect sperm membrane for the cryoinjury during cryopreservation.

HSP70 has an essential role during the interaction of sperm-oocyte *in vitro* fertilization in bovine (Matwee, 2001) and swine (Spinaci *et al.,* 2006). Research is being conducted to evaluate the effect of HSPs on oxidative stress and indicator transduction pathways, the mechanism of its activity is being established, but the relationship between HSPs and antioxidant activity along with the consequences regarding cell damage has not been explored. The initial target for stress is the cell membrane and subsequent loss of function and integrity of the cell (Török *et al.,* 2014).

HSP70 has a vital role in maintaining suitable protein conformation, contributing to translocation and transferring protein over the membranes and stabilizing unfolded precursor proteins (Gething and Sambrook, 1992). It also has a defensive role against stressing actions, plays a positive role in improving sperm integrity, it has the ability to re-

localises after acrosome reaction and capacitation (Spinaci *et al.,* 2005). These activities of HSP70 were shown in the present study through the positive high expression level of HSP70 in the acrosome region, middle piece and tail which could be due to the cooperative effect of the antioxidants to protect the sperm parts during cryopreservation. It has been reported that the cryopreservation causes a reduction in the expression of HSP70 mRNA in post-thaw bull sperm (Zhang *et al.,* 2015), and distribution of HSP70 in buffalo spermatozoa (Varghese *et al.*, 2016). Moein-Vaziri *et al.* (2014) stated that HSP70 increases sperm plasma membrane liquidity which provided uncapacitated spermatozoa in presence of cholesterol. Therefore, antioxidant supplementation (PF + PT) could reduce the harmful effect of ROS during cryopreservation and preserve the activity of HSP70.

Holt *et al.* (2015) reported that HSPA8 (as a member of HSP70) has the ability to improve bovine sperm cryopreservation procedures through pre-freezing addition of HSP70 which decreased membrane permeability and reduced sperm apoptosis and early degeneration when used in combination with cholesterol-loaded cyclodextrin. It has been shown that HSP70 has a protective effect and improves ram, bull, and boar sperm viability *in vitro* (Elliott *et al.,* 2009; Lloyd *et al.,* 2012). This effect of HSP70 was shown in the present study through the significant correlation (r^2 = 0.612) between the expression level in the sperm head and the proportion of ROS <50, and also through the high correlation (r^2 = 0.603) between the expression level and low level of LPO on the post-acrosome region of sperm in samples that were treated with antioxidants (Table 6.6). This effect demonstrates the importance of antioxidants (1.0 mg/ml cysteine or taurine) to maintain the activity of HSP70 which can preserve the activity of protein kinase and prevent cell death with increase the resistance of cell (Kennedy *et al.*, 2014), and the reduction of lipid peroxidation of sperm membrane (Su *et al.,* 1999), and consequently improve the integrity of frozen-thawed ram sperm.

It has been stated the HSP90 has the ability to initiate ATP depletion through freezing-thawing which provides a rest state of sperm and reduces the dynamic of sperm and consequently decreases the loss of energy and reduce the damage of oxidative stress (Wang *et al.,* 2014). Zhang *et al.* (2015) stated that sperm parameters such as motility, acrosome integrity, and viability were decreased in frozen-thawed bull spermatozoa and that it was combined with a decrease on the level of HSP90 expression over the incubation time compared with fresh samples which had the highest expression level. Additionally, the decline in expression level during the incubation time in the present study could be due to leaking of some proteins including HSPs from the sperm membrane to the extracellular medium during cryopreservation of ram spermatozoa, as observed in bull and boar semen (Harrison and White, 1972). Therefore, the preserved HSP expression and significant values of sperm parameters in the present study could be related to the presence of

antioxidants (taurine or cysteine). These antioxidants have the ability to maintain cell functions and provide a protective effect on the sperm membrane including proteins from the effect of ROS, and that could reduce the degradation of proteins and maintain the activity of HSPs. Furthermore, the method used for antioxidants supplementation (PF + PT) in the present study could also aid this activity.

HSP90 protects the process of ATP metabolism within the sperm (Prodromou *et al.,* 1997), therefore supporting sperm motility, and acts by reducing the effect of oxidative stress on sperm membrane, stabilizing and preserving the integrity of the cell membrane by interacting with the lipid component of the cell membrane and maintaining the integrity and function under stress environments (Welker *et al.,* 2010; Török *et al.,* 2014). HSP90 has been shown to be in possession an attendant ATPase as part of the protein structure, which is fundamental for the activity of actual dependent proteins *in vivo* (Pearl and Prodromou, 2000). These research confirmed the critical role of HSPs for sperm integrity.

Wang *et al.* (2014) found that HSP90 has the ability to modulate the fluidity of the membrane through the association with phospholipid. This effect of HSPs may clarify the increase resistance and maintain sperm integrity under stress environments (Kwon *et al.*, 2002). Furthermore, it has been stated that HSP90 in frozen bull sperm were used to synthesize other proteins and enzymes to protect sperm from the stresses during cryopreservation (Zhang *et al.,* 2015). This could explain the high expression level of HSPs on treatment groups in the present study compared with the control group. This effect was also represented through the significant results of the proportion of ROS < 50 for the groups treated with cysteine, taurine and control respectively (46.067±1.118: 40.885±1.467; 30.697±1.125). Therefore, high sperm integrity could be related to the synergistic effect of antioxidants and HSPs. It has been stated that HSP90 was highly sensitive to H_2O_2 and reversibly oxidized (as described in chapter 5), and that could be explained by transformation of cysteine substance either into sulphonic acids and sulphenyl-amide linkages or into internal disulphides and mixed disulphides with glutathione (Zhang *et al.,* 2018).

We propose that antioxidants aid to prevent damage to HSPs and consequently avoid the decrease of HSPs expression thus reducing the effect of ROS, and play a significant role to maintain sperm integrity through their protection. These results showed that there is a relationship between sperm parameters and the expression of HSP90, and also indicated that it might be possible to effectively maintainthe viability of spermatozoa by preserving the level of HSPs in spermatozoa before cryopreservation through treatment with antioxidants such as taurine or cysteine. The cryopreservation process including freezing and thawing increases the production of ROS, and that leads to reduced sperm integrity and fertilizing ability (Guthrie and Welch 2012). The presence of high amounts of polyunsaturated fatty

acid in the membrane of sperm makes the membrane highly sensitive to lipid oxidation (LPO) leading to reduced sperm integrity due to the attacks by ROS (Hu *et al.,* 2010).

This was in agreement with the present results that showed the expression level of HSP90 decreased during incubation time, and that was associated with decreased sperm parameters and increased proportion of ROS (Table 6.2), with increased the level of LPO of sperm and for SP for control, taurine and cysteine respectively after 60 min incubation. This might be related to that proteins degrade over time due to natural proteases in the seminal plasma (Cao *et al.,* 2003).

It has been hypothesized that high levels of HSP90 expression could form a protective covering preventing membrane damage and cell death (Zhang *et al.,* 2018), and the HSPs expression was increased due to the response of lipid peroxidation in animal cells (Zhang *et al.,* 2015).

In the present study, the highest level of expression of HSP90 was on the tail and middle piece (r^2 = 0.624; r^2 = 0.637) respectively. Increased sperm motility was also associated with high HSP90 both here and in Sagare-Patil *et al.* (2017). Therefore, it is possible that the mechanism leading to increase in HSP90 expression could be a way of preserving sperm motility during oxidative stress conditions. This could also be related to Ca^{+2} levels in the sperm cell which is an essential substrate in the metabolic pathway that regulates sperm motility (Darszon *et al.*, 2001). Cryopreservation reduces the sperm membrane permeability thus affecting calcium transport with associated reduction in the level of HSP90 expression. It has been reported that a high level of ROS during cryopreservation effects the level of intracellular calcium (Peris *et al.*, 2008). In addition, HSP90 has the ability to protect cells from the effect of ROS (Fukuda *et al*. 1996), and also reported that the ATP level is reduced post cold shock inferring HSP90 could be involved in ATP metabolism (Prodromou *et al*., 1997).

Therefore, by adding antioxidants such as taurine or cysteine to cryopreserved semen, it is possible to promote HSP90 expression level thus helping protect and regulate membrane permeability, maintain Ca^{+2} transport and ATP levels and which leads to improve sperm integrity.

The results in the present study show the high expression of HSP70 in other sperm regions such as (acrosome, post-acrosomal, and full head) (Figures 6.4 and 6.5). The increased expression in these regions followed cysteine supplementation protects the sperm membrane. Cysteine supplementation is also associated wirth the increase of the antioxidant glutathione in the sperm cell (Gungor *et al.*, 2017).

These results may suggest that antioxidant supplementation (taurine or cysteine) have an essential effect on regulating the role of HSPs through protecting the sperm membrane from the stress, and that was apparent with the significant results on sperm integrity such as motility and viability. This is inconsistent with previous studies that stated HSP90 has an important role in folding of the protein, and it is localized in the tail of sperm in all species and related with sperm motility and fertility (Wang *et al.*, 2012).

Sperm parameters declined during incubation time. These findings propose that a positive association exists between the levels of expression of HSP90 and HSP70 on different sperm regions and an improvement in sperm parameters (Table 6.6). The results in the present study found a high association between the expression level of HSP90 and HSP70 on sperm regions and the integrity of frozen-thawed ram spermatozoa in presence the antioxidants (cysteine or taurine). Therefore, due to sperm cells being unable to respond to stress with an enhanced HSP synthesis and only consuming the already synthesized HSP pool, antioxidants supplementation can improve and maintain the functions of HSP90 and HSP70 in the frozen-thawed ram spermatozoa and consequently improve fertility rates.

6.5. Conclusion

The current study confirmed that treatment with 1.0 mg/ml pre-freeze and post-thaw (PF + PT) of cysteine or taurine have a significant effect on reducing the oxidative stress and improving the integrity of frozen-thawed ram sperm. Cysteine or taurine (PF + PT) supplementation improved the expression level of HSP70 and HSP90 in the sperm regions (acrosome, post-acrosomal, full head, middle piece and tail), and this was related to improved sperm functions. The highest effect was with cysteine supplementation and the intermediate effect was with taurine. However, the lowest effect was in the control group which had no treatment. The results of this study proposed that the antioxidants supplementation with 1.0 mg/ml of cysteine or taurine have a protective role from the oxidative stress and maintained the activity of HSP70 and HSP90.

Chapter 7.1. General Discussion

This research aimed to improve post-thaw ram sperm integrity by reducing the negative impact of cryopreservation on sperm, and achieve higher fertility rates during artificial insemination. A reduction in fertility has been associated with an inability of FT spermatozoa to migrate through the female tract, and the increased time required for FT spermatozoa to reach the oviduct (Soleilhavoup *et al.,* 2014). Therefore, this research investigated through the first study, the effect of supplementing fresh and frozen-thawed ejaculated and epididymal ram sperm with seminal plasma protein (SPP) fractions. The second study was to investigate the effect of antioxidants (vitamin C, cysteine, or taurine) on the integrity of the frozen-thawed (FT) ram sperm. The third study aimed to determine the impact of oxidative stress using H_2O_2 on sperm functions and the expression of HSP70 and HSP90. The fourth study aimed to identify the relationship between the antioxidants (taurine and cysteine) supplementation and HSP90 and HSP70 expression on reducing oxidative stress of frozen-thawed ram spermatozoa.

The sperm membrane can be considered as the first and main component of the cell that could be affected by external stress such as oxidative stress, which effects the activity and integrity of sperm. Thus, maintaining sperm membrane permeability could improve sperm functions and consequently improve fertility rates. Cryopreservation has a negative impact on sperm membrane and reduces the integrity. Overall, the results obtained in Chapter 3 have demonstrated that SPP fractions improve sperm motility and viability, reduce acrosome damage, and enhance the ability of spermatozoa to penetrate cervical mucus *in vitro* when added to the semen extender. Four fractions were included in the investigation: Whole SP (WSP), >100kDa, 30-100kDa and <30kDa. Although all SPP treatments improved sperm function, WSP and <30kDa were more effective than other fractions.

Most previous studies have shown the beneficial effect of SPP on fresh and FT spermatozoa in the ram and the bull (Maxwell *et al.,* 2007; Juyena and Stelletta, 2012; Rickard *et al.,* 2014), but there are limited studies that discuss the effect of SPPs on the integrity of fresh and frozen-thawed epididymal spermatozoa.

SPPs supplementation protected and repaired ram sperm membrane integrity of fresh ejaculated, fresh epididymal and FT epididymal spermatozoa. Bernardini *et al.* (2011) reported that SP not only has protective proteins but also plays an important role in maintaining metabolism for sperm motility This effect could be due to the impact of the main components of SP such as sugar, lipids and proteins which have the ability to restore the sperm membrane (Leahy and de Graaf, 2012; Druart *et al.*, 2013). The low molecular weight fraction can easily pass across the membrane, aiding protection of the sperm membrane and improving sperm motility such as fraction <30kDa (RSVP 20, RSVP 24) (Barrios *et al.*,

2005; Xie *et al.*, 2016). Furthermore, low molecular proteins such as 30.05kDa was identified at a high level in extracts of liquid stored semen (Paul *et al.*, 2017). This could be considered an indicator of the essential role of the low molecular proteins to reduce the destabilization and any decrease in plasma membrane integrity. Therefore, the long exposure period of spermatozoa to SPP, specifically low molecular weight fractions (17kDa and 23kDa), showed a significant role in increasing the ability of sperm to penetrate the cervical mucus (El-Hajj Ghaoui *et al.*, 2007; Bernardini *et al.*, 2011; Gwathmey *et al.*, 2018).

Additionally, tris citrate glucose (TCG) was considered to be essential to maintain sperm motility when only interacting SPP fractions were supplemented (Naing *et al.*, 2011).

Antioxidants that have a low molecular weight, such as vitamin C, cysteine, and taurine, can enter the sperm membrane and reduce the effect of ROS (Çoyan *et al.*, 2011; Topraggaleh *et al.*, 2014). It has been stated that some types of antioxidants such as GPx and SOD are recognised with low molecular weight SPP (14kDa and 20kDa) are primarily involved in the reduction of cold shock (Marti *et al.*, 2007).

Cryopreservation causes osmotic stress (Wu *et al.*, 2015), damage to sperm membrane and reduces the components such as lipids and proteins (Bergeron *et al.*, 2005), and increases lipid peroxidation by ROS due to a decrease in the concentration of antioxidants (Trinchero *et al.*, 1990; Aitken *et al.*, 1998). These antioxidants are necessary to maintain sperm integrity and prevent the lipid peroxidation of spermatozoa (Gungor *et al.*, 2017). It has been stated that a high level of ROS during cryopreservation increases the level of intracellular calcium and reduces the integrity of sperm membrane permeability (Peris *et al.*, 2008). The concentration of antioxidants in defensive systems are limited (Aitken, 1995), and they differ with the type of ejaculation, species, and season (Ollero *et al.*, 1996).

The membrane of ram spermatozoa contains a high quantity of polyunsaturated fatty acids which increases the susceptibility to lipid peroxidation by ROS (Osipova *et al.,* 2016; Tvrdá *et al.*, 2016). ROS have a damaging effect on lipid peroxidation of sperm membranes which includes loss of intracellular enzymes, DNA damage, loss of sperm functions (Bansal and Bilaspuri, 2011), and consequently leakage in sperm-oocyte fusion (Aitken *et al.,* 1998).

It has been found that superoxide dismutase eliminates superoxide radical producing hydrogen peroxide as the primary type of ROS (Mennella and Jones, 1980), which is subsequently converted by catalase (Lapointe *et al.*, 2005) or by glutathione peroxidase (Sinha *et al.,* 1996). Thus, supplementation of sperm extender with antioxidants such as vitamin C, cysteine, or taurine during pre-freezing and post-thawing of semen could aid the elimination of free radicals (Uysal and Bucak, 2007; Bucak *et al.,* 2007), and so reduce

losses of sperm integrity and DNA damage, related to the accumulation of malondialdehyde (Vishwanath and Shannon, 1997; Barbato *et al.*, 2017).

As described in Chapter 4, the study aimed to determine the optimal time and optimial concentration of antioxidants to improve FT ram sperm integrity. Therefore, vitamin C, cysteine, or taurine were supplemented at different concentrations pre-freeze and post-thaw (PF+PT) to reduce the effect of ROS on the quality of frozen-thawed ram sperm.Vitamin C supplementation (as described in Chapter 4.A) showed no effect ($P > 0.05$) on sperm function and integrity, which could be related to the low ability of vitamin C to protect the cell membrane from osmotic stress during cryopreservation and thus did not improve membrane stability (Ball, 2008; Li *et al.,* 2010; Martínez-Páramo *et al.*, 2012). , Improved sperm penetrability and reduced ROS production was significant as a result of the effects of vitamin C, and most likely due to the prevention of endogenous oxidative processes and scavenging superoxide anions in the TRIS semen extender (Silver *et al.*, 2005; Ménézo *et al.*, 2007; Michael *et al.,* 2007; Piomboni *et al.*, 2008; Cabrita *et al.*, 2011).

The post-thaw ram sperm integrity such as motility, viability and penetrability into artificial mucus significantly improved ($P < 0.001$) with 1.0 mg/ml (PF+PT) of cysteine supplementation, and reduced ROS production on treatment samples compared to the control, as described in Chapter 4.B. The effect of cysteine could be due to the presence of a thiol group in cysteine, which is an intracellular glutathione precursor. Furthermore, cysteine has a low molecular weight that makes it easy to penetrate the sperm membranes and protect the spermatozoa from the effect of ROS. In addition, glutathione (GSH) and the other thiol (-SH) compounds are essential components to protect cells from DNA damage (Topraggaleh *et al.,* 2014). However, cysteine had no effect in protecting acrosome integrity, and this effect was similar to previous studies that also did not show cysteine protecting acrosome integrity from the damaging effect of ROS (Michael *et al.,* 2007; Bucak *et al.,* 2008; Tuncer *et al.,* 2010). This effect on the acrosome could be related to the effect of the cold shock during cryopreservation, which reduces cellular function leading to a loss of the selectivity of plasma membrane permeability (Medeiros *et al.* 2002). Moreover, it has been stated that the acrosome reaction is more sensitive to oxidative damage than other sperm functions (Whittington and Ford, 1998).

Additionally, cryopreservation with high levels of ROS prompted a capacitation-like effect, which increases the level of intracellular calcium and membrane permeability (Peris *et al.,* 2008). This effect causes severe damage (include swelling and breaking) in the acrosome region of sperm (Aisen *et al.*, 2005). Cysteine supplementation cannot recover this damage. Therefore, using another type of antioxidant, such as taurine, that has the ability to reduce the effect of cryopreservation and regulate sperm membrane permeability could improve post-thaw ram sperm integrity as described in Chapter 4.C.

The results in Chapter 4.C reveal that taurine supplementation 1.0 mg/ml (PF+PT) had a positive effect ($P < 0.001$) on sperm integrity and reduced ROS production. Taurine has the ability to re-arrange the proteins and lipids of the sperm membrane, which improves membrane fluidity (Yang *et al.,* 2010), and regulates energy metabolism in the cell (Agarwal *et al.,* 2004). In addition, taurine has the ability to protect and improve the antioxidant enzymes system such as catalase (Perumal *et al.*, 2013), and superoxide dismutase (Higuchi *et al.,* 2012), glutathione peroxidase (Nonaka *et al.*, 2001), and thioredoxin reductase (Yildirim *et al.*, 2007). This effect of taurine with the method used (PF+PT) could provide some protection and maintain the antioxidant system of the sperm cell to resist the harmful effect of ROS (Holmes *et al.,* 1992; Bucak *et al.,* 2007; Paál *et al.*, 2018).

Taurine has two mechanisms to protect the cells. A direct effect as an antioxidant via its ability to detoxify and decrease some volatile intermediates such as hypochlorous acid created by myeloperoxidase, and an indirect effect by introducing taurine into the cell membrane and stabilizing it (Schaalan *et al.,* 2018). In the present study, taurine supplementation improved sperm functions including sperm penetrability and reduced the proportion of ROS.

Furthermore, taurine has an amine group that plays an important role through its association with nucleic acids and can subsequently decrease the generation of ROS, and reducing the damage to DNA (Zhang *et al.*, 2004; Sokól *et al.*, 2009). Thus, taurine in pre-freeze supplementation could play an essential role as an osmoregulator and protect ram sperm membrane from the effect of cold shock damage (Sturman and Hayes, 1980), and through post-thaw supplementation could act as antioxidant and support the systemic enzymes of the sperm to reduce the effect of ROS and oxidative stress (Yang *et al.*, 2010). Taurine supplementation, as shown previously on tambaqui sperm by Lopes *et al.* (2018) regulates and transports Ca^{2+} in the sperm membrane and is required to produce sperm activity. This positive effect of taurine on sperm membrane could also explain its ability to maintain integrity and improve sperm penetrability.

In summary, the most beneficial effect of antioxidants on sperm function was through using 1.0 mg/ml (PF+PT) supplementation of cysteine or taurine to improve motility, structural and functional membrane integrity, acrosome integrity, viability and penetrability in artificial mucus with reducing ROS%.

ROS comprises many types such as hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide (O^2) and hydroxyl radical (OH). Due to the severe damaging effect of ROS on sperm integrity, it was important to determine the effect of H_2O_2 as a primary type of ROS on the integrity of fresh ram semen and lipid peroxidation. In addition, it was essential to understand how oxidative stress may induce sperm damage and affect the expression of

heat shock proteins, which appear important for sperm function (as described in Chapter 5).

The results showed at post 60 min incubation, the detrimental effect of ROS on sperm integrity, using 15mM H_2O_2 was substantial compared to the moderate effect with 5mM and the lowest effect on control samples which did not receive any treatment with H_2O_2. The intracellular level of LPO of sperm and SP increased due to the presence of high levels of ROS production in sperm, which increased by the exogenous addition of H_2O_2. This t has a high ability to penetrate the cell membrane and cause severe damage in sperm DNA (Halliwell and Aruoma, 1991).

From the results of Chapter 5, it can be concluded that H_2O_2 is the primary type of ROS causing an adverse effect on ram sperm integrity and reducing the expression of HSP70 and HSP90. In addition, the level of HSP70 and HSP90 expression was measured as an indicator for sperm cell membrane response to external stress. HSPs are proteins that have the ability to protect sperm cells from stress including oxidative stress (Beere and Green, 2001). Welker *et al.* (2010) and Török *et al.* (2014) found that HSPs have the ability to stabilize and preserve the integrity of the cell membrane through interacting with the lipid of the cell membrane and maintaining the integrity and function under stress environments. Wang *et al.* (2014) found that HSP90 has the ability to modulate the fluidity of the membrane through the association with phospholipid, and also has a vital role in protecting sperm motility during cooling or after thawing (Cao *et al.,* 2003; Martin *et al.,* 2007). HSP70 can sustain the conformation of the proteins and is involved in protein movement through the intracellular membranes (Zhang *et al.,* 2015). These proteins have an essential role in fertilization (Spinaci *et al.*, 2006), and the motility of frozen-thawed sperm (Wang *et al.,* 2014; Zhang *et al.,* 2015).

Therefore, the oxidative stress on sperm membrane causes a reduction and disturbance in the activity and roles of these proteins. Specifically, the high level of ROS causes protein degradation and reduces the activity of HSPs to maintain sperm membrane integrity (Cao *et al.*, 2003). This effect was confirmed in Chapter 6, that antioxidants supplementation (taurine or cysteine) has a vital effect on preventing ROS production and regulating the role of HSPs through protecting the sperm membrane from stress. Supplementation was clear through the significant results of sperm integrity such as motility and viability.

The level of HSP70 and HSP90 expression of treatment samples on different sperm regions decreased post 60 min at 37ºC and was associated with decreased sperm integrity such as motility and increased level of LPO in sperm and SP. The main reason for these results that could be due to the high stress by H_2O_2 and incubation time, which caused protein deterioration and decreased the activity of HSPs through their role to reduce the effect of

H_2O_2 and maintain the sperm membrane integrity (Cao *et al.,* 2003). It has been stated that the level of HSP70 and HSP90 decreased gradually during incubation time and this effect was related with decreased sperm integrity and motility (Wang *et al.,* 2014; Zhang *et al.,* 2015). Plessis *et al.* (2010) reported that exogenous H_2O_2 causes high levels of free electrons to leak from the mitochondria and consequently increased levels of ROS in caused in the sperm cell.

As the primary objective of the study was to improve frozen-thawed ram sperm integrity, a reduction, in the levels of ROS and LPO on sperm and SP, and maintenance of activity of the sperm membrane proteins, such as HSPs, could be the main influential factors improving sperm integrity. This protection method could be achieved by using optimal timing and concentration of antioxidants such as 1.0 mg/ml (PF+PT) of cysteine or taurine supplementation on ram semen samples which can improve post-thaw ram sperm integrity (as described in Chapter 6), and identify the relationship with the level of HSP70 and HSP90 expression.

As discussed previously, the role of HSPs in reducing oxidative stress and the relationship between the antioxidants and the action of HSPs is not yet fully understood. The results in Chapter 6 showed a significant ($P < 0.001$) effect on sperm treatments parameters such as motility, viability, acrosome integrity, and penetrability compared to the control. The highest levels of HSP70 and HSP90 expression on most sperm regions were found in post-thawed sperm treatment samples with cysteine compared to the lowest expression in the control samples. This effect could be as a response to the protective effects of antioxidants (cysteine or taurine) and reduce the adverse effect of cryopreservation. This increase in HSP90 and HSP70 expression was linked to improved sperm parameters. HSP90 and HSP70 are known to play a role in the production of proteins and enzymes that act to protect the sperm membrane (Zhang *et al.,* 2015). The effect of HSPs in the presence of antioxidants, which inhibit oxidative stress, could have an inpact in in inhibiting phospholipase and modulating the fluidity of the membrane through their association with phospholipids (Wang *et al.,* 2014). It has been stated that HSPA8 (HSP70) has ability to interact with the plasma membrane and prevents adverse variations in membrane lipid construction through the interaction with the lipid membranes in numerous related ways that comprise of connecting to sulphated galactolipids (Mamelak *et al.*, 2001), the creation of ion channels (Arispe and De Maio, 2000), and the reduction of lipid peroxidation of sperm membrane (Su *et al.*, 1999). Consequently, improve sperm integrity and reduce the harmful effect of lipid peroxidation during cryopreservation (Chatterjee and Gagnon 2001; Guthrie and Welch, 2012). The pre-freeze addition of HSP70 on bull spermatozoa reduces sperm apoptosis and membrane permeability (Holt *et al.,* 2015), and improves sperm viability *in*

vitro (Elliott *et al.*, 2009; Lloyd *et al.*, 2012), and liberates ATP in the cell, which confirms the role of HSP70 in protecting sperm motility (Ha and McKay, 1995).

The method, which is used for antioxidant supplementation (PF+PT) and providing more protection to membrane components of frozen-thawed ram sperm during cryopreservation and post-thawing could improve the activity of HSPs and consequently increase the level of expression. This effect could be due to the ability of cysteine or taurine to protect the sperm membrane as they amino acids with low molecular weights that can eliminate lipid oxidation by inhibiting the generation of ROS (Coyan *et al.,* 2011). Post-thaw supplementation may provide support to the activity of glutathione peroxidase during the post-thaw incubation time of spermatozoa (Zhu *et al.,* 2017). Consequently, the post-thaw addition in the present study could increase the glutathione level which improves sperm integrity through scavenging ROS (Bansal and Bilaspuri 2011). Therefore, this method (PF+PT) of antioxidants supplementation on ram semen extender is considered a novel method of semen cryopreservation protocol because it contributes to improving the post-thaw ram sperm integrity.

The results of the present study showed high expression of HSP90 and HSP70 in the acrosome region, middle piece and tail which could be due to the synergistic effect of cysteine or taurine in protecting these regions during cryopreservation. However, cryopreservation causes a reduction in the expression of HSP70 and HSP90 in post-thaw bull sperm (Varghese *et al.,* 2016; Zhang *et al.,* 2015), and HSP70 distribution in buffalo spermatozoa (Varghese *et al.,* 2016). Therefore, antioxidant supplementation could reduce the harmful effect of cryopreservation and preserve the activity of HSP90 and HSP70. The results of these two studies Chapter 5 and 6 suggest that cysteine and taurine are effective in reducing ROS and thereby protecting HSP70 and HSP90 against ROS such as H_2O_2.

Moreover, the precise observation of high level of expression of HSP70 and HSP90 in sperm regions, specifically the middle piece and tail, could be due to protection of the mitochondria activity from stress and increased energy production during glycolysis (Prodromou *et al.,* 1997; Ashraf *et al.,* 2013; Zhang *et al.,* 2015). It has been reported that the axoneme on the middle piece is enveloped by mitochondria that produce the energy and which are responsible for sperm motility (Sariözkan *et al.*, 2009). This could explain the high motility of samples treated by cysteine or taurine compared to the control. In addition, the high expression level of HSP90 on the tail and middle piece could have arisen in an attempt to produce ATP and maintain the action of flagellar fibres to improve motility.

It has been stated that the level of lipid peroxidation is 2-3 times more in FT semen than in fresh bull semen (Slaweta *et al.,* 1988). This finding could explain the role of antioxidant supplementation with cysteine or taurine to reduce the effects of oxidative stress and

preserve the high expression of HSP90 and HSP70 in different regions of FT ram sperm. It could also explain the reduced level of HSP90 and HSP70 expression in fresh semen after exposure to H_2O_2 as an external stress to induce ROS production, when incubated in the absence of antioxidants supplementation. Additionally, it has been reported that H_2O_2 causes increase ribosome activity related with key mRNAs, which may be converted rapidly and reduce the oxidative stress, therefore, the high level H_2O_2 can cause reduction of protein-membrane synthesis (Shenton *et al.*, 2006).

Furthermore, it has been found that there is no gene expression in the headpiece as the genes are condensed into chromosomes (Ren *et al.*, 2017), and the gene expression in the mitochondria limited to energy production for motility (Anderson *et al.*, 1981). Therefore, the ability of HSPs to protect the integrity of FT ram sperm is limited, and that could explain the low expression level of HSPs on the head sperm region. This could also be considered as the main reason for the high expression of HSPs and improved FT ram sperm integrity in the current study through cysteine or taurine supplementation. This in turn could maintain the activity of HSPs and protect them from the effect of cryopreservation and degradation by the high level of ROS.

These observations, together with the current findings in this study, raise the possibility that HSP90 and HSP70 may participate in regulating sperm functions in a variety of ways. The results in Table 6.6 show that sperm parameters declined during the incubation time. These findings suggest that there is a positive relationship between the level of expression of HSP90 and HSP70 and the integrity of sperm parameters, such as sperm motility, acrosome integrity, viability, and penetrability, level of ROS and LPO over the incubation time (as described in Figure 7.1 and 7.2). As such, expression levels of HSP90 and HSP70 could be considered as useful parameters to evaluate the integrity of ram spermatozoa post-thaw.

Better sperm function and integrity can mean better fertility and development for sheep AI. As known, AI in the sheep farming industry by cervical insemination, is limited due to the low fertility rates of frozen-thawed semen. Therefore, improving the main factors of frozen-thawed ram sperm parameters can improve success of AI and increase fertility rates. David *et al.* (2015) stated that the sperm that contributed to the fertilization process must be able to rapidly penetrate the female reproductive tract to the fertilization site (which requires penetrability of cervical mucus) and enter the membranes of the oocyte (which requires acrosomal integrity), and contribute to creation of an embryo (which requires DNA integrity).

Variable methods, including functional and non-functional assessments of sperm, have been suggested to assess the various parameters of the sperm cell. The functional parameters are the sperm's ability to penetrate the cervical mucus, ability to penetrate the

zona pellucida, and *in vitro* fertilization test, which is related to the sperm fertilization capacity (Gadea, 2005). The non-functional assessments are the evaluation of seminal plasma proteins and chromatin integrity (Evenson and Jost, 2004), evaluation of sperm motility (mass motility) related with ATP (Amann and Katz, 2004), the acrosome and plasma membrane integrity, and the proportion of abnormal or dead sperm (Malo *et al.,* 2004).

These assessments of motility, acrosome integrity, viability and penetrability related tothe effect of low molecular SP proteins have been conducted in the present study. The main improvement to the integrity of frozen-thawed ram spermatozoa was observed through the control on the proportion of ROS production by detecting the optimal timing and concentration of antioxidants supplementation using cysteine or taurine. Additionally, the main effect of these antioxidants on the activity of HSP70 and HSP90 on the sperm membrane is to have an important role in maintaining sperm functions such as capacitation and regulation calcium and tyrosine phosphorylation (Li *et al.*, 2014). These findings taken together could be used to provide a more reliable fertilizing capacity of frozen-thawed ram sperm and improve the AI in sheep the industry.

7.2. General conclusion

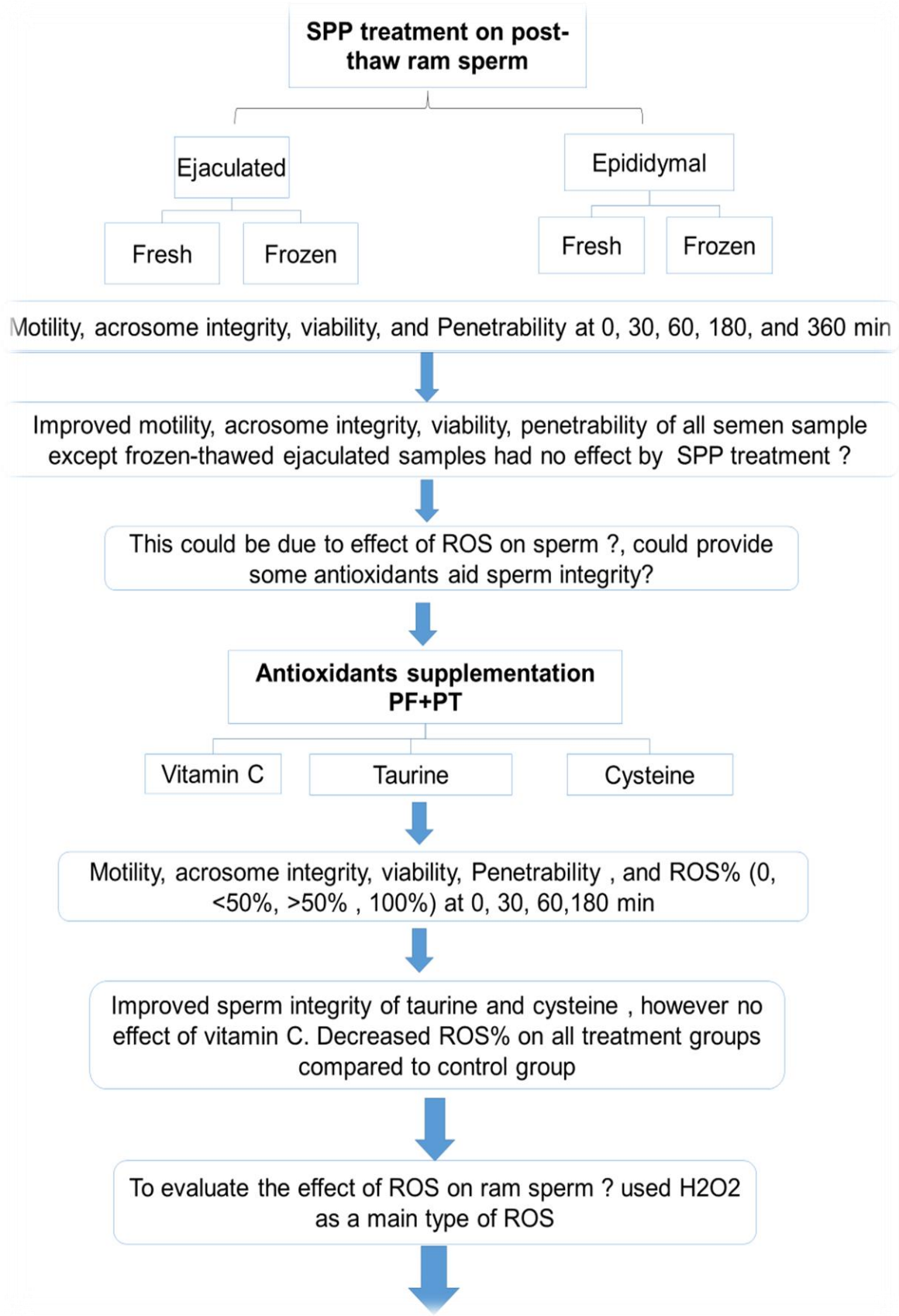
The results obtained from this thesis show some novel findings of the effect of SPP on fresh, FT ejaculated and epididymal spermatozoa. Antioxidants supplementation provided some protection to FT ram sperm. Artificial reproduction requires that ROS production is minimised during the preparation of sperm, particularly as the antioxidants system of semen is reduced from sperm and SP. The primary outcomes obtained from this research can be summarized as:

- The effect of WSP was similar to the <30KDa fraction on improving function and integrity of fresh and FT epididymal spermatozoa.
- The effect of WSP and other fractions, particularly <30KDa, have a positive effect on fresh and FT ejaculated spermatozoa; however, the results of motility and penetrability of ejaculated FT were not significant.
- Cysteine or taurine supplementation (1.0 mg/ml) PF + PT improved FT ram sperm parameters such as motility, acrosome integrity, viability, and penetrability and reduced ROS production. However, acrosome integrity did not improve with cysteine supplementation.
- Vitamin C supplementation (PF + PT) did not improve FT ram sperm integrity, however, showed a significant effect in reducing ROS production.
- The addition of H_2O_2 to fresh ram semen samples reduced HSP90 and HSP70 protein expression on the sperm membrane and increased ROS production and reduced sperm integrity with high levels of LPO in SP and sperm.
- 1.0 mg/ml of cysteine or taurine supplementation protected the activity of HSP90 and HSP70 and reduced the effect of ROS and LPO on SP and sperm, with improved ram sperm integrity.

7.3. Recommendations for further work

- Evaluate whether the positive impact of antioxidant supplementation on sperm parameters *in vitro* results in higher fertility rates compared to untreated semen *in vivo.* It would be worthwhile investigating the impact of antioxidants on fertility rates using cervical AI to determine whether the imporved sperm function and increased HSP expression aids sperm transport in vivo and improves fertility.

- Investigate the effect of HSPs on sperm integrity in the female reproductive tract in sheep and relate that to fertility rates.


SPP treatment on post-thaw ram sperm
Ejaculated
Epididymal
Fresh
Frozen
Fresh
Frozen
Motility, acrosome integrity, viability, and Penetrability at 0, 30, 60, 180, and 360 min
Improved motility, acrosome integrity, viability, penetrability of all semen sample except frozen-thawed ejaculated samples had no effect by SPP treatment ?
This could be due to effect of ROS on sperm ?, could provide some antioxidants aid sperm integrity?
Antioxidants supplementation PF+PT
Vitamin C
Taurine
Cysteine
Motility, acrosome integrity, viability, Penetrability , and ROS% (0, <50%, >50% , 100%) at 0, 30, 60,180 min
Improved sperm integrity of taurine and cysteine , however no effect of vitamin C. Decreased ROS% on all treatment groups compared to control group
To evaluate the effect of ROS on ram sperm ? used H2O2 as a main type of ROS

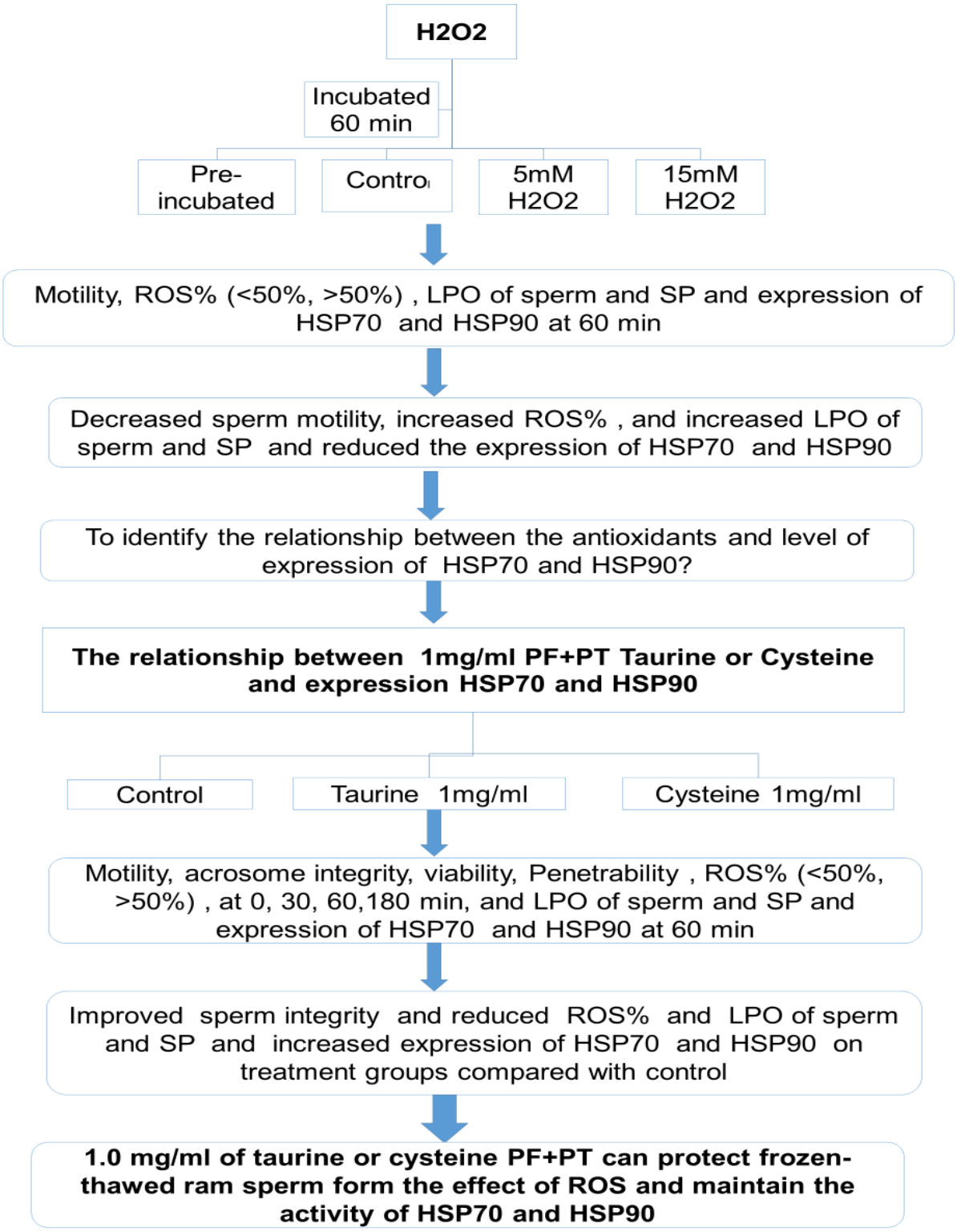


Figure 7. 1. Flow diagram of outline of the experimental design and main findings of the PhD research.

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