REACTIVE OXYGEN SPECIES AND THEIR EFFECTS ON BOAR SPERMATOZOA
FUNCTION, TYROSINE PHOSPHORYLATION AND MAPK SIGNALING

A Thesis
Presented to
The Faculty of Graduate Studies
of
The University of Guelph

by
BASIM J. AWDA

In partial fulfilment of requirements

For the degree of
Doctor of Philosophy
September, 2008

© Basim J. Awda, 2008
NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Canada
ABSTRACT

REACTIVE OXYGEN SPECIES AND THEIR EFFECTS ON BOAR SPERMATOCZOVA FUNCTION, TYROSINE PHOSPHORYLATION AND MAPK SIGNALING

Basim J. Awda
University of Guelph, 2008

Advisor
Dr. Mary M. Buhr

Boar spermatozoa are very susceptible to Reactive Oxygen Species (ROS) due to their high content of polyunsaturated fatty acids (PUFAs). This study hypothesized that ROS modulate signal transduction during capacitation of fresh boar spermatozoa through the extracellular signal-regulated kinases (ERKs) of mitogen-activated protein kinase (MAPK) pathway, and that cryopreservation damage to sperm involves ROS.

All experiments analysed multiple ejaculates. Flow cytometry using dual fluorescent dyes measured sperm viability, intracellular levels of $O_2^{-\cdot}$, $H_2O_2$, membrane lipid peroxidation and PLA activity. Western blotting of extracted proteins from boar spermatozoa detected and quantified MAPKs (Raf1, MEK1/2 and ERK1/2) and phosphotyrosine in boar spermatozoa after 0 and 4hr incubation in capacitating or non-capacitating media, in sperm pre-exposed for 30 minutes to buffer or an ROS generating system. The ROS generating system had multiple effects: increased fresh sperm intracellular level of $H_2O_2$; completely inhibited sperm motility; increased the percentage of sperm undergoing acrosome reactions; increased lipid peroxidation in the viable, dead and average sperm populations, and; inhibited tyrosine phosphorylation of proteins with high molecular weights.
Phosphorylated forms of the ERK pathway components Raf-1, MEK1/2, and ERK1/2 are present in boar sperm. Their protein profiles, quantified using Image Quant software, differed with capacitation and ROS generating system. Boar sperm capacitated as before and preincubated with a specific inhibitor of ERK1/2, but not with inhibitors of Raf1 or MEK1/2, significantly inhibited tyrosine phosphorylation of spermatozoa proteins of 172, 97 and 66 kDa. Combining ERK1/2 inhibitor with the ROS generating system greatly increased inhibition of tyrosine phosphorylation of these three proteins (P<0.002) and also a 111 kDa protein (P<0.028). Inhibiting MEK1/2 in the presence of the ROS generating system subsequently inhibited capacitation-induced tyrosine phosphorylation of proteins of 187(P<0.01) and 112 kDa (P<0.04).

This study indicates that H₂O₂ is the major free radical mediating the damage of boar spermatozoa and ROS have physiological roles regulating protein tyrosine phosphorylation of capacitating boar spermatozoa. The ERK pathway regulates tyrosine phosphorylation in boar spermatozoa through its ERK1/2 component and ROS mediate cAMP-dependent PKA and ERK pathway signaling during sperm capacitation, through phosphorylation/dephosphorylation of specific proteins.
ACKNOWLEDGEMENTS

To reach your goal after 3 years of hard work and effort as you planned, would be wonderful, and to add something new to the science when you reach this goal, would be great. However, it was not possible to reach the goal and to add something new to the science without the help, the encouragement, and the support of others. Those others are the people who have left their fingerprints in my life.

Dr Mary Buhr was not just the wonderful advisor or the colleague or the friend that gives the support, the encouragement and the suggestions during hard times, she was our family always. Many thanks Dr Mary for all of that.

Many thanks to my advisory committee, Dr Muhammad Anzar, Dr Jacob Thundathil, and Dr Cathy Gartly for their help, comments and support, and my examining committee for their helpful comments. Sincere thanks to Dr Margaret Quinton and Dr Michelle Edward for their help and advice in doing the statistical analysis and Richard Avery for all the long distance conferences during all my advisory committee meetings. I greatly appreciate the excellent help of my colleagues, Meghan Bell, Rachel Mixer, Katie Hickey, and Paul Dyce during doing the experiments. Also I would thank my colleagues Ian Mahony, Kalaivani Anpalakan (Vani), Adam Colley, Dr Nipa Kakuda and Dr Laura Graham for their cooperation. Special thanks to my family, my relatives, and my friends for their support and encouragement.

Finally, I would gratefully acknowledge NSERC, OMAFRA, Bioniche, Semex Alliance and L’Alliance Boviteq, who financially supported this research.
4.3.1. Semen handling

4.3.1.1. Semen collection

4.3.1.2. ROS generating system

4.3.2. Protein extraction, SDS-PAGE and western blotting

4.4. Statistical analysis

4.5. Results

4.6. Discussion

5. Extracellular signal-regulated kinases (ERKs) family of mitogen-activated protein kinase (MAPK) pathway and reactive oxygen species are involved in the regulation of tyrosine phosphorylation in boar spermatozoa

5.1. Abstract

5.2. Introduction

5.3. Materials and methods

5.3.1. Semen handling

5.3.1.1. Semen collection

5.3.1.2. ROS generating system

5.3.2. Experiment 1: The role of ERK signaling pathway and ROS on modulating tyrosine phosphorylation

5.3.2.1. Treatments

5.3.2.2. Protein extraction, SDS-PAGE and western blotting

5.3.3. Experiment 2: The effect of inhibiting ERK signaling pathway components on boar sperm tyrosine phosphorylation

5.3.3.1. ERK pathway components inhibitors
5.3.3.2. Treatments ................................................................. 94
5.3.3.3. Protein extraction, SDS-PAGE and western blotting ............ 94

5.4. Statistical analysis .......................................................... 95

5.5. Results .............................................................................. 96

5.5.1. Experiment 1: The role of ERK signaling pathway and ROS on modulating tyrosine phosphorylation ............................................ 96

5.5.2. Experiment 2: The effect of inhibiting ERK signaling pathway components on boar sperm tyrosine phosphorylation ..................... 107

5.5.2.1. Raf inhibitor (ZM336372) .............................................. 107

5.5.2.2. MEK1/2 inhibitor (U0126) ............................................ 110

5.5.2.3. ERK 1/2 inhibitor (ERK Activation Inhibitor Peptide I, Cell-Permeable) ................................................................. 115

5.6. Discussion ......................................................................... 119

6. General discussion ............................................................... 131

6.1. Sperm intracellular levels of ROS and response to an ROS generating system ................................................................. 131

6.2. ROS generating system affects sperm functions ....................... 135

6.2.1. Motility ........................................................................ 135

6.2.2. Acrosome reaction ......................................................... 136

6.2.3. Lipid peroxidation .......................................................... 137

6.2.4. Phospholipase A activity .................................................. 139

6.3. Tyrosine phosphorylation signal transduction ......................... 140

6.4. ERK pathway and tyrosine phosphorylation signaling ................ 143
6.5. ROS and sperm cryopreservation .................................................. 146
6.6. Summary and conclusions ............................................................. 146
Literature cited .................................................................................. 149
Appendices ....................................................................................... 167
List of Figures

Figure 3.1. The effect of freezing, thawing, and washing on the % of boar sperm motility and viability .................................................................41

Figure 3.2. Flow cytometric 2-dimensional dot-plots fluorescence intensity of YO-PRO-1 and ethidium of fresh and frozen-thawed boar sperm..............42

Figure 3.3. Ethidium fluorescence intensity of viable fresh and frozen-thawed boar spermatozoa.................................................................43

Figure 3.4. The % of viable fresh and frozen-thawed boar sperm sperm measured by three different methods.......................................................44

Figure 3.5. The effect of incubating boar spermatozoa in BTS ± ROS generating system on the % of motile and viable sperm.................................45

Figure 3.6. Flow cytometric 2-dimensional dot-plots fluorescence intensity of YO-PRO-1 and ethidium (viability and O$_2^-$) of boar sperm after incubation ± ROS generating system.........................................................46

Figure 3.7. The % of viable, moribund, and dead boar spermatozoa after incubation of fresh sperm ±ROS generating system and subsequent stained with YO-PRO-1/HE (viability and O$_2^-$) and measured by flow cytometer.......47

Figure 3.8. Ethidium fluorescence intensity (intracellular level of O$_2^-$) of viable, moribund, dead and average boar spermatozoa incubated ± ROS generating system.........................................................................................48

Figure 3.9. Flow cytometric 2-dimentional dot-plots fluorescence intensity of PI and DCF (viability and H$_2$O$_2$) of boar sperm after incubation in BTS ± ROS
Figure 3.10. The % of viable sperm after exposure to BTS ± ROS generating system and subsequently stained with YO-PRO-1/HE (viability and O$_2^{-}$) or PI/H$_2$DCFDA (viability and H$_2$O$_2$) and measured by flow cytometer.

Figure 3.11. DCF fluorescence intensity (intracellular level of H$_2$O$_2$) of viable, dead, and average boar sperm incubated in BTS ± ROS generating system.

Figure 3.12. The % of acrosome reacted boar sperm preincubated ± ROS generating system.

Figure 3.13. BODIBY581/591 C11 fluorescence intensity of viable, dead, and average boar spermatozoa incubated in BTS ± ROS generating system.

Figure 3.14. bis-BODIBY-FL C11 fluorescence intensity of viable, dead and average boar spermatozoa incubated in BTS ± ROS generating system.

Figure 4.1. Effect of preincubating boar sperm ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent capacitation.

Figure 4.2. Effect of preincubating boar sperm ± ROS generating system on intensity of tyrosine-phosphorylated proteins present during subsequent capacitation.

Figure 4.3. Effect of pH on tyrosine phosphorylation of boar sperm proteins.

Figure 5.1. Effect of preincubating boar sperm ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent capacitation.
Figure 5.2. Effect of pre-incubating boar sperm ±ROS generating system on phospho-Raf-1 proteins detected subsequent capacitation ................. 99

Figure 5.3. Effect of preincubating boar sperm ± ROS generating system on intensity of phospho-Raf proteins present during subsequent capacitation ............ 100

Figure 5.4. Effect of preincubating boar sperm ± ROS generating system on phospho-MEK1/2 proteins detected subsequent capacitation ............... 102

Figure 5.5. Effect of preincubating boar sperm ± ROS generating system on intensity of phospho-MEK1/2 proteins present subsequent capacitation ............... 103

Figure 5.6. Effect of preincubating boar sperm ± ROS generating system on phospho-ERK1/2 proteins detected subsequent capacitation ................. 105

Figure 5.7. Effect of preincubating boar sperm ± ROS generating system on intensity of phospho-ERK1/2 proteins present during subsequent capacitation ........ 106

Figure 5.8. α-tubulin protein content during capacitation of boar sperm pre-incubated ± ROS generating system and then capacitated ..................... 107

Figure 5.9. Effect of preincubating boar sperm ± ZM336372 (C-Raf inhibitor; 6μM) ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent capacitation ........................................ 108

Figure 5.10. Effect of preincubating boar sperm ± ZM336372 (C-Raf inhibitor; 6μM) ± ROS generating system on intensity of tyrosine-phosphorylated proteins present during subsequent capacitation ...................... 109

Figure 5.11. Effect of preincubating boar sperm ± U0126 (MEK1/2 inhibitor; 1μM) ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent capacitation ........................................ 111
Figure 5.12. Effect of preincubating boar sperm ± U0126 (MEK1/2 inhibitor; 1μM) ± ROS generating system on intensity of tyrosine phosphorylated proteins present during subsequent capacitation ........................................ 112

Figure 5.13. Effect of preincubating boar sperm ± U0126 (MEK1/2 inhibitor; 1μM) ± ROS generating system on intensity of tyrosine phosphorylated proteins present during subsequent capacitation in capacitating medium .................. 114

Figure 5.14. Effect of preincubating boar sperm ± ERK inhibitor (20 μM) ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent capacitation......................................................... 116

Figure 5.15. Effect of preincubating boar sperm ± ERK inhibitor (20μM) ± ROS generating system on intensity of tyrosine-phosphorylated proteins present during subsequent capacitation ......................................................... 117

Figure 5.16. Effect of preincubating boar sperm ± ERK inhibitor (20μM) ± ROS generating system on intensity of tyrosine phosphorylated proteins present during subsequent capacitation in capacitating medium .................. 118

Figure 6.1. Hypothetical mechanism by which ROS generating system impacts membrane lipid peroxidation, motility, PLA activity, acrosome reaction and modulating signal transduction of tyrosine phosphorylation ... 145

Figure A3.1. Measuring bis-BODBY-FL C11 incorporation into boar sperm ............. 172
1. Literature Review

1.1. Introduction

Artificial insemination (AI) using fresh liquid semen is standard practice in the modern swine industry. However, the use of frozen thawed boar spermatozoa in AI is very limited due to the low fertility rates achieved in comparison with fresh semen (Breininger et al., 2005). In addition to damaging the membrane and cellular functions, cooling and freezing semen is associated with the production of reactive oxygen species (ROS; Bilodeau et al., 2000; Chatterjee and Gagnon, 2001) which lead to lipid peroxidation of sperm plasma membranes (Brouwers et al., 2005), DNA damage (Lopes et al., 1998; Bennetts and Aitken, 2005; Peris et al., 2007), inhibition of sperm-oocyte fusion (Aitken et al., 1989) loss of sperm motility (de Lamirande and Gagnon, 1992; Bilodeau et al., 2002), and inducing capacitation-like changes (Satorre et al., 2007).

Boar spermatozoa are very sensitive to peroxidative damage due to the high content of unsaturated fatty acids in their plasma membranes and the low antioxidant capacity of seminal plasma (reviewed in Breininger et al., 2005). ROS are implicated in sperm capacitation, tyrosine phosphorylation (human, de Lamirande and Gagnon, 2002; O'Flaherty et al., 2006; bovine, Rivlin et al., 2004; equine, Baumber et al., 2003) and acrosome reaction (human, Luconi et al., 1998b; Liguori et al., 2005). Moreover, ROS have been associated with the components of the extracellular signal regulated kinase (ERK) family of mitogen-activated protein kinase (MAPK) pathway, which regulate the signaling of tyrosine phosphorylation during capacitation in human spermatozoa (O'Flaherty et al., 2005).
A better understanding of how ROS affect fresh and frozen-thawed boar sperm in terms of membrane integrity, motility, viability, capacitation and the acrosome reaction will help improve fertility. Knowing the precise steps in the signaling pathways that control capacitation and the acrosome reaction, is essential to that goal. Such knowledge can then be applied to improve boar sperm preservation and the swine AI industry.

1.2. Sperm morphology

The sperm is a highly polarized and motile cell that is the end product of spermatogenesis, which occurs in the seminiferous tubules within the testes. During this process extensive morphological and functional alteration occurs as the spermatogonia transform into spermatocytes (by mitosis) and spermatids (by meiosis). The round spermatids are transformed into spermatozoa by a series of progressive morphological changes called “spermiogenesis” and subsequently released to the lumen of the seminiferous tubules with a haploid number of chromosomes. (Garner and Hafez, 2000; Senger, 2003).

Most animal spermatozoa are composed of two components, the head and flagellum (Eddy, 2006). The head of the mammalian spermatozoon has a shape characteristic for each species (supported by a cytoskeleton), and contains an oval nucleus, acrosome, cytoskeletal structures, and a small amount of cytoplasm. The nucleus contains the haploid number of paternal chromosomes, and is capped anteriorly by the acrosome, which is a double walled cytoplasmic vesicle situated between the plasma membrane and the anterior of the nucleus. The acrosome contains hydrolytic enzymes such as acrosin,
hyaluronidase, zona lysine, estrases, and acid hydrolases, which are required to penetrate the cellular investments and the zona pellucida of the ovulated oocyte (Senger, 2003; Eddy, 2006).

The spermatozoa flagellum is joined to the spermatozoa head by the connecting piece and divided into midpiece, principle piece and end piece regions. The flagellum contains a central complex of microtubules forming the axoneme (Eddy, 2006). The central core of the middle piece together with the entire length of the tail comprises the axoneme. The axoneme component is composed of 9 pairs of microtubules arranged radially around two central filaments. In the middle piece this 9+2 arrangement of microtubules is surrounded by the nine outer dense fibers that are associated with the nine doublets of the axoneme. The axoneme and the associated dense fibers of the midpiece are covered by the mitochondria which are the source of energy for sperm motility (Garner and Hafez, 2000; Senger, 2003).

Even as a highly differentiated cell, the entire spermatozoan is still a single cell that is covered by a contiguous heterogeneous plasma membrane (Garner and Hafez, 2000; Eddy, 2006). This plasma membrane is composed of a phospholipid bilayer that contains integral and peripheral proteins and cholesterol (Nicholson, 1972). The plasma membrane plays a very important role in sperm function and the acquisition of fertilizing ability during epidermal maturation and interaction with the female genital tract, influencing such functions as motility (De Leeuw et al., 1991), capacitation and acrosome reaction (Stojanoff, 1988), and the binding to the oocyte (Yanagimachi, 1981). The spermatozoa
released from Sertoli cells by spermiation into the lumen of the seminiferous tubules are immotile and unable to fertilize (Williams et al., 1991); they have to undergo extensive changes and maturation both in the epididymis and the female reproductive tract within a specific time to acquire the ability to fertilize an oocyte (Yanagimachi, 1981).

1.3. Fertilization

Fertilization is the process that involves a set of complex events including species-specific interaction between egg and sperm activating a serious of events that lead to formation of a zygote (Naz and Rajesh, 2004).

Before ejaculation, spermatozoa acquire the potential for motility and fertility by the action of different secretions from epithelial cells and luminal fluids in the epididymis. At ejaculation spermatozoa become motile but are not able to fertilize an oocyte (Hafez and Hafez, 2000). After deposition in the female reproductive tract, mammalian spermatozoa migrate to the site of fertilization, the ampulla of the oviduct, and gradually undergo a serious of biochemical, physiological and membranous changes termed capacitation, which enable spermatozoa to be hyperactivated, reach and bind to the zona pellucida, undergo the acrosome reaction, penetrate the zona pellucida and fuse with the oocyte (de Lamirande et al., 1997; Visconti et al., 2002).
1.3.1. Capacitation

In order to fertilize an oocyte, Chang (1951) and Austin (1951) discovered that ejaculated spermatozoa require residence in the female reproductive tract for a specific time. During this time the female reproductive tract induces maturational and structural alterations (Baldi et al, 1996). These alterations and modifications are called capacitation.

Capacitation can be defined as a series of functional, biochemical and biophysical cellular modifications that spermatozoa undergo during their migration in the female reproductive tract. During their migration from the site of semen deposition to the site of fertilization, spermatozoa reach and bind to the zona pellucida, undergo the acrosome reaction and bind to the oolemma (Baldi et al., 1996; de Lamirande et al., 1997; Aitken, 1997). Also this process can be induced in vitro under appropriate conditions (Yanagimachi, 1994), including the use of defined media (Harrison, 1996).

During capacitation, extensive changes occur in all sperm compartments including head and flagellum; membrane, cytosol, and cytoskeleton. Some of these modifications are cholesterol efflux from the plasma membrane, re-organization of membrane lipids and proteins, increase in intracellular calcium (Ca^{2+}), bicarbonate (HCO_3^-), potassium (K^+), protein phosphorylation, increase in intracellular pH (Parrish et al., 1999; Visconti et al., 1999; Flesch and Gadella, 2000) and control of the generation of ROS (de Lamirande et al., 1997b). These molecular events are required for the subsequent induction of hyperactivation, acrosome reaction and sperm-egg fusion (Visconti et al., 2002).
As mentioned above, the migration of spermatozoa from the site of semen deposition to the site of fertilization is associated with many membrane changes that ready spermatozoa to fertilize. Decapacitation factors, which are materials coating the sperm membrane that originate from the epididymis (de Lamirande et al., 1997a; Visconti and Kopf, 1998) and seminal plasma (Cross, 1996) are removed from the sperm surface. This helps expose the sperm membrane's components to interact with capacitating agents in the female reproductive tract fluid such as serum albumin, high density lipoproteins and glycosaminoglycans (Cross, 1998). Changes in the distribution and composition of the plasma membrane lipids and phospholipids (Yanagimachi, 1994) such as cholesterol removal that decreases the membrane cholesterol:phospholipid ratio, increase sperm plasma membrane fluidity. Increasing sperm membrane fluidity and activation of ion channels (Fraser and McDermott, 1992; Okamura et al., 1993) leads to the increase of intracellular concentration of \( \text{HCO}_3^- \) and \( \text{Ca}^{2+} \) (Hoshi et al., 1990, Benoff, 1993). Also capacitation causes redistribution of membrane proteins (Cross and Overstreet, 1987).

It has been suggested that serum albumin may be responsible for the cholesterol removal from sperm plasma membrane (Benoff, 1993; Cross, 1998). Also, capacitation is associated with increasing phospholipid methylation, synthesis of phosphatidylcholine from phosphatidylethanolamine (Llanos and Meizel, 1983), and increasing levels of phosphatidylinositol and lysophosphatidylcholine (porcine; Snider and Clegg, 1975). Such changes may prepare the sperm for the acrosome reaction.
Spermatozoa transiting through the male and female reproductive tracts are exposed to variations of extracellular ion concentrations and osmolarity. In the cauda epididymis, sperm are stored in an environment that contains high K\(^+\), low Na\(^+\) and very low HCO\(_3^-\) concentrations (Setchell et al., 1994). However, the female reproductive tract exposes semen (sperm plus seminal plasma) to significantly lower K\(^+\) concentrations and significantly higher Na\(^+\) and HCO\(_3^-\) concentrations (Yanagimachi, 1994). These changes in extracellular ion concentrations are important to trigger the modulations of intracellular ion concentrations in sperm which are important to the process of capacitation through altering membrane potential of the sperm plasma membrane (Visconti et al., 2002). A rise in intracellular pH has been also reported during capacitation of bovine spermatozoa (Vredenburgh-Wilberg and Parrish, 1995), and in mammalian sperm in general an important role of HCO\(_3^-\) in capacitation has been shown. It is believed that the rise of HCO\(_3^-\) concentration during capacitation may be related to its role in stimulation of adenyl cyclase (Ac) activity (Visconti et al., 1995ab).

Ca\(^{2+}\) is another very important ion for sperm capacitation. Modification of intracellular concentrations of this ion is arguably the most important biochemical event during capacitation. Extracellular Ca\(^{2+}\) is necessary for the completion of spermatozoa capacitation, and the increase of intracellular Ca\(^{2+}\) is required for sperm hyperactive motility (Visconti et al., 1995a; Luconci et al., 1996). Various mechanisms are known to regulate Ca\(^{2+}\) concentration in spermatozoa such as, Ca\(^{2+}\)-ATPase (Fraser and McDermtt, 1992), Ca\(^{2+}\)/H\(^+\) exchange system, Na\(^+\)/Ca\(^{2+}\) antiport in the plasma membrane (Fraser, 1995), and by intracellular Ca\(^{2+}\) stores (Blackmore, 1992).
The lower portion of the oviduct (isthmus) serves as a reservoir for sperm (Hunter et al., 1991). In addition, the sperm attachment to the oviduct extends their lifespan (Ellington et al., 1991; Suarez et al., 1998). During this time sperm develop the ability to undergo hyperactivation, acrosome reaction and fertilization of the oocyte (Arnoult et al., 1999; Fazeli et al., 1999). Sperm interaction and binding to the oviductal mucosal epithelium is mediated by carbohydrate; there is a carbohydrate-binding molecule on the surface of the sperm (Suarez, 2002), such as the PDC-109 or BSP protein in cattle (Ignotz et al., 2001). The attachment of bull spermatozoa to the epithelial cells of the oviduct may stimulate important protein synthesis (Ellington et al., 1993), whose secretion is suggested to be important for capacitation. Also, Ca\(^{2+}\) dependent bovine oviductal proteins were found to bind strongly to the spermatozoa and were associated with long term maintenance of sperm motility and cell membrane stability (Lapointe and Sirard, 1996).

1.3.2. Hyperactivation

In mammalian oviducts, sperm become extremely active (hyperactivated) after ovulation occurs. Capacitated sperm display a distinct motility pattern called hyperactivation, which is characterized by a vigorous swimming pattern and high flagellar bend amplitude and beat asymmetry (Katz and Yanagimachi, 1980; Yanagimachi, 1994; Baldi et al., 1996). Hyperactivation in the female reproductive tract can be stimulated by different physiological materials, such as hormones and ions (Hunter et al., 1991). The oocyte enters the oviduct accompanied by the cumulus oophorus and follicular fluid. These have been shown to influence sperm hyperactive motility perhaps by the release of
progesterone, which is an active factor enhancing hyperactivation (Suarez, 2002). Calcium ions have a major regulatory role in sperm hyperactivation by interacting with the axoneme of the flagellum to switch on hyperactivation (Suarez and Ho, 2003).

Sperm hyperactivation has multiple functions, aiding spermatozoal detachment from the epithelium of the oviduct, its approach toward the egg (Suarez and Dai, 1992), and penetration of the mucous substances and finally the zona pellucida surrounding the oocyte (Stauss et al., 1995; Suarez, 2002). In addition, hyperactivation is considered as one of the first events that indicate capacitation is in progress (Yanagimachi, 1994).

1.3.3. Acrosome reaction, sperm-zona pellucida binding and penetration

The sperm acrosome is a structure forming a cap over the anterior region of the nucleus. This structure contains many hydrolytic enzymes (Yanagimachi, 1994). Capacitated and hyperactivated spermatozoa acquire the ability to undergo the acrosome reaction in response to some physiological stimuli such as the attachment to the zona pellucida proteins (particularly ZP3; Senger, 2003; Florman and Ducibella, 2006), and exposure to the high concentrations of progesterone found during capacitation (Suarez and Ho, 2003).

The acrosome reaction (AR) is an exocytotic (structural and biochemical) event where the outer acrosomal membrane fuses with the head plasma membrane. The P25b protein in the bovine sperm acrosome plays a role in sperm zona pellucida interaction (Sullivan, 2004). This protein is synthesized by the epididymal epithelium and is added to the sperm surface during epididymal transit (Parent et al., 1999). Low amounts of P25b in bull semen are associated with low fertility (Sullivan, 2004).
The sperm AR is necessary to penetrate the ZP and fertilize an oocyte when both the sperm and the oocyte reach the site of fertilization in the oviduct. In mammalian sperm, the equatorial segment of the sperm head fuses with the zona (Wassarman, 1992).

Sperm have to pass through two layers of egg-coatings before they can contact the oocyte directly. The first outermost layer is the cumulus oophorus, which consists of granulosa cells (Dandekar et al., 1991), and the second is the zona pellucida, which consists of the glycoproteins ZP1, ZP2, and ZP3 (Green, 1997). ZP1 is the zona's protein backbone held together by inter-molecular disulphide bonds. Extending from it, and attached to it, are ZP2 and ZP3 subunits that are rich in terminal carbohydrate (Green, 1997). Lytic enzymes on the sperm surface, such as hyaluronidase (Lin et al., 1994) and acrosomal acrosin may facilitate the passage of sperm through the cumulus and ZP (Dandekar et al., 1992) by dissolution of chemical bonds; hyperactivated motility provides physical power (Suarez and Ho, 2003).

1.4. Signal transductions regulating sperm capacitation, tyrosine phosphorylation, hyperactivation and acrosome reaction

The process of sperm capacitation is regulated by several specific signal transduction pathways (Visconti et al., 1998; O'Flaherty et al., 2006ab; de Lamirand and O'Flaherty, 2008) that have been studied in a variety of species, including murine (Visconti et al., 1995b), human (Leclerc et al., 1996; O'Flaherty et al., 2006ab; de Lamirande and O'Flaherty, 2008), bovine (Galantino-Homer et al., 1997) and porcine (Kalab et al., 1998; Flesch et al., 2000; Bravo et al., 2005). These pathways include protein kinase A (PKA), its substrates, and the cAMP/PKA-dependent tyrosine phosphorylation of fibrous sheath
proteins (Visconti et al., 1995b; Leclerc et al., 1996; Visconti et al., 1997; Aitken et al., 1998; Harrison, 2004; O'Flaherty et al., 2004), protein kinase C (PKC) (Thundathil et al., 2002), protein tyrosine kinases (PTK) (Visconti et al., 1995b; Leclerc et al., 1996; Visconti et al., 1997; Aitken et al., 1998; Harrison, 2004; O'Flaherty et al., 2004), and components of the The extracellular signal-regulated kinase (ERK) family of mitogen-activated protein kinase (MAPK) pathway (Thundathil et al., 2002; de Lamirande and Gagnon, 2002; O'Flaherty et al., 2005, 2006ab; de Lamirande and O'Flaherty, 2008).

During capacitation, these pathways regulate the serine/threonine and tyrosine phosphorylation of spermatozoa proteins (Visconti and Kopf, 1998; Urner and Sakkas, 2003; Naz and Rajesh, 2004). Protein phosphorylation is very important in regulating numerous cellular activities, and tyrosine phosphorylation is very important as a signal transduction pathway indicator in a cell (Naz and Rajesh, 2004).

The protein phosphorylation/dephosphorylation is regulated by protein kinases and phosphatases (Urner and Sakkas, 2003; Naz and Rajesh, 2004). Protein kinases, such as serine/threonine (Ser/Thr) kinases including cAMP-dependent PKA, Calcium/calmodulin-dependent kinase (CaMK), and PKC, control protein phosphorylation by the addition of a phosphate group on the amino acids Ser or Thr of a protein substrate (reviewed in Tardif et al., 2001; Urner and Sakkas, 2003; Naz and Rajesh, 2004). The protein Ser/Thr phosphorylation during sperm capacitation by the activation of cAMP-dependent PKA kinase, leads to protein tyrosine phosphorylation through the activation of PTK, inactivation of protein tyrosine phosphatase (PTP), or both (Visconti and Kopf,
1998; Breitbart, 2002; Urner and Sakkas, 2003; Naz and Rajesh, 2004). The ERK component ERK1/2, which is also a Ser/Thr kinase, is present in human spermatozoa (de Lamirande and Gagnon, 2002).

The PTKs include two classes, the receptor tyrosine kinases (RTKs) and non-receptor protein tyrosine kinases (non-RTKs). The RTKs are transmembrane proteins presenting an extracellular ligand binding domain and an intracellular tyrosine kinase domain that phosphorylate proteins on tyrosine residues (Tyr; Urner and Sakkas, 2003; Naz and Rajesh, 2004). The PTK TK-32 identified in pig spermatozoa is activated concomitant with capacitation (Tardif et al., 2003).

In addition to the RTKs and non-RTKs, there are dual-specificity protein kinases able to phosphorylate both Tyr and Ser/Thr residues (Letwin et al., 1992) such as MEK1/2 kinases in the ERK pathway (Windmann et al., 1999). These kinases have been found in mammalian spermatozoa, and they mediate the signaling cascades regulating such sperm functions as motility and fertilization (Rotem et al., 1990; Visconti et al., 1997; Ignotz and Suarez, 2005; Marin-Briggiler et al., 2005) and zona pellucida recognition (Leyton and Saling, 1989; Naz and Ahmed, 1994., Burks et al., 1995).

The protein tyrosine kinases are very important mediators of sperm tyrosine phosphorylation that are associated with hyperactivated motility, zona pellucida binding and acrosome reaction (Urner and Sakkas, 2003; Naz and Rajesh, 2004). The increase in protein tyrosine phosphorylation during capacitation has been shown to be regulated by a
cAMP-dependent pathway involving PKA in sperm of various species including mouse (Visconti et al., 1995b), hamster (Visconti et al., 1999), boar (Kalab et al., 1998; Bravo et al., 2005), bull (Galantino-Homer et al., 1997, 2004), stallion (Pommer et al., 2003), monkey (Mahony and Gwathmey, 1999) and human (Leclerc et al., 1996., Osheroff et al., 1999).

The Ac/ cAMP/PKA pathway induces protein tyrosine phosphorylation as a late event of the process of sperm capacitation (Visconti et al., 1995b; Brewis et al., 2005) in the presence of bovine serum albumin (BSA), \(\text{Ca}^{2+}\) and \(\text{HCO}_3^-\) in defined capacitating medium (Visconti et al., 1995b). BSA acts as a sink to promote cholesterol removal from the plasma membrane and increase its fluidity, while the entry of \(\text{HCO}_3^-\) into the sperm cell activates soluble adenylyl cyclase (sAc), and results in increasing cAMP levels which in turn activates PKA (Chen et al., 2000; Visconti et al., 2002). \(\text{HCO}_3^-\) influx has been associated with an increase in intracellular pH (Baldi et al., 2002; Naz and Rajesh, 2004) that is observed during capacitation, along with regulation of cAMP levels, reversible change in the architecture of plasma membrane lipids and phospholipids, and hyperpolarization of the sperm plasma membrane (Naz and Rajesh, 2004). These changes increase in the membrane fluidity and intracellular \(\text{Ca}^{2+}\) and alters membrane architecture occurs concomitantly with the increase in tyrosine phosphorylation (Brewis et al., 2005). An increase in the concentration of \(\text{Ca}^{2+}\) during capacitation has been demonstrated in several mammalian species (Harrison et al., 1993). \(\text{Ca}^{2+}\) influx into sperm during capacitation maybe associated with the activation of Ac (Tash and Means, 1983), PKC and CaMK (Harayama, 2003).
In boar spermatozoa, HCO$_3^-$ and Ca$^{2+}$ are the major players in the capacitation process and in the signaling pathway of Ac/cAMP/PKA (Gadella et al., 2000; Harrison et al., 2000). HCO$_3^-$ activates sperm Ac (Okamura et al., 1991), increases sperm plasma membrane fluidity (Wolf et al., 1986) and stimulates Ca$^{2+}$ influx. In boar spermatozoa, phospholipid scrambling and cholesterol ejection from the membrane are downstream events from the Ac/cAMP/PKA pathway (Flesch et al., 2001). A late event in capacitation is the phosphorylation of tyrosine residues on membrane proteins (Flesch et al., 1999).

MAPKs or ERKs exist in all eukaryotic cells and are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence (Windmann et al., 1999; Kolch, 2000). The ERK activity is regulated by a phosphorylation cascade initially triggered by the GTP-binding protein Ras or PKC. The MAPK-kinase-kinase (Raf), which is the first enzyme of this cascade, phosphorylates MAPK-kinases (MEK1/2) on Ser/Thr (dual-specificity protein kinase), which in turn phosphorylates MAPK (ERK1/2) on Thr and Tyr residues (Windmann et al., 1999; Kolch, 2000). The ERK1/2 is Ser/Thr kinase, involved in human sperm motility, capacitation (de Lamirande and Gagnon, 2002) and acrosome reaction (Luconi et al., 1998). ERK1/2 may phosphorylate proteins that influence protein tyrosine phosphorylation indirectly (de Lamirande and Gagnon, 2002).

During capacitation the cAMP/PKA pathway phosphorylates several sperm proteins on their tyrosine residues (Visconti et al., 1995b; Galantino-Homer, et al., 1997). Although
sperm capacitation is associated with some flagellum tyrosine phosphorylated proteins that are involved in hyperactivation (Si and Okuno, 1999; Naaby-Hansen et al., 2002), studies have shown one can occur without the other in some species under certain conditions (Ho and Suarez, 2001). For instance, during hamster sperm capacitation, protein tyrosine phosphorylations can occur hours before the onset of hyperactivated motility (Si and Okuno, 1999).

In boar spermatozoa, activating PKA by incubating spermatozoa with Sp-5, 6-dichloro-1-β-dribofuranosyl-benzimidazole-3',5'-monophosphorothioate (cBiMPS, a cell permeable cAMP analog) stimulated PKCs in the connecting piece and led to hyperactivation of flagellar movement (Harayama and Miyake, 2006). Moreover, the cBiMPS-induced hyperactivation was suppressed by specific PKC inhibitors suggesting that cAMP-PKA signaling controls calcium-sensitive PKCs involved in hyperactivation of flagellar movement in boar spermatozoa. In addition, cAMP may have a unique role as the up-regulator of PKCs during the expression of fertilizing ability in boar spermatozoa (Harayama and Miyake, 2006). Thus, the relationship of sperm capacitation, tyrosine phosphorylation and hyperactivation could be species-specific.

Capacitated spermatozoa have the ability to undergo the process of acrosome reaction and subsequently fuse to and fertilize an oocyte. Ca$^{2+}$, progesterone, and binding to the ZP3, are the major factors initiating the signal transductions associated with the acrosome reaction.
The attachment of the sperm to the zona-enclosed oocyte at ZP3, coupled with progesterone, may trigger the signaling of the acrosome reaction. The binding of ZP3 to its receptors on the surface of the sperm plasma membrane leads to aggregation and tyrosine phosphorylation of sperm proteins. Progesterone can bind to its receptors on the sperm membrane (Flesch and Gadella, 2000). This binding stimulates Ca\(^{2+}\) influx (Shah et al., 2003) and tyrosine phosphorylation of sperm proteins (Calogero et al., 2000), causing hyperactivation (Calogero et al., 1996) with an increase in cAMP levels (Parinaud and Milhet, 1996). The tyrosine kinase-associated progesterone receptor is responsible for the effect of progesterone on hyperactivated motility and the acrosome reaction (Shah et al., 2003).

Progesterone has been associated with increasing plasma membrane fluidity, capacitation and tyrosine phosphorylation of human spermatozoa (Naz and Rajesh, 2004). Also ZP3 and progesterone binding to the sperm membrane increase pH via activation of G proteins and depolarization of the plasma membrane potential (Flesch and Gadella, 2000). The direct effect of increasing intracellular pH and depolarization of the plasma membrane is increasing the entry of Ca\(^{2+}\) via a T-type voltage dependent calcium channel. Ca\(^{2+}\) may play a role in the fusion events in the sperm membrane (Watson et al., 1995), possibly through Ca\(^{2+}\)-dependent activation of phospholipase C (PLC) which has been translocated to the sperm membrane during capacitation. This enzyme cleaves phosphatidylinositol-4, 5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP3). Increasing intracellular calcium also activates phospholipase A\(_2\) (PLA\(_2\)) which increases the level of lysophosphatidylcholine (LPC) and free fatty acids
by degrading phosphatidylcholine. The combination of increasing DAG, FFA, and LPC levels activates PKC which, in the presence of high concentrations of calcium, facilitates fusion of the plasma membrane with the underlying acrosomal membrane and eventually the release of acrosomal enzymes (Watson et al., 1995; Flesch and Gadella, 2000).

The AR allows the release of a variety of enzymes such as acrosine (hydrolyses zona proteins), and proacrosine which has strong affinity for the zona as the acrosomal reaction proceeds. The hyperactive motility helps to push the sperm through the zona (Suarez and Ho, 2003). When the sperm completely penetrates the zona and reaches the perivitelline space, it settles into a bed of microvilli formed from the oocyte plasma membrane. Following that, the plasma membrane of the oocyte fuses with the membrane of the sperm equatorial segment and the fertilizing spermatozoon is engulfed (McAvey et al., 2002; Senger, 2003).

After fusion, the oocyte undergoes a serious of changes, such as, the cortical reaction, which enables the cortical granules to move to the periphery of the oocyte cytoplasm, undergo exocytosis and release their contents (mucopolysaccharides, proteases activator, plasminogen activator, acid phosphates, and peroxidase) into the perivitelline space. This process leads to the zona block that prevents polyspermy. Finally, after the sperm nucleus has entered the cytoplasm of the egg, it becomes the male pronucleus (Senger, 2003; Stein et al., 2004).
1.5. Cryopreservation of boar spermatozoa and artificial insemination

Ejaculated spermatozoa are catabolic cells. Their viability in vitro at normal body temperature is limited to a few hours for most species, while in the oviduct sperm can remain viable for one to a few days in most mammalian species (Foot and Parks, 1993).

Sperm viability in vitro can be extended for several hours by diluting sperm with a variety of physiological media. However, long-term sperm preservation involves cooling and freezing in special media. This process includes steps which alter sperm viability and fertilizing ability, such as cooling, cellular dehydration, freezing and thawing (Foot and Parks, 1993).

The process of sperm cryopreservation alters sperm membrane integrity, such as, changing membranes’ sulfhydryl group proteins (bull, Chatterjee et al., 2001; boar, Gadea et al., 2005), losing transmembrane proteins in bull sperm (Ollero et al., 1998), a decrease in sperm content of antioxidants such as superoxide dismutase (bull, Bilodeau et al., 2000) and glutathione (bull, Bilodeau et al., 2000; boar, Gadea et al., 2005), generation of ROS (Alvarez and Storey 1992; Bilodeau et al., 2000) and damage to membrane lipids (Maldjian et al., 2005) including lipid peroxidation (Brouwers et al., 2005; Neild et al., 2005). Cryopreservation alters sperm capacitation (Bailey et al., 2000; Mederios et al., 2002; Guthrie and Welch, 2005) and acrosome reaction (Guthrie and Welch, 2005; Maldjian et al., 2005), decreases sperm life span, interferes with their ability to interact with the female reproductive tract and reduces their fertilizing ability (Bailey et al., 2000; Mederios et al., 2002). In addition, cryopreservation causes damage to the mitochondria and to the sperm tail (Wooley and Richardson, 1978).
Unlike the cryopreserved semen widely used in bovine breeding, frozen-thawed boar semen is not generally used for artificial insemination programs. The poor post-thaw survival, low farrowing rates and smaller litter sizes obtained after insemination with frozen thawed semen compared with fresh or liquid-preserved semen makes cryopreserved semen insufficiently productive for the swine industry (Johnson et al, 2000; Cerolini et al., 2001). Sperm cryopreservation is associated with cold shock, osmotic stress, intracellular ice formation and, premature onset of capacitation-like changes (Watson, 2000; Mederios et al., 2002). Boar spermatozoa are very susceptible to cold shock and to peroxidative damage because of their high plasma membrane content of unsaturated fatty acids and low antioxidant capacity of seminal plasma (reviewed in Breininger et al., 2005).

1.5.1. Plasma membrane changes
In order to fertilize an oocyte, a sperm must have an intact and functional plasma membrane. However, plasma membrane destabilization during cooling, freezing and thawing causes functional alteration to the sperm plasma membrane. Membranes are the primary site for structural and functional chilling injury in sperm (Canvin and Buhr, 1989; Hammerstedt et al., 1990; Watson, 1995). Membrane chilling injury is dependent on the biophysical properties of the membrane. Phospholipids with their fatty acids are the major structural components of biological membranes and affect both biophysical and function properties of the cell (Stubbs and Smith, 1984). Freezing and thawing alter osmolarity and water volume of sperm cells, which induces mechanical stress on the cell membrane (Bailey et al., 2000; Watson, 2000, Holt, 2000; Mederios et al., 2002; Meyers, 2005). Membrane destabilization due to mechanical stress occurs when the membrane
undergoes phase transition, from the fluid phase to the gel phase, when temperature is decreased (Buhr et al., 1989; Purdy and Graham, 2004). One consequence of the membrane disruption is the loss of the intracellular components, such as metabolic enzymes and ATP, which eventually leads to cell death (Graham and Moce, 2005).

The processes of cooling, freezing and thawing change sperm membrane lipid composition, which is an important structural factor in the resistance to thermal stress and organization of sperm plasma membrane structure (Hinkovska-Galchera et al., 1989; Buhr et al., 1994). Alteration of sperm plasma membrane lipid composition disturbs normal sperm membrane functions by affecting the specific lipid-lipid and lipid-protein interactions in the plasma membranes (Bailey et al., 2000).

The cholesterol level and the ratio of unsaturated/saturated fatty acids present in the plasma membrane are highly associated with sperm resistance to cold shock and phase transition. Spermatozoa with low levels of cholesterol and high ratio of unsaturated/saturated membrane fatty acids, such as boar, bull and ram sperm, are more sensitive to cold temperature than sperm with high levels of cholesterol and low ratio of unsaturated/saturated membrane fatty acids, such as, rabbit, human and rooster sperm, (Bailey et al., 2000; Mederios et al., 2002).

Also sperm membrane content of cholesterol and unsaturated/saturated fatty acids are associated with sperm membrane fluidity (Watson, 1995) and permeability (Rodolph and Crowe, 1985).
1.5.2. Capacitation-like changes phenomenon

Exposing spermatozoa to low temperature shortens their capacitation time, relating to chilling-induced changes in membrane lipid architecture, membrane permeability, and to reduced efficiency in homeostatic enzymes (Watson, 1995, 2000; Melissa et al., 2003). These changes resemble capacitation, and reduce sperm viability, alter sperm motility and the natural interaction between spermatozoa and epithelial cells in the female reproductive tract. They also affect the ability to regulate Ca\(^{2+}\) in ovine (Robertson and Watson, 1986) and bovine spermatozoa (Bailey and Buhr, 1994) possibly by altering the ability of sperm membrane to maintain Na\(^{+}\)/K\(^{+}\) ATPase activity and can lead to lethal increases in intracellular Ca\(^{2+}\)-concentration (Zhao and Buhr, 1995).

The phenomenon of cryopreservation-induced capacitation-like changes is a consequence of increasing intracellular calcium level during cryopreservation. The increased intracellular calcium level triggers the internal cascade that is associated with capacitation (Holt, 2000; Watson, 2000; Cormier and Bailey, 2003). Freezing and thawing bull spermatozoa are associated with the presence of tyrosine phosphorylated proteins (Bailey et al., 2000). It has been suggested, that the mechanism by which protein tyrosine phosphorylation is regulated in cryopreserved bull sperm is different from the regulatory mechanisms that regulate protein tyrosine phosphorylation of heparin-sensitive, incubation-dependent changes in fresh sperm. While two phosphotyrosine-containing proteins (56-PP and 114-PP) appear by incubating fresh spermatozoa for 5 hours with heparin, there was only one (56-PP) in cryopreserved spermatozoa at 0 hour post thaw from the same bull ejaculate (Cormier and Bailey, 2003). This could be due to
the high concentration of intracellular calcium in thawed sperm triggering capacitation signal transduction pathways and inducing protein tyrosine phosphorylation (Cormier and Bailey, 2003).

1.5.3. Reactive oxygen species generation and their modifications and damaging actions:

The process of cryopreservation induces the formation of Reactive Oxygen Species (ROS; Alvares and Storey, 1992) in different species (bovine, Bilodeau et al., 2000; equine, Ball et al., 2001). Excessive formation of ROS by spermatozoa has been associated with plasma membrane lipid peroxidation (Brouwers et al., 2005), DNA damage (Lopes et al., 1998; Bennett and Aitken, 2005), inhibition of sperm-oocyte fusion (Aitken et al., 1989), and the loss of sperm motility (de Lamirande and Gagnon, 1992; Bilodeau et al., 2002).

ROS disrupt sperm function by the peroxidation of the polyunsaturated fatty acids present in the sperm plasma membrane (Aitken, 1994, 1995). Lipid peroxidation damages membrane phospholipids, disrupting membrane structure and function (Sevanian et al., 1988; Aitken, 1995). Peroxidation-induced cytotoxicity may be exacerbated by elevated intracellular Ca$^{2+}$ resulting from increased Ca$^{2+}$ permeability. The binding of Ca$^{2+}$ to membrane phospholipids decreases lipid bilayer fluidity, and initiates structural changes to non-bilayer arrangements such as lipid phase separation, formation of heterogeneous structural domains, and membrane fusion (reviewed in Salgo et al., 1993).
Losing membrane fluidity as a consequence of lipid peroxidation also disrupts membrane-bound enzymes, such as Ca\(^{2+}\)-Mg\(^{2+}\) ATPase, which leads to a loss of their capacity to regulate the internal concentrations of ions that control such sperm functions as motility (Aitken, 1995).

Increasing the extra or the intracellular PLA\(_2\) activity as a result of the coupled action of lipid peroxidation and Ca\(^{2+}\) on membrane phospholipids alters membrane structure and initiates membrane degradation (reviewed in Salgo et al., 1993). In addition, cell membrane lysis and cell death could occur if this enzyme is extensively activated (Shier, 1979).

DNA is one of the cell components that is very susceptible to ROS damage. ROS, particularly HO', attacks nitrogenous bases and the sugar phosphate backbone, causing hydroxylation, ring opening, fragmentation (Buxton et al., 1988), transitory cross-linking of protein-DNA (Oleinick et al., 1986), and strand breaks that may be mutagenic or lethal for the cell (Friedberg et al., 2006).

In addition to lipid peroxidation and DNA damage, ROS inhibit sperm-oocyte fusion. Aitken and Clarkson (1987) reported that human spermatozoa subjected to lipid peroxidation developed refractoriness to calcium signals and were inhibited from fusing with oocytes in the presence of the calcium ionophore A23187. Lipid peroxidation changed plasma membrane properties, which led to a kind of refractoriness. The mechanism responsible for the impairment of sperm-oocyte fusion is believed to be the
reduction of plasma membrane fluidity and disruptions of membrane bound enzyme activities, like ion channels (Aitken et al., 1989).

ROS also impairs sperm motility. Baumber et al (2000) suggested that hydrogen peroxide (H$_2$O$_2$) causes perturbations in important biochemical functions, including formation of oxidized intracellular sulfhydryl, decreasing ATP levels and depressing glycolytic flux. Another study (de Lamirand and Gagnon, 1992) showed that ROS inhibited sperm motility by depleting ATP and thereby decreasing phosphorylation of axonemal proteins required for sperm motility. ROS also inhibit one or more enzymes of oxidative phosphorylation and glycolysis, limiting ATP generation by sperm. Lipid peroxidation products, such as malondialdehyde and 4-hydroxynonenal (4HN), could contribute to the loss of sperm motility. Peroxidation products have the ability to inhibit cellular enzyme functions, anaerobic glycolysis, DNA, RNA and protein synthesis (Comporti, 1989).

1.6. Reactive oxygen species and sperm function

ROS are reduced forms of oxygen and their reaction products with other molecules, such as superoxide anion (O$_2^-$), H$_2$O$_2$, HO, and peroxyl (ROO·). ROS can be highly chemically reactive free radicals, due to the unpaired parallel spin electrons they contain (Halliwell and Gutteridge, 1989; reviewed in Ford, 2004).

ROS are generated in aerobic biological systems as byproducts during metabolic activity (Halliwell and Gutteridge, 1989). Incubating spermatozoa under aerobic conditions can generate such ROS as O$_2^-$ (Aitken and Clarkson, 1987; Alvarez et al., 1987) which
dismutates to $H_2O_2$ and is not very harmful, because it has low reactivity and a short, 1$\mu$s, half life. However, the more stable $H_2O_2$ (half-live 1ms) also has the ability to diffuse through cellular membranes because it is uncharged, unlike $O_2^{--}$. Thus $H_2O_2$ has higher oxidant potential (Halliwell and Gutteridge, 1989).

The extremely reactive HO can be formed through at least two reactions. These are:
1) between $O_2^{--}$ and $H_2O_2$ (Hrber-Weiss reaction):

$$H_2O_2 + O_2^{--} = HO + OH^- + O_2$$

2) between $H_2O_2$ and ferrous ions (Fenton-type reaction):

$$H_2O_2 + Fe^{2+} = HO + OH^- + Fe^{3+}.$$ 

This HO free radical has an extremely short life (1ns), reacts with all cell components and has highly toxic effects (Halliwell and Gutteridge, 1989).

High concentrations of ROS, mainly $H_2O_2$, were shown to have detrimental effects on human (Aitken and Clarkson, 1987; Alvarez et al., 1987; de Lamirande and Gagnon, 1995), and bull spermatozoa (Rivlin et al, 2004). However, when produced in very low and controlled concentrations, they regulate cell signalling and functions (Ford, 2004).

1.6.1. Capacitation, tyrosine phosphorylation and acrosome reaction

The binding of different ligands, such as cytokines, growth factors, and hormones to the cell membrane, generate ROS in most mammalian species (Meier et al., 1989; Lo and Cruz, 1995., Sundaresan et al., 1995). In spermatozoa, low and controlled concentrations of ROS have physiological roles in inducing capacitation, tyrosine phosphorylation and
the acrosome reaction. Sperm capacitation is an oxidative process associated with the production of small amounts of ROS such as O$_2^{-}$, H$_2$O$_2$, and NO' (O’Flaherty et al., 2006a) that may promote signal transduction pathways associated with capacitation. ROS at very low concentrations regulate protein tyrosine phosphorylation of spermatozoa (Ford, 2004) through cAMP/PKA and the ERK pathways as two signaling mechanisms acting independently. In human spermatozoa, ROS contribute to the modulation of protein tyrosine phosphorylation required to achieve capacitation (O’Flaherty and de Lamirande, 2005).

H$_2$O$_2$ induces cellular tyrosine phosphorylation in endothelial cells (Natarajan et al., 1998), and ROS at physiological concentrations may be natural initiators of capacitation (Ford, 2004). In human (de Lamirande et al., 1997b), equine (Baumber et al., 2003), and bull (Rivlin et al., 2004) spermatozoa, ROS at physiological concentrations, in concert with such factors as HCO$_3^-$, loss of membrane cholesterol and increasing intracellular Ca$^{2+}$, increase intracellular O$_2^{-}$, activating Ac (rat sperm; Lewis and Aitken, 2001), and thereby increasing cAMP concentrations. Increased cAMP activates PKA, activating tyrosine kinases and inhibiting tyrosine phosphatases. H$_2$O$_2$ can directly activate the kinase and inhibit the phosphatases, and indeed, H$_2$O$_2$ can replace HCO$_3^-$ in activating the cyclase in bull sperm (Rivlin et al., 2004). Lipid peroxidation resulting from this low concentration of ROS promoted sperm binding to the zona pellucida (de Lamirande et al., 1997b). ROS have been associated with the components of the ERK in activation of tyrosine phosphorylation during capacitation in human spermatozoa (O’Flaherty et al., 2006).
2. Research Objectives

ROS generation during sperm cryopreservation is associated with damage that impairs sperm function and fertilizing ability by interaction with various, species-specific signalling pathways. The mechanism(s) of action in sperm are not well understood, and knowledge in boar spermatozoa is particularly lacking. This research hypothesised that ROS impacts the functions of frozen thawed and fresh boar spermatozoa through the signalling pathway of the mitogen-activated protein kinase family. Specific hypotheses were:

1. Cryopreservation of boar spermatozoa, and exposing fresh boar spermatozoa to Xanthine/Xanthine Oxidase (XA/XO) ROS generating system, generate ROS, induce membrane lipid peroxidation and phospholipase A (PLA) activity, and impair such whole sperm functions as motility, viability, and onset of the acrosome reaction.

2. ROS modulate signal transduction during capacitation of fresh boar sperm.

3. The extracellular signal-regulated kinases (ERK) family of mitogen-activated protein kinase (MAPK) pathway are associated with capacitation and tyrosine phosphorylation of fresh boar spermatozoa and ROS modulate the signal transduction of this pathway during sperm capacitation.

The experiments designed to test these hypotheses had specific goals:

1. To evaluate the relative intracellular levels of O$_2^-$ and H$_2$O$_2$ in fresh and frozen-thawed boar sperm

2. To determine how generating ROS in boar spermatozoa would affect sperm function
(motility, viability and acrosome integrity), sperm intracellular levels of $\text{O}_2^{-}$ and $\text{H}_2\text{O}_2$, lipid peroxidation, and PLA activity.

3. To determine how exposure to an ROS generating system affects tyrosine phosphorylation in boar spermatozoa incubated in capacitating or non-capacitating media.

4. To determine:
   a) the presence or absence of the phosphorylated forms of ERK pathway components (Raf, MEK1/2, and ERK1/2) in fresh boar spermatozoa
   b) the impact of ROS on these elements.
   c) the impact of ROS on tyrosine phosphorylation of capacitated and uncapacitated boar spermatozoa previously exposed to the inhibitors of Raf, MEK1 and 2, and ERK1/2.

This research was designed to increase the knowledge of ROS involvement in capacitation signalling pathways in boar spermatozoa and in cryodamage of boar spermatozoa.
3. Reactive oxygen species and boar sperm function

3.1. Abstract

Boar spermatozoa are very susceptible to Reactive Oxygen Species (ROS) due to their high content of polyunsaturated fatty acids (PUFAs). This study hypothesized that cryopreservation of boar spermatozoa, and exposing fresh boar spermatozoa to Xanthine/Xanthine Oxidase (XA/XO) ROS generating system, would generate ROS, induce membrane lipid peroxidation and phospholipase A (PLA) activity, and impair such whole sperm functions as motility, viability, and onset of the acrosome reaction. Boar spermatozoa content of superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) were assessed by flow cytometry using hydroethidine (HE) and 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) respectively; sperm lipid peroxidation and PLA activity were determined by flow cytometry by labelling sperm with BODIPY 581/591 C11 and bis-BODIPY FL C11 respectively. Cryopreservation of intact sperm significantly reduced total and progressive sperm motility and viability of thawed sperm in comparison with fresh sperm. Fresh viable sperm produced more O$_2^-$ (P<0.0001) than the viable frozen-thawed boar spermatozoa; there was no difference in H$_2$O$_2$ intracellular levels. Fresh boar spermatozoa exposed to the ROS generator for 30 minutes had more intracellular level of H$_2$O$_2$ than did control sperm (P<0.0003), both averaged over the whole population (total; viable plus dead sperm) and in viable sperm alone. The ROS generating system similarly induced a greater percentage of acrosome reaction under capacitating conditions and increased lipid peroxidation in the viable, dead and average sperm populations, but did not affect sperm intracellular production of O$_2^-$$. While PLA activity in viable sperm was unaffected by the ROS generating system, dead sperm had
higher PLA activity (P<0.0002). The XA/XO ROS generating system completely inhibited sperm motility (P<0.001), but did not affect (P>0.05) sperm viability.

In summary, H$_2$O$_2$ rapidly increases in boar sperm exposed to the XA/XO ROS generating system, and sperm simultaneously become immotile, undergo the acrosome reaction and there is extensive lipid peroxidation. This generation of ROS does not affect sperm viability or PLA activity. Cryopreservation decreased sperm intracellular level of O$_2$~$^-$~ and had no effect on H$_2$O$_2$ even although frozen-thawed sperm had significantly poorer sperm motility and viability. Therefore H$_2$O$_2$ is the major free radical mediating the damage of boar spermatozoa through the XA/XO ROS generating system. Boar sperm motility, acrosome integrity and lipid peroxidation are more sensitive indicators of oxidative stress than viability and PLA activity.

3.2. Introduction

Sperm cryopreservation is not common in swine artificial insemination programs. Frozen thawed boar spermatozoa have poor fertility in comparison with fresh or cooled semen (Johnson et al, 2000), for reasons not yet completely understood. Boar spermatozoa are susceptible to cold shock (White, 1993), possibly relating to the lipid composition of the membrane which contains a high concentration of PUFA (White, 1993; Cerolini et al., 2000). Sperm cryopreservation significantly increases ROS production (Alvares and Storey, 1992) in different species (bovine, Bilodeau et al., 2000; equine, Ball et al., 2001). ROS have dual effects on sperm function. Low concentrations are beneficial in inducing sperm capacitation (Leclerc et al., 1997), hyperactivation (de Lamirande and
Gagnon, 1993a), acrosome integrity (Aitken et al., 1995) and sperm-oocyte fusion (Aitken et al., 1989). However, excessive amounts of ROS damage DNA (Lopes et al., 1998; Bennett and Aitken, 2005), inhibit sperm-oocyte fusion (Aitken et al., 1989), and reduce motility (human, deLamirande and Gagnon, 1992; equine, Baumber et al., 2000; porcine, Guthrie and Welch, 2006).

DNA is very susceptible to ROS damage, as oxidants like HO' attack nitrogenous bases and the sugar phosphate backbone, causing hydroxylation, ring opening, fragmentation (Buxton et al., 1988), transitory cross-linking of protein-DNA (Oleinick et al., 1986), and strand breaks that may be mutagenic or lethal for the cell (Friedberg et al., 2006), and certainly decrease fertilization rates (Jiang et al., 2007). ROS also cause sperm to become refractory to calcium signals and inhibit ionophore-induced sperm-oocyte fusion (Aitken and Clarkson, 1987), perhaps by stimulating peroxidation that reduces plasma membrane fluidity and interferes with membrane bound enzymes and/or ion channels (Aitken et al., 1989). ROS impair motility, perhaps depleting ATP by H2O2 inhibiting oxidative phosphorylation and/or glycolysis thereby limiting ATP replenishment (de Lamirande and Gagnon, 1992) or by depressing glycolytic flux (Baumber et al., 2000) or by decreasing phosphorylation of axonemal proteins required for sperm motility (de Lamirande and Gagnon, 1992), or by the products of lipid peroxidation, such as malondialdehyde and 4-hydroxynonenol (4HN), inhibiting anaerobic glycolysis, DNA, RNA and protein synthesis (Comporti, 1989).
Boar spermatozoa are sensitive to peroxidative damage due to the high content of unsaturated fatty acids in the phospholipids of the plasma membrane (Parks and Graham, 1992; White 1993; Cerolini et al., 2000), which are preferred substrates for ROS and HO generation in membranes (Brouwers et al., 2005), coupled with the relatively low antioxidant capacity of boar seminal plasma (reviewed in Breininger et al., 2005). Lipid peroxidation disrupts membrane structure and function (Aitken, 1994, 1995) by disordering membrane phospholipid structure (Sevanian et al., 1988) and changing membrane fluidity. Extracellular Ca$^{+2}$ binding to membrane phospholipids decreases lipid bilayer fluidity, further damaging bilayer structure through inducing lipid phase separation, formation of heterogeneous structural domains, and membrane fusion (reviewed in Salgo et al., 1993), increasing membrane permeability and affecting ion transport (Aitken, 1995). Lipid peroxidation also disrupts mitochondrial enzymes eventually uncoupling respiration associated with oxidative phosphorylation (Hogberg et al., 1973).

Increasing extra or intracellular phospholipase A$_2$ (PLA$_2$) activity as a result of the coupled action of lipid peroxidation and Ca$^{2+}$ on membrane phospholipids, ultimately alters membrane structure and initiates membrane degradation (reviewed in Salgo et al., 1993). Cell membrane lysis and cell death could occur if this enzyme is extensively activated (Shier, 1979).

Despite knowing how ROS affect sperm function in different species, there is very little known about the influence of ROS on boar sperm function. This current study hypothesizes that (1) the process of freezing and thawing boar spermatozoa increases
ROS content, and (2) altered ROS levels affect sperm motility, viability, acrosome integrity, lipid peroxidation and Phospholipase A activity. The specific goals were to evaluate relative \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \) in fresh and frozen-thawed boar sperm and determine how ROS generated in boar spermatozoa would affect sperm function (motility, viability and acrosome integrity), sperm intracellular levels of \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \), lipid peroxidation, and PLA activity.

3.3. Materials and Methods

3.3.1. Semen handling

3.3.1.1. Semen collection:

Boars of proven fertility were housed according to the requirements of the Canadian Council on Animal Care. The sperm rich fraction was collected using the gloved-hand method into a 35°C Thermos®️, immediately assessed for motility, and only ejaculates with motility of greater than 70% were used. Sperm concentration was determined by a calibrated spectrophotometer (Spectronic 20, Milton Roy, Rochester, New York) at an excitation of 550 nm.

3.3.1.2. Sperm freezing and thawing:

The sperm rich fraction (one ejaculate from each of four boars) was assessed for volume, sperm concentration, motility (CASA) and viability and ejaculates with more than 70% motile sperm were used. The ejaculate was cooled down gradually from 35 °C to 25 °C at a cooling rate of 0.1 °C/ minute using a programmable cell freezer (Ice cube 14s-A, Minitube Canada). At 25 °C, the sperm rich fraction was centrifuged (800g; 10 min; 25
°C; Jouan-CT 422, Jouan, Inc. Winchester, VA 22601) and the pellet was re-suspended with a small amount of 25 °C BTS (205.37mM Glucose (dextrose), 20.4 mM Sodium citrate dehydrate, 14.88 mM Sodium bicarbonate, 3.36 mM Disodium ethylenediamine tetra acetate (EDTA), 10.01 mM potassium chloride, and Dihydrostreptomycin (770mg/g activity); pH 7.4; (Pursel and Johnson, 1975) and the concentration re-determined and re-suspended to 12× 10⁸ spermatozoa/ml with BF5 extender fraction A [177.62 mM Glucose (dextrose), 16.51 mM Tris (hydroxymethyl) amino methane, 52.35 mM TES-Tris (hydroxymethyl) Methyl 2 amino ethane sulphonic acid, 20% egg yolk (vol:vol), 0.33% Orvus ES Paste, streptomycin (770 mg/gm activity), pH 7.3-7.4; (Pursel and Johnson, 1975)]. The extenders’ pH was adjusted using Accument® pH Meter 925 (Fisher Scientific, ON, Canada) by adding drops of NaOH or HCl solution. The sperm were cooled in the cell freezer to 5 °C at 0.1 °C/minute, and then diluted 1:1 (vol:vol) with BF5 fraction B (Fraction A containing 6% glycerol; final concentration 3% glycerol; 6× 10⁸ spermatozoa/ml). The extended semen was sealed in 0.5 ml straws (IMV Technologies, France) with commercial straw filler (IMV) and frozen in the cell freezer at -30 °C/minute from 5 °C to -80 °C, when the straws were plunged and stored in liquid nitrogen. When needed, sperm straws were thawed in 60 °C water bath for 5 seconds and the straw contents emptied into 37 °C tubes and either immediately mixed with BTS or centrifuged (300g, 3 min., 37 °C) to remove the egg yolk extender and then the pellet mixed with BTS (final concentration, 3×10⁷ spermatozoa/ml). Motility, progressive motility and viability were then assessed on the washed and/or unwashed sperm.
3.3.2. Sperm function:

3.3.2.1. Motility: Sperm motility was assessed using computer assisted sperm analysis (CASA; IVOS System Software, Hamilton Thorn Bioscience, Massachusetts, USA). The sperm samples were diluted to the concentration of $1 \times 10^7$ spermatozoa/ml with BTS extender and BF5 fraction-A in ratio of 3:1 (BTS: BF5; vol: vol). Motility and progressive motility of 200 sperm cells was assessed in one chamber of a four chamber standard count analysis slide (Leja products B.V. Luzernestraat 10, 2153 GN Nieuw-Vennep, The Netherlands), at 37 °C (Appendix 1).

3.3.2.2. Viability: The percentage of live sperm was determined with the Live: dead sperm viability kit 200-1000 assay (cat. no. L-7011, Molecular Probes, Inc., Eugene, OR) on sperm diluted to $1 \times 10^7$ spermatozoa/ml with BTS extender. A total of 2 x 100 spermatozoa were counted at 400× magnification with a fluorescence microscope (Leitz, Laborlux S, Germany), fitted with the blue filter at 450-490 nm (Appendix 2).

3.3.2.3. Capacitation and acrosome reaction: To determine capacitation status, 15μl aliquots of the samples to be assessed were smeared on glass slides and air dried for 15 minutes, fixed with 100% ethanol (-20°C, 20 secs) and then air dried for 15 minutes. Smears were stained with Coomassie blue (1 g Coomassie blue, 50% methanol, 40% water, and 10% Glacial Acetic acid) for 2 minutes (Larson and Miller, 1999) and then washed thoroughly with distilled water to remove the excess stain, air dried for 15 minutes, and covered with cover slips. Sperm were examined under bright field
microscopy (400 X) and 100 sperm had their acrosomes assessed as intact or reacted (totally or partially).

3.3.3. Sperm intracellular levels of $O_2^{-\cdot}$ and $H_2O_2$

Hydroethidine (HE; cat. no. D-1168, Molecular Probes, Inc., Eugene, OR) and 2', 7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; cat. no. D-399, Molecular Probes, Inc., Eugene, OR) detected $O_2^{-\cdot}$ and $H_2O_2$ respectively as described by Guthrie and Welch (2006). When HE is exposed to $O_2^{-\cdot}$, it is oxidized to ethidium (Carter et al., 1994), which emits fluorescence at 610 nm in response to 488 nm excitation (Guthrie and Welch, 2006). The diacetate form of H$_2$DCFDA is non-fluorescent and cell-permeable, and so readily incorporates into cells. Living cells contain an esterase (Bass et al., 1983), that will cleave the acetate moiety, leaving a non-fluorescent 2', 7'-dichlorodihydrofluorescein form that, when exposed to $H_2O_2$, is oxidized to dichlorofluorescein (DCF), which, when excited at 488 nm, emits fluorescence at 530 nm at an intensity that correlates to the amount of $H_2O_2$ present. Working solutions of 20 mM HE and H$_2$DCFDA in DMSO were aliquoted and stored in the dark at -20 °C. On the day of the experiment, 3x10$^7$ fresh sperm in BTS were mixed with HE or H$_2$DCFDA (final concentrations: 1 x 10$^6$ sperm/ml; 4 μM HE or 200 μM H$_2$DCFDA) and YO-PRO-1 [YO-PRO-1 iodide (491/509) in DMSO; cat. no. Y3603, Molecular Probes, Inc., Eugene, OR; final concentration, 0.05 μM] was added to HE-treated sperm and PI (Molecular Probes Inc.; final concentration, 9.6 μM) was added to H$_2$DCFDA-treated sperm; YO-PRO-1 and PI monitor sperm viability (Guthrie and Welch, 2006). Sperm were incubated for 60
min at 25 °C and then fluorescence was assessed in a FACS Calibur flow cytometer (BD Bioscience, Mississauga, ON).

A total of 10000 individual sperm-sized events were selected with forward (FSC) and side scatter (SSC) gates. Using a 488 nm excitation source, the fluorescence selected sperm stained with HE and YO-PRO-1 and collected them in the fluorescence detector 3 (FL-3; 650 long pass (LP) filter) and fluorescence detector 1 (FL-1; 530/30 nm filter) respectively, while the fluorescence of sperm stained with H$_2$DCFDA and PI was collected in FL1 (530/30 nm filter) and FL3 (650 LP filter), respectively. The sperm populations were subsequently gated to live and dead populations based on low and high YO-PRO-1 or PI; and the mean fluorescence intensity and number of sperm in each population identified by ethidium or DCF was calculated in the statistical region of FACS.

3.3.4. Effects of ROS on boar sperm

To generate O$_2^-$ and H$_2$O$_2$ in boar spermatozoa, an ROS generating system of xanthine and xanthine oxidase (Sigma, Oakville, ON, Canada) was used (Guthrie and Welch, 2006). Xanthine (20 mM in 1M NaOH) was diluted to 2 mM in water (pH 7.5) and 0.2 U of xanthine oxidase was added to 0.5 ml of 2 mM xanthine. The pH was adjusted using Accument® pH Meter 925 (Fisher Scientific, ON, Canada) by adding drops of NaOH or HCl solution. This adjustment was carried out repeatedly over two hours while the medium was stirred, which resulted in the medium’s pH remaining stable; without this lengthy pH adjustment, the medium’s pH rose within 5 minutes.
Freshly-collected sperm (n=1 ejaculate from each of 5 boars) were mixed with either BTS alone or ROS generating system (final concentrations: $3 \times 10^7$ sperm; and either 0 or 1 mM xanthine + 0.1 U xanthine oxidase per ml) and incubated (30 min, 38 °C). Sperm were then cooled to room temperature over 10 minutes, and stained with HE and YOPRO-1 or H2DCFDA and PI and assessed by flow cytometry for $O_2^-$, $H_2O_2$, and viability. Four separate ejaculates were similarly incubated ± ROS generating system and assessed for motility (CASA) and viability (SYBR-PI).

To assess the effects of ROS on acrosome integrity, four separate ejaculates were incubated as above ± ROS generating system (30 min, 38 °C), at which time aliquots were diluted into BTS or capacitating medium (4.8 mM Potassium chloride KCl, 1.2 mM Potassium phosphate KH2PO4, 95 mM sodium chloride NaCl, 5.55 mM Glucose (Dextrose), 25 mM Sodium Bicarbonate NaHCO3, 2 mM Calcium chloride CaCl2, 2 mM Pyruvate, 0.4% BSA (fatty acid free, fraction V); pH 7.4; Tardif et al., 2001; 5 $\times$ 10^6 spermatozoa/ml), and incubated for four hours at 36 °C, 5% CO2 and 100% humidity. After both the 30 min and 4 hour incubation periods, aliquots were taken of all treatments (control and ROS-treated in BTS and capacitating) and received either DMSO or the Ca$^{2+}$ ionophore A23187 (0.4 mM in DMSO; Molecular Probes), incubated for 30 minutes (36 °C, 5% CO2 and 100% humidity), and acrosomal status assessed.

The pH of the capacitating medium was carried out repeatedly over two hours while the medium was stirred, which resulted in the medium’s pH remaining stable; without this lengthy pH adjustment, the medium’s pH rose within 5 minutes.
3.3.5. Lipid modification by ROS

To assess the effect of ROS generation on lipid peroxidation in boar spermatozoa, sperm (n = 1 ejaculate from each of 5 different boars) were exposed to the fluorescent probe BODBY 581/591 C11 (4, 4-difluoro-5-(4-phenyl-1, 3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid; Molecular Probes), which is a fatty acid analogue that incorporates into cell membranes and changes its fluorescence irreversibly from red to green on exposure to ROS (Brouwers and Gaddella, 2003). As before, freshly-ejaculated sperm in BTS were incubated (30 min, 38 °C) with BTS or the ROS generating system (final concentration, 2 x 10^7 spermatozoa/ml), and then 2 μM (final concentration) of BODBY 581/591 C11 was added and incubated (30 min, 25 °C). Then PI (24 μM, final concentration) was added to the sperm and incubated for an extra 15 min at 25 °C. The degree of lipid peroxidation was analyzed by flow cytometer (excitation 488 nm, emission detected with 650 LP filter (FL-3)) to detect PI, and a separate 530/30 nm filter (FL-1) to detect BODBY 581/591 C11.

Sperm from the same ejaculates as used for peroxidation assessment, also had their phospholipase A (PLA) activity measured using the Probe bis-BODIPY FL C11 (B-7701, Molecular Probes, Inc., Eugene, OR). The two BODIPY-labeled C11 fatty acids are linked to the 1- and 2- positions of phosphatidylcholine (PC) and their proximity promotes energy transfer that quenches fluorescence. The presence of endogenous PLA₁ or PLA₂ cleaves off the BODIPY group and increases fluorescence emission at 530 nm (Meshulam et al., 1992).
Unlike previous studies that required incorporation of the probe into liposomes for delivery to the target cells (Meshulam et al., 1992), the probe here was directly incubated with boar spermatozoa (20 min, 37 °C), since a preliminary experiment demonstrated that these conditions supported significant uptake of the probe by the cells (Appendix 3). Following the 30 min incubation ± the ROS generating system, sperm received BODIPY and PI (8 and 24 μM final concentration) and were incubated for 20 minutes at 37°C. The PLA activity of sperm samples were analyzed by flow cytometer (excitation 488 nm, emission with 650 LP filter) to detect PI fluorescence, and a separate 530/30nm filter detected the fluorescence of BODIPY (PLA activity).

3.4. Statistical analysis

Data were analyzed using the SAS 9.1 (SAS 9.1 for windows TS Level 1M3, The SAS Institute Inc., Cary, NC, USA). General Linear Model Procedure (GLM) and Multiple Comparison Tukey’s Test were used to determine the differences of Least Squares Means between treatment (sperm preincubated with ROS generating system) and control (sperm preincubated with BTS only) of all assays. Differences with values of P<0.05 were considered to be statistically significant.

3.5. Results

3.5.1. Sperm function:

3.5.1.1. Motility: Fresh sperm had more motile sperm than either washed or unwashed frozen thawed spermatozoa (P<0.0001; Fig. 3.1) and also more progressively motile
sperm (P<0.0001; 65.5 ± 6.6 vs. 19.5 ± 4.1 or 30 ±1.8 % progressively motile for fresh, washed, vs. unwashed frozen-thawed spermatozoa, respectively).

3.5.1.2. Viability: Fresh sperm viability that was assessed by SYBR-PI and fluorescence microscopy was higher than that in washed or unwashed frozen-thawed spermatozoa (Fig. 3.1).

Figure 3.1. The effect of freezing, thawing, and washing on the % of motile, viable boar spermatozoa. Motility was measured by CASA and viability by SYBR14-PI fluorescence microscopy. Values (n=4) are means ± SE. a, b: columns with different superscripts differ (P<0.05).
3.5.2. Sperm intracellular levels of $\text{O}_2^{--}$ and $\text{H}_2\text{O}_2$

The flow cytometric 2-dimensional dot-plots (Fig. 3.2) showed fresh sperm had three populations based on YO-PRO-1 fluorescence (live, dead, and moribund, corresponding to low, high and intermediate YO-PRO-1 fluorescence, respectively being 91.4%, 6.2%, and 2.4% of the total sperm population), while both washed and unwashed frozen-thawed sperm showed only live and dead populations.

Figure 3.2. Flow cytometric 2-dimensional dot-plots of fluorescence intensity of YO-PRO-1 and ethidium of fresh and frozen-thawed boar sperm. (A) fresh, (B) frozen-thawed unwashed and (C) washed boar sperm stained with YO-PRO-1 and HE dyes for 60 minutes at 25 °C to detect sperm viability and sperm intracellular level of $\text{O}_2^{--}$. 
Frozen-thawed sperm had lower O$_2^-$ level of the viable (P<0.0009; Fig. 3.3), but not the dead or overall average sperm population; washing frozen-thawed sperm had no significant effect on O$_2^-$ content.

![Figure 3.3](image)

Figure 3.3. Ethidium fluorescence intensity of viable fresh and frozen-thawed boar spermatozoa. Boar spermatozoa (n=4 ejaculates) incubated with YO-PRO-1 and HE dyes for 60 minutes at 25 °C to detect sperm viability and sperm intracellular level of O$_2^-$: a, b values differ from (P<0.05).

The DCF fluorescence demonstrated no significant difference between the H$_2$O$_2$ level of fresh or frozen-thawed sperm, either considering the average over the total sperm populations, or just the viable sperm (13.09 ± 2.26, 15.56 ± 2.41, and 12.52 ± 2.05 fluorescence units for viable fresh, viable frozen thawed washed and viable frozen thawed unwashed respectively; P>0.05).

Comparing measurements of % viable sperm, fresh sperm stained with H$_2$DCFDA-PI apparently had fewer % viable sperm than measured with either HE-YoPro-1 or SYBR-PI staining methods (Fig. 3.4; P<0.0002 and P<0.0007). There were no differences
(P>0.05) between the % viable sperm population measured among frozen-thawed sperm populations by any method.

Figure 3.4. The % of viable fresh and frozen-thawed boar sperm measured by three different methods. Values are means ± SE; n=4 ejaculates. a, b: columns with different superscripts differ (P≤0.05).
3.5.3. Effects of ROS on boar sperm

Exposing boar spermatozoa to ROS generating system for 30 minutes at 38 °C completely inhibited sperm motility (P<0.01) but had no effect on the percent of viable sperm (Fig. 3.5).

![Graph](image)

Figure 3.5. The effect of incubating boar spermatozoa in BTS ± ROS generating system on the % of motile and viable sperm. Control: sperm incubated with BTS only, ROS: sperm incubated with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml) for 30 minutes at 38 °C. Values are means ± SE; n=4 ejaculates. a, b: % motile sperm reduced by ROS generating system (P≤0.05).

Incubation for 30 min in an ROS generating system did not change the % of live, dead, or moribund sperm (Fig. 3.6, 3.7), nor did the ROS generating system affect the O₂⁻ intracellular level in the live, moribund, dead or overall average sperm populations (Fig. 3.8).
Figure 3.6. Flow cytometric 2-dimensional dot-plots of fluorescence intensity of YO-PRO-1 and ethidium (viability and $O_2^-$) of boar sperm after incubation ± ROS generating system. (A) sperm incubated without or (B) with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml) for 30 minutes at 38 °C and then stained with YO-PRO-1 and HE dyes for 60 minutes at 25 °C to detect sperm viability and sperm intracellular level of $O_2^-$. 
Figure 3.7. The % of viable, moribund, and dead boar spermatozoa after incubation of fresh sperm ± ROS generating system and subsequent staining with YO-PRO-1/HE (viability and CV"), as measured by flow cytometer. Values are mean ± SE; n=5; ROS generating system did not affect the number of sperm within a viability category. (Control: 81.7%, 14.8%, and 3.5%, live, dead and moribund respectively; ROS: 63.5%, 25.7%, and 10.8%, live, dead and moribund respectively). a, b: columns with different superscripts differ (P<0.05).
However, live sperm, whether in BTS or the ROS generating system, had significantly more $O_2^{\cdot-}$ than did the dead sperm (Fig. 3.8, $P<0.05$).

Figure 3.8. Ethidium fluorescence intensity (intracellular level of $O_2^{\cdot-}$) of viable, moribund, dead and average boar spermatozoa incubated ± ROS generating system. Viability is based on fluorescence of Yo-Pro-1. Values are means SE; $n=5$ ejaculates. a, b: bars with no superscript in common differ ($P<0.05$).
When assessing H$_2$O$_2$ intracellular level, the viability stain PI differentiated sperm into two populations, of either high or low fluorescence (dead or live, respectively), visible in the flow cytometric 2-dimensional dot-plots (Fig. 3.9).

![Flow cytometric 2-dimensional dot-plots](image)

*Figure 3.9. Flow cytometric 2-dimensional dot-plots of fluorescence intensity of PI and DCF (viability and H$_2$O$_2$) of boar sperm after incubation in BTS ± ROS generating system. (A) Sperm incubated with BTS or (B) ROS generating system (1mM xanthine + 0.1 U xanthine oxidase per ml; 30 minutes at 38 °C) and then stained with PI and H$_2$DCFDA dyes (60 min; 25 °C) to detect sperm viability and sperm intracellular level of H$_2$O$_2$."

The % viable sperm was similar in the presence or absence of the ROS generating system (P>0.05; 64.2 ± 9.4, and 52 ± 5.0). There was no difference (P>0.05) between the viable % of sperm stained with YOPRO-1/HE or PI/H$_2$DCFDA (Fig. 3.10). Although YOPRO-1 detected a moribund population that was not detected by PI, there was no difference (P>0.05) between the % of viable sperm detected by YOPRO-1 or PI (Fig. 3.10)
Figure 3.10. The % of viable sperm after exposure to BTS ± ROS generating system and subsequently stained with YO-PRO-1/HE (viability and O₂⁻) or PI/H₂DCFDA (viability and H₂O₂) and measured by flow cytometer. Values are means ± SE (n=5 ejaculates).

The ROS generating system did increase the H₂O₂ content of the average sperm population (P< 0.0004; Fig. 3.11). Furthermore, ROS caused the appearance of three populations of live sperm containing 3.9, 10.0, and 86.0% of the total viable sperm population) with distinct H₂O₂ intracellular level, the most numerous of which had a significantly higher H₂O₂ intracellular level than did the single population of control sperm.
Figure 3.11. DCF fluorescence intensity (intracellular level of $\text{H}_2\text{O}_2$) of viable, dead, and average boar sperm incubated in BTS ± ROS generating system. Fresh boar sperm incubated ± ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml; 30 min; 38 °C) and stained with H$_2$DCFDA and PI (60 min; 25 °C) dyes to detect sperm intracellular level of H$_2$O$_2$ and sperm viability; n=5. Bars with no superscripts in common differ; $P \leq 0.05$. 
The Ca$^{2+}$ ionophore A23187 did not affect acrosomal status regardless of incubation time or conditions (P>0.05), so the data of % of reacted acrosomes of sperm incubated in DMSO and Ca$^{2+}$ ionophore were pooled for assessment of other effects. More sperm had reacted acrosomes after 4hr incubation in capacitation medium than after 4hr in BTS (P < 0.025; Fig.3.12) Exposing sperm to ROS for 30 min greatly increased the % of acrosome-reacted sperm after 4hr incubation in capacitation medium, compared to no ROS exposure ( P<0.0001 ), or compared to any 30 min values (Fig.3.12).

![Figure 3.12. The % of acrosomes reacted boar sperm preincubated ± ROS generating system. Sperm were first incubated in BTS ± ROS generating system (XA/XO; 1mM xanthine + 0.1mM xanthine oxidase per ml; 30 min; 38 C') and then incubated for 4h (36 °C; 5% CO2; 100% humidity) in either BTS or capacitating medium. Values are mean ± SE; n=4 ejaculates. a, b, c, d: bars with no superscripts in common differ (P≤0.05).](image-url)
3.5.4. Lipid modification by ROS

ROS generation significantly increased the intensity of BODIPY 581/591 C11 fluorescence, indicating a significantly greater extent of lipid peroxidation in the overall sperm, and in each of the live and dead sperm populations (P<0.0016, P<0.0036, and P<0.0004, respectively; Fig. 3.13). Dead sperm also had significantly more lipid peroxidation than found in either the live or average sperm population after 30 min incubation with ROS generation.

Figure 3.13. BODIPY581/591 C11 fluorescence intensity of viable, dead, and average boar spermatozoa incubated in BTS ± ROS generating system. Fresh boar spermatozoa (n=5 ejaculates) were incubated in BTS or ROS generating system (XA/XO; 1mM xanthine+0.1mM xanthine oxidase per ml; 30 min, 38 °C) and then 2 μM of BODIPY 581/591C11 and PI were added to measure lipid peroxidation and viability, prior to assessment by flow cytometer. Values are mean ± SE; a, b, c: bars with different superscripts differ (P≤0.05).
Sperm from the same incubations as used for lipid peroxidation measurements were assessed for PLA activity. Exposure to the ROS generator for 30 min did not affect PLA in live sperm, but interestingly, dead sperm following exposure to the ROS generator had more PLA than the dead control sperm (P<0.0002). Also, PLA activity in dead sperm was higher than that in either the overall average, or the viable sperm populations (Fig. 3.14; P<0.0001).

Figure 3.14. bis-BODIPY-FL C11 fluorescence intensity of viable, dead and average boar spermatozoa incubated in BTS ± ROS generating system. Fresh boar spermatozoa (n=5 ejaculates) were incubated in BTS or ROS generating system (XA/XO; 1 mM xanthine + 0.1 mM xanthine oxidase per ml; 30, 38 °C) then 8 μM of bis-BODIPY-FL-C11 and PI were added and incubated for 20 minutes at 37 °C to measure PLA activity and viability, prior to assessment by flow cytometer of sperm samples were analyzed by flow cytometer. Values are means± SE; a, b, c: bars with different superscripts differ (P≤0.05).
3.6. Discussion

Unexpectedly, cryopreserved sperm had less $O_2^{\cdot-}$ than fresh sperm, and similar amounts of hydrogen peroxide. As expected, cryopreserved sperm were less viable and motile than fresh sperm. A careful examination of the response of fresh sperm to the generation of ROS confirmed that there were few changes in intracellular level of $O_2^{\cdot-}$ but a significant increase in intracellular level of $H_2O_2$. Unique and novel findings include that the acrosome reaction and membrane lipid peroxidation were greatly increased after exposure to the XA/XO generating system and that dead sperm had significantly inflated levels of phospholipase A. Relating these ROS changes to sperm function will help elucidate fertilizing steps in boar sperm.

Cryopreservation effects: As expected, motility, progressive motility and viability of fresh spermatozoa were significantly higher than that of frozen-thawed sperm (whether the frozen-thawed sperm were washed free of extender or evaluated immediately after thawing, in the condition they would be at the insemination time). This reduction in functional abilities could be due to cold shock (White, 1993), intracellular ice formation and/or osmotic stress (Watson, 1995), which damage the sperm membrane, the mitochondria, the acrosome and the sperm tail (Wooley and Richardson, 1978). Cryopreservation is known to reduce motility (Maldjian et al., 2005), with the loss of mitochondrial function (Henry et al., 1993) potentially interrupting ATP availability to the tail filaments (Mahadevan et al., 1997). Concomitant loss of membrane integrity (Henry et al., 1993) would both increase PI permeability and ultimately kill the cells.
Contrary to the hypothesis that cryopreservation would increase ROS, frozen-thawed sperm had unchanged intracellular level of H$_2$O$_2$, and viable frozen-thawed sperm had significantly less intracellular level of O$_2$•⁻ than did fresh spermatozoa. Somewhat similarly, Guthrie and Welch (2006) reported that boar sperm had low basal concentrations of the ROS species O$_2$•⁻ and H$_2$O$_2$, which did not change after cryopreservation. The cryopreservation-induced decrease in O$_2$•⁻ documented here could be related to the alteration of mitochondrial function. Oxidative phosphorylation is required for mitochondrial ATP production and this process is coupled with electron transport and ROS formation (reviewed in Guthrie and Welch, 2006). Moreover, this process is a major source of endogenous ROS production (Thannickal and Fanburg, 2000). Thus, cryopreservation could be associated with decreasing oxidative phosphorylation, ATP synthesis, ROS generation and sperm motility in this study.

Since cryopreservation had unexpected effects on the boar sperm content of two well-known ROS species and yet, as expected, interfered with normal sperm function, it was important to directly explore the impact of ROS on boar sperm.

The XA/XO reaction is known to form O$_2$•⁻ and H$_2$O$_2$ (McCord and Fridovich, 1968). Incubating boar spermatozoa for 30 minutes with this ROS generating system significantly increased their intracellular level of H$_2$O$_2$ (Fig. 3.11) but not O$_2$•⁻ (Fig. 3.8), as has been found before with boar (Guthrie and Welch, 2006), human (Aitken et al., 1993) and stallion spermatozoa (Baumber et al., 2000). These results concur with H$_2$O$_2$ being a major ROS causing oxidative damage in boar spermatozoa (Guthrie and Welch, 2006), which could result from the high intracellular (mitochondrial and cytoplasmic)
levels of SOD activity in boar sperm (Mennella and Jones, 1980) scavenging $\text{O}_2^{\cdot-}$ by a rapid dismutation reaction to $\text{H}_2\text{O}_2$ (McCord and Fridovich, 1969). Boar semen is extremely low in CAT (Foote, 1962) in comparison with ram and rabbit semen (White, 1959) which resistance to $\text{H}_2\text{O}_2$ (Wales et al., 1959). Therefore, the high $\text{H}_2\text{O}_2$ intracellular level detected after XA/XO exposure is due to the very low boar sperm content of CAT that is not sufficient to convert all $\text{H}_2\text{O}_2$ to water and oxygen. Moreover, the addition of CAT to the freezing extender alone or in combination with SOD reduces post thaw ROS generation, improving motility and viability of boar spermatozoa (Roca et al., 2005), supporting the fact that boar spermatozoa have low content of CAT and are very sensitive to $\text{H}_2\text{O}_2$.

The presence of three populations of viable fresh sperm with differing DCF fluorescence after 30 min incubation with ROS generating system (Fig. 3.11) reflects their having differing intracellular levels of $\text{H}_2\text{O}_2$ (Carter et al., 1994). Since the most numerous populations had the highest $\text{H}_2\text{O}_2$ level, it is tempting to speculate that there are sub-populations of sperm in an ejaculate that differ in their endogenous content of SOD to create $\text{H}_2\text{O}_2$, or in their amounts of catalase or other systems that would rapidly reduce any $\text{H}_2\text{O}_2$ created.

Different DNA-binding probes provided different assessments of sperm viability. Flow cytometry with YO-PRO-1 detects 3 sperm populations (live, moribund, and dead) in fresh sperm with or without exposure to ROS generating system, while PI detects only two populations (Live and Dead) in all fresh and frozen-thawed sperm. Both YO-PRO-1
and PI are nucleic acid stains, but while viable cells with intact cell membrane exclude both YO-PRO-1 and PI (Plantin-Carrenard et al., 2003), live and apoptotic cells are impermeant to PI but apoptotic cells take up YO-PRO-1 in FACS analysis (Idzorek et al., 1995) and fluorescence microscopy (Choucroun et al., 2001). Thus, YO-PRO-1 here has detected 3 populations with different fluorescence intensity depending on the sperm membrane permeability to YO-PRO-1, probably representing live, apoptotic (moribund) and dead cells in fresh sperm. This probe detects only two populations in frozen-thawed sperm, because freezing and thawing reduce sperm viability (Maldjian et al., 2005), probably causing the death of most apoptotic sperm. In addition, the percentage of viable sperm detected by PI in flow cytometry was significantly (Fig. 3.4) lower than that measured with either YO-PRO-1 or SYBR-PI with fluorescence microscopy in fresh but not frozen thawed sperm. These results are in agreement with Li et al (2007) who found significant difference between PI and YO-PRO-1 using the flow cytometer to measure the viability of bacteria and fungi. Chen and Li (2005) found 3μM PI with 15 minutes incubation gave optimal separation of the viable and non viable cells, but while high concentrations of PI did not separate properly the viable and non viable bacterial populations, YO-PRO-1 very clearly separated the two populations without any effect of the incubation time or the dye concentrations. So, it is possible that the PI concentration (9.6 μM) that was used in our study, coupled with the incubation time, affected the separation between the live and dead fresh sperm populations and underestimated the percentage of viable sperm, while the freezing and thawing extender prevented this effect on the percentage of viable frozen-thawed sperm.
Regardless of PI-assessed viability, this brief incubation (30 min; 38 °C) with the ROS generating system completely inhibited boar sperm motility (Fig.3.5), as did similar incubations with human spermatozoa (de Lamirande and Gagnon, 1992). However, Guthrie and Welch (2006) found 33% of boar sperm were motile after similar exposure to the XA/XO generating system. Their sperm were in BTS extender containing 0.3% BSA, while our BTS extender was BSA-free; when we diluted spermatozoa with capacitating medium containing 0.4% BSA and then incubated with the ROS generating system up to 10% of the sperm remained motile, presumably due to the antioxidant properties of albumin (Alvarez and Story, 1983). Regardless of the exact extent, sperm obviously quickly become immotile when exposed to the XA/XO generating system. The H$_2$O$_2$ may oxidize intracellular sulphydryl moieties, depressing glycolytic flux and decreasing ATP levels (Baumber et al., 2000). Spermatozoal ability to generate ATP may also be limited by inhibiting one or more enzymes of oxidative phosphorylation and/or glycolysis (de Lamirande and Gagnon, 1992), thereby decreasing phosphorylation of axonemal proteins. Lipid peroxidation products could contribute to loss of sperm motility, releasing PUFAs from sperm plasma membrane to alter membrane fluidity, permeability and cellular capacity to regulate the intracellular ions that control sperm motility (Baumber et al., 2000). Lipid peroxidation of membrane phospholipids was significantly higher in all sperm exposed to XA/XO-induced ROS generation similar to that recently found when boar sperm were incubated with FeSO$_4$/Na ascorbate ROS-generating system (Guthrie and Welch, 2007), and the resultant loss of membrane PUFAs and production of cytotoxic aldehydes such as malondialdehyde and 4HN could inhibit anaerobic glycolysis, in addition to altering membrane architecture (Comporti, 1989).
ROS at low concentrations are known to induce sperm capacitation and acrosome reactions in sperm from other species (Aitken et al., 1995; Leclerc et al., 1997), and the current results demonstrate for the first time that there is a time-dependent significant increase in the percentage of acrosome reactions seem in sperm exposed to an ROS generating system and then incubated in capacitating medium (Fig. 3.12).

These results could be related to the fact that ROS significantly increased sperm membrane phospholipid peroxidation (viable and dead sperm) and PLA activity in dead sperm, as is demonstrated here for the first time. This could disorder membrane phospholipids structure (Sevanian et al., 1988), change membrane fluidity and elevate intracellular Ca\(^{+2}\) resulting from increased Ca\(^{+2}\) permeability. Thus, the coupled action of lipid peroxidation and Ca\(^{+2}\) on membrane phospholipids may activate extra- or intracellular PLA\(_2\) (Salgo et al., 1993) that can create cis-unsaturated free fatty acids and lysophospholipids, induce membrane instability and fusion (Karnovsky et al., 1982) such as in fertilization (Fleming and Yanagimachi, 1981) and the acrosome reaction of hamster sperm (Meizel and Turner, 1983).

The bis-BODIPY FL C11 probe measures PLA activity by the presence of endogenous PLA\(_1\) or PLA\(_2\) which cleaves off the BODIPY group and increases fluorescence emission at 530 nm, and therefore does not differentiate between cleavage of acyl chains at the 1 and 2 positions (Meshulam et al., 1992). Interestingly, viable sperm had the same PLA activity whether they were from control or XA/XO exposed sperm; dead sperm from both groups had significantly higher PLA activity than the live sperm; and ROS generation
resulted in dead sperm with more PLA activity than dead sperm in simple BTS. PLA\textsubscript{2} is known to be involved in signal transduction pathways in animal cells (Liscovitch and Cantley, 1994), and after being activated by either G proteins, phosphorylation or Ca\textsuperscript{2+} (Axelrod, 1990; Liscovitch and Cantley, 1994), PLA hydrolyses membrane phospholipids to lysophospholipids and free fatty acids; PLA\textsubscript{2} preferentially targets phosphatidylcholine with arachadonic acid at the sn2 position and so the most common PUFA released is arachidonic acid (Smith et al., 1991). The high levels of H\textsubscript{2}O\textsubscript{2} documented to have been produced may have increased intracellular calcium concentration (Golconda et al., 1993) through oxidative stress and lipid peroxidation, which could support PLA activation and help translocate it from the cytoplasm to the membrane (Schalkwijk et al., 1996). Other studies suggest that membrane lipid peroxidation increased activity of cPLA\textsubscript{2} due to an increase in substrate availability for cPLA\textsubscript{2} and implicated the high cPLA\textsubscript{2} activity in cell death (Hornfelt et al., 1999), and certainly sperm with excessive levels of PLA were membranes compromised. However the brief ROS generation did not increase the numbers of dead sperm, so boar sperm do not appear to be highly sensitive to PLA-induced cell death.

Although PI was used to detect the % of viable and dead sperm that were exposed to ROS generation and detecting their content of lipid peroxidation and PLA activity, flow cytometric 2-dimensional dot-plots of fluorescence intensity showed that sperm populations were very well separated into live and dead populations indicating the reliability of these results.
In conclusion, the frozen-thawed boar sperm had significantly less O$_2$$^\cdot$-, and H$_2$O$_2$ is the major free radical mediating the damage of boar spermatozoa through the XA/XO ROS generating system. Boar sperm motility, acrosome integrity and lipid peroxidation are more sensitive indicators of oxidative stress than viability and PLA activity.
4. Regulation of tyrosine phosphorylation in boar spermatozoa by reactive oxygen species.

4.1. Abstract

This study hypothesized that Reactive Oxygen Species (ROS) modulate signal transduction during capacitation of fresh boar sperm. The objective of this study was to determine how tyrosine phosphorylation was affected when boar spermatozoa were exposed to an ROS generating system and subsequently incubated with or without capacitation medium.

Boar spermatozoa were incubated for 30 min either with an ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml) or in BTS, then either diluted in BTS or capacitating medium (5×10^6 spermatozoa/ml) and incubated under capacitating conditions for four hours. Proteins from 5×10^6 sperm were extracted after 30 min and 4 hours incubation, electrophoresed, electrotransferred, immunoblotted using anti-phosphotyrosine antibody and protein intensities were quantified using Image Quant software. Incubating boar spermatozoa with capacitation medium for four hours induced tyrosine phosphorylation of proteins with 174, 105, 94, 58, 48, 39, 35, 28, and 23 kDa; of these proteins, only two (39 and 35 kDa) were prominent in boar spermatozoa incubated with BTS buffer for either 30 minutes or four hours, although occasional slight evidence of phosphorylated proteins at 105, 48, and 31 kDa was detected in these non-capacitating conditions. ROS specifically affected capacitation-related signaling as indicated by tyrosine phosphorylation. Boar spermatozoa that were exposed to ROS for 30 minutes
and incubated in capacitating medium for four hours had significantly less tyrosine phosphorylation of the 174 kDa protein (P<0.0352).

In conclusion, ROS have physiological roles in regulating protein tyrosine phosphorylation of capacitated boar spermatozoa. This signaling specificity inhibits tyrosine phosphorylation of high molecular weight proteins. ROS may mediate these actions through the cAMP-dependent PKA pathway, or through other signaling pathways, such as the extracellular signal regulated kinase (ERK) family of mitogen-activated protein kinase (MAPKs) pathway, to phosphorylate/dephosphorylate different proteins. These potential pathways have yet to be identified.

4.2. Introduction

Capacitation is the process in which mammalian spermatozoa undergo many biochemical and functional changes to acquire the ability to fertilize an oocyte (de Lamirande et al., 1997a; Visconti et al., 2002). Biochemical changes associated with sperm capacitation include the increase of protein serine/threonine and tyrosine phosphorylation (Leclerc et al., 1996; O'Flaherty et al., 2004) and intracellular changes of calcium concentration (Baldi et al., 1991), while functional changes include alteration of hyperactivated motility and onset of the acrosome reaction (de Lamirande et al., 1997a).

Protein tyrosine phosphorylation has been implicated in the regulation of sperm hyperactivated motility, zona pellucida binding, acrosome reaction (Pukazhenthhi et al., 1998) and sperm-oocyte fusion. Hyperactivated motility, which is required to penetrate
the zona pellucida of the oocyte (Urner and Sakkas, 2003), is associated with tyrosine phosphorylation of flagellar proteins (Si and Okuno, 1999; Naaby-Hansen et al., 2002). Tyrosine phosphorylation accompanies sperm capacitation in different mammalian species such as human (Baldi et al., 2002), cattle (Galantino-Homer et al., 1997), mouse (Visconti and Kopf, 1998), and boar (Kalab et al., 1998; Flesch et al., 1999; Bravo et al., 2005). In boar spermatozoa, capacitation is associated with tyrosine phosphorylation of 32 kDa proteins (Tardif et al., 2001; Tardif et al., 2003). Various specific signaling pathways mediating protein tyrosine phosphorylation during sperm capacitation include protein kinase A (PKA), protein kinase C (PKC) (Thundathil et al., 2002), cAMP/PKA-dependent tyrosine phosphorylation of fibrous sheath protein and protein tyrosine kinase (PTK; Visconti et al., 1995b; Leclerc et al., 1996).

The cAMP-dependent PKA regulates tyrosine phosphorylation in spermatozoa directly or indirectly by affecting the enzymes of tyrosine kinase or tyrosine phosphatases (Visconti et al., 2002). Protein tyrosine kinases (receptor and non-receptor) mediate signal transductions of many extra and intracellular signals, such as cell growth and differentiation (Natarajan et al., 1998). They phosphorylate specific targets on tyrosine residues to regulate their function, while protein phosphatases dephosphorylate the molecules and control protein phosphorylation (Tardif et al., 2001). The PKA, PKC, and calcium/calmodulin-dependent kinase (CaMK) are serine/threonine kinases (Visconti et al., 1997; Tardif et al., 2001; Ignotz and Suarz, 2005; Marin-Briggiler et al., 2005) and have been found in mammalian spermatozoa mediating pathways regulating sperm motility and fertilization (Visconti et al., 1997; Ignotz and Suarz, 2005; Marin-Briggiler
et al., 2005). The increase of PKA activity is dependent on increased cAMP. The A-kinase anchoring proteins (AKAPs) are implicated in the regulation of tyrosine phosphorylation, anchoring PKA to specific compartments by binding its regulatory unit, thereby tethering these enzymes to their target proteins (e.g. for flagellar tyrosine phosphorylation; Urner and Sakkas, 2003).

Reactive Oxygen Species (ROS) induce cellular tyrosine phosphorylation in association with receptor or non receptor tyrosine kinases (Natarajan et al., 1998), and ROS at physiological concentrations may be natural initiators of capacitation (Ford, 2004). Human (de Lamirande and Gagnon, 1993ab), equine (Baumber et al., 2003), and bovine (Rivlin et al., 2004) spermatozoa use ROS at physiological concentrations to induce sperm capacitation, acrosome reaction and tyrosine phosphorylation, although H$_2$O$_2$ at high concentration in bull spermatozoa inhibits tyrosine phosphorylation (Rivlin et al., 2004) and ROS have been implicated in damaging sperm (Lopes et al., 1998). In concert with such factors as bicarbonate, loss of membrane cholesterol and increasing intracellular Ca$^{2+}$, ROS can increase intracellular superoxide which activates adenyl cyclase in rat sperm (Lewis and Aitken, 2001), increasing cAMP concentrations. Increased cAMP activates PKA, activating tyrosine kinase and inhibiting tyrosine phosphatase. Hydrogen peroxide can directly activate the kinase and inhibit the phosphatases, and indeed, H$_2$O$_2$ can replace bicarbonate in activating the cyclase in bull sperm (Rivlin et al., 2004). Lipid peroxidation resulting from this low concentration of ROS promoted sperm binding to the zona pellucida (de Lamirande et al., 1997b).
The extracellular signal regulated kinase (ERK) family of mitogen-activated protein kinase (MAPKs) form a signaling pathway separate from PKA that contributes to protein tyrosine phosphorylation during capacitation (de Lamirande and Gagnon, 2002; O'Flaherty et al., 2006a). ROS have been associated with the components of the ERK in activation of tyrosine phosphorylation during capacitation in human spermatozoa (O'Flaherty et al., 2005).

Although the effects of ROS generation on sperm capacitation and tyrosine phosphorylation have been studied in many mammalian species, the physiological role of ROS in regulation of protein tyrosine phosphorylation in boar spermatozoa has not yet been investigated. This study hypothesized that exposing boar spermatozoa to an ROS generating system would modulate capacitation-associated tyrosine phosphorylation.

4.3. Materials and methods

4.3.1. Semen handling

4.3.1.1. Semen collection: Boars of proven fertility were housed according to the requirements of the Canadian Council on Animal Care. The sperm rich fraction was collected using the gloved-hand method into a 35 °C Thermos®, immediately assessed for motility, and only ejaculates with motility of greater than 70% were used. Sperm concentration was determined by a calibrated spectrophotometer (Spectronic 20, Milton Roy, Rochester, New York) at an excitation of 550 nm.
4.3.1.2. **ROS generating system:** To generate $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ in boar spermatozoa, an ROS generating system of xanthine and xanthine oxidase (Sigma, Oakville, ON, Canada) was used (Guthrie and Welch, 2006). Xanthine (20 mM in 1M NaOH) was diluted to 2 mM in water (pH 7.5) and 0.2 U of xanthine oxidase was added to 0.5 ml of 2 mM xanthine. The pH was adjusted using Accumet® pH Meter 925 (Fisher Scientific, ON, Canada) by adding drops of NaOH or HCl solution. This adjustment was carried out repeatedly over two hours while the medium was stirred, which resulted in the medium’s pH remaining stable; without this lengthy pH adjustment, the medium’s pH rose within 5 minutes.

Freshly-collected sperm (n=1 ejaculate from each of 4 boars) were mixed with BTS (205.37 mM Glucose (dextrose), 20.4 mM Sodium citrate dihydrate, 14.88 mM NaHCO$_3$, 3.36 mM EDTA, 10.01mM KCl, containing 770 mg/g (activity) of Dihydrostreptomycin; pH 7.4; Pursel and Johnson, 1975) with or without the ROS generating system (final concentrations: $3 \times 10^7$ sperm; and either 0 or 1 mM xanthine + 0.1 U xanthine oxidase per ml) and incubated (30 min, 38 °C).

4.3.2. **Protein extraction, SDS-PAGE and western blotting**

To assess the effects of ROS on tyrosine phosphorylation, four separate ejaculates were incubated as above ± ROS generating system (30 min, 38 °C), at which time aliquots were either diluted into BTS to the concentration of $5 \times 10^6$ spermatozoa/ml and their proteins were extracted immediately or diluted into BTS or capacitating media (4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 95 mM NaCl, 5.55 mM Glucose (Dextrose), 25 mM NaHCO$_3$, 2
mM CaCl$_2$, 2 mM Pyruvate, and 0.4% BSA (bovine serum albumen, fatty acid free, fraction V; Sigma, Oakville, ON, Canada); pH 7.4; Tardif et al., 2001; final concentration $5 \times 10^6$ spermatozoa/ml), and incubated for four hours at 36 °C, 5% CO$_2$ and 100% humidity and then their proteins were extracted. As with ROS generating system, the pH of the capacitating medium was adjusted repeatedly over two hours while the medium was stirred, which resulted in the medium’s pH remaining stable; without this lengthy pH adjustment, the medium’s pH rose within 5 minutes.

Proteins were extracted by adding 20 μl of 10 mM Na$_2$VO$_3$ (Fisher, Mississauga, ON, Canada) to 1 ml sperm aliquot ($5 \times 10^6$ spermatozoa/ml), centrifugation (13000g, 10 min, room temperature), and sperm pellets re-suspended with 50 μl of 0.2 mM Na$_2$VO$_3$ and 12.5 μl of 5x sample buffer containing 0.350g dithiothreitol (DTT), 0.5g SDS, 2.0 ml Tris (1 M; pH 6.8), 2.5 ml of 50% glycerol, and 150 μl of 2.5% Bromophenol Blue and boiled for 5 minutes. The boiled sperm preparation was centrifuged (13000g, 5 min, room temperature) and the supernatant kept on ice until used for SDS-PAGE.

The extracted proteins were subjected to SDS-PAGE on 4% polyacrylamide stacking gel/10% running gel for 15 minutes at 75V first and then at 100V at room temperature over 150 minutes or until the dye front reached the bottom of the gel. The gel was equilibrated in transfer buffer (Bio-Rad; Mississauga, ON, Canada; containing 25 mM Tris, 192 mM Glycine and 20% (v/v) methanol; pH 8.3) for 30 minutes at room temperature and then transferred electrophoretically (100V) to Immobilon polyvinylidene difluoride (PVDV) transfer membrane (Millipore, ON, Canada) for 60 minutes at 4 °C using transfer buffer.
Non-specific binding was blocked by incubating membranes with a 5% (w/v) solution of skim milk powder in Tris (20 mM, pH 7.8)-buffered saline containing 1% Tween 20 (Fisher, Mississauga, ON Canada; TTBS; 0.1% v/v) overnight at 4 °C with shaking. After blocking, membranes were incubated with a monoclonal antibody (1:2000 in TTBS supplemented with 1% Tween 20) developed in mouse against phosphotyrosine proteins (Anti-Phosphotyrosine (4G10™), HRP conjugate; Upstate, NY 12946, USA) for 2 hours at room temperature with shaking. Membranes were then washed with TTBS (3×10 minutes) and twice with milliQ water and positive immunoreactive bands were detected using the enhanced chemiluminescence detection system. Bands detected and their volume, area and kDa were measured using Image Quant TL.lnk software (Amersham Bioscience) and then band intensities were calculated.

4.4. Statistical analysis

Data were analyzed using The Mixed Procedure in SAS 9.1 (SAS 9.1 for Windows TS Level 1M3, The SAS Institute Inc., Cary, NC, USA). Band intensities were analyzed as a split-plot in which the 4 boars represented the blocks and treatment (sperm preincubated with ROS generating system) versus control (sperm preincubated with BTS only) was the main plot factor. Image Quant band intensities after 30 minutes and after 4 hours incubation either with capacitating medium or BTS (control and treatment), were the subplot factor. All data were log-transformed prior to analysis. The differences of Least Squares Means, Tukey-Kramer test were used to detect the differences among different treatments and controls. Differences with values of P<0.05 were considered to be statistically significant.
4.5. Results

Incubating boar spermatozoa for four hours under capacitating conditions resulted in tyrosine phosphorylation of many proteins with different molecular weights, mainly proteins of 173.48±9.76 (~174), 104.75±2.26 (~105), 93.61±3.91 (~94), 57.55±1.85 (~58), 47.71±0.26 (~48), 38.75±0.19 (~39), 35.4±0.20 (~35), 28±0.03 (~28), and 23±0.31 (~23) kDa (mean ± SE: Fig. 4.1, lane 4, and Fig. 4.2). Of these proteins, only two (39 and 35 kDa) were prominent in boar spermatozoa incubated with BTS buffer for either 30 minutes or four hours, although occasional slight evidence of phosphorylated proteins at 105, 48, and 31 kDa was detected in these non-capacitating conditions (Fig. 4.1, lanes 1 and 3; Fig. 4.2).
Figure 4.1. Effect of preincubating boar sperm ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent capacitation. Boar spermatozoa were treated either with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml) or BTS and incubated (30 min, 38 °C) and then diluted in either BTS or capacitating medium and incubated for four hours. Proteins from $5 \times 10^6$ sperm were extracted by sample buffer, loaded in each well, electrophoresed, electrotransferred, and immunoblotted using the anti-phosphotyrosine antibody as described under Materials and Methods. Lane 1: sperm incubated for 30 min in BTS only (BTS-30 min; control), lane 2: sperm incubated in ROS generating system for 30 min (ROS-30 min; treated), lane 3: Control sperm incubated for four hours in BTS only (BTS-4h), lane 4: Control sperm incubated for four hours in capacitating medium (Cap.-4h), lane 5: treated sperm incubated in BTS for four hours (BTS-ROS-4h), lane 6: treated sperm incubated in capacitating medium for four hours (Cap-ROS-4h). The molecular masses of standard proteins are indicated at the left side of the gel.
Tyrosine phosphorylation differed due to ROS pre-exposure and the nature of media in subsequent incubations (Fig.4.2). Only sperm incubated in capacitating medium demonstrated a tyrosine-phosphorylated protein at 174 kDa, and protein intensity of sperm not treated with ROS was higher than that exposed to ROS (P<0.035). High variability in the amount of the 105 and 94 kDa phosphoproteins detected in sperm in capacitating medium resulted in a numerical, but not significant decline in its presence due to ROS. Sperm incubated in capacitating medium for four hours had more 94 kDa phosphotyrosine protein than sperm incubated in BTS (P<0.0003); exposure to the ROS generating system had no additional effect. Only sperm incubated in capacitating medium for four hours showed tyrosine phosphorylation of 58 kDa proteins. Exposure to the ROS generating system prior to the capacitating incubation reduced, but did not eliminate, the appearance of the 58 kDa phosphoprotein. The tyrosine phosphorylations of the lower molecular mass proteins (48, 39, 35, 31, 28, and 23 kDa) were not affected by ROS, incubation time or medium.

Overall, exposing boar spermatozoa to an ROS generating system for 30 minutes and then incubating four hours in capacitating medium inhibited tyrosine phosphorylation of proteins that otherwise were phosphorylated under these capacitating conditions. ROS decreased tyrosine phosphorylation of proteins with kDa of 174 (P<0.035) and had no effect on tyrosine phosphorylation of proteins 39 and 35 kDa (Fig. 4.2).
Figure 4.2. Effect of preincubating boar sperm ± ROS generating system on intensity of tyrosine-phosphorylated proteins present during subsequent capacitation. Proteins were extracted from boar sperm treated ± ROS generating system after 30min as detailed in figure 4.1 legend and incubated for four hours in BTS medium or capacitating medium (Cap). Intensity of immunoblotted bands was quantified by Image Quant software. Values are means (SE: see appendix 4); n=4 ejaculates. a, b, c: bars within a kDa with different superscripts differ (P<0.05). □ Sperm incubated in BTS for 30 min only (control; BTS-30 min), ▪ sperm exposed to ROS for 30 min (treated; ROS-30 min), ▲ sperm incubated in BTS only for 4h (control; BTS-4h), ▼ sperm exposed to ROS for 30 min and then incubated in BTS for 4h (treated, BTS-ROS-4h), ▼ sperm incubated in capacitation medium for 4 h (control; Cap-4h), ▼ sperm exposed to ROS for 30 min and then incubated in capacitation medium for 4 h (treated; Cap-ROS-4h).
Interestingly, tyrosine phosphorylated proteins were very different when the pH was not very carefully controlled. The pH of this system consistently and readily rose to $\sim 7.9 - 8$ unless it was controlled by periodic addition of HCl. Incubating boar sperm in capacitating medium (regardless of pre-exposure to ROS generating system) when its pH was allowed to naturally rise, induced tyrosine phosphorylation of boar sperm, generating a distinctly different phospho-protein profile than that obtained when the pH was kept at $\sim 7.4$ (Fig. 4.3).

Figure 4.3. Effect of pH on tyrosine phosphorylation of boar sperm proteins. Two immunoblots of the same boar's sperm: A) sperm incubated for 4hr in capacitating medium or BTS ± pre-exposure to ROS generating system, pH uncontrolled, (B) sperm incubated for 4hr in capacitating medium or BTS ± pre-exposure to ROS generating system, pH controlled to ($\sim 7.5$). Proteins from $5 \times 10^6$ sperm were extracted by sample buffer, electrophoresed, and immunoblotted using the-anti-phosphotyrosine antibody as described under Material and Methods. Lane 1: sperm incubated for 30 min in BTS only (BTS-30 min; control), lane 2: sperm incubated in ROS generating system for 30 min (ROS-30 min; treated), lane 3: Control sperm incubated for four hours in BTS only (BTS-4h), lane 4: Control sperm incubated for four hours in capacitating medium (Cap.-4h), lane 5: treated sperm incubated in BTS for four hours (BTS-ROS-4h), lane 6: treated sperm incubated in capacitating medium for four hours (Cap-ROS-4h). The molecular masses are indicated at the left side of the gel.
4.6. Discussion

Unlike sperm of other species, exposing boar spermatozoa to the ROS generating system xanthine/xanthine oxidase followed by four hours incubation in capacitating medium (Figure 4.1, 4.2) inhibited tyrosine phosphorylation of proteins of high molecular mass, particularly proteins of 174 kDa. Protein tyrosine phosphorylation/dephosphorylation has been implicated in the regulation of different cellular functions (Hubbard and Till, 2000), and in boar sperm, increasing the degree of tyrosine phosphorylation of protein is associated with sperm capacitation (Kalab et al., 1998; Flesch et al., 1999; Tardif et al., 2001, 2003; Bravo et al., 2005). Furthermore, our recent findings indicate that this ROS generating system has been associated with increasing acrosome reaction of capacitated boar sperm (Chapter 3).

The present study confirmed that this capacitating system tyrosine-phosphorylated several proteins in sperm incubated under capacitating conditions for four hours. Non-capacitated sperm had phospho-tyrosine on proteins of 105, 48, 39, 35, and 31 KDa, while sperm incubated for 4hr under capacitating conditions also displayed phosphorylation of proteins of 174, 105, 94, 58, 48, 39, 35, 28 and 23 kDa, all of which are similar to the kDAs of proteins identified by Kalab et al. (1998) in capacitated boar spermatozoa. The p34 kDa in the Kalab et al. (1998) study is likely identical to the p35 identified here. The present p39 might contain three separated proteins of 36/38, 40 and 44 kDa (Berruti and Martegani, 1989; Kalab et al., 1998). Both these studies only presented and discussed results from one individual gel of an individual ejaculate, so comparing those results to these obtained with the much more rigorous approach taken
here of quantifying protein profiles from multiple ejaculates from different boars, is difficult. The multiple ejaculate approach, with the amount and kDa of each protein present statistically analysed through unbiased Image Quant analysis of bands and their intensity, more closely represents the phospho-protein profile of sperm from all boars, while reducing the attention paid to possibly minor proteins unique to an individual boar. This population-type of investigation is more likely to generate an understanding of how sperm from a species respond, which was the rationale for this rigorous experimental design.

An enhanced presence of the 32 kDa protein has been noted repeatedly in capacitated boar sperm (Tardif et al., 2001; 2003; Bravo et al., 2005; Dube et al., 2005), but both the current results and those of Kalab et al. (1998) showed no increased amount of this protein in capacitated boar sperm. Although the 32 kDa tyrosine phosphorylation is calcium-dependent, it is not a pre-requisite for capacitation in boar sperm (Tardif et al., 2003). Furthermore, capacitation-dependent phosphorylation of an approximately 32 kDa protein was obtained when pH was not controlled and consequently rose above physiological levels (Fig. 4.3A, lane 4). Methodological differences (Tardif et al., 2001) such as capacitation conditions (the components of capacitating medium, incubating temperature, pH), protein extraction method (reduced or non reduced SDS-PAGE (with/without β-mercaptoethanol), the % of polyacrylamide gel used, blocking buffer and many other possible experimental circumstances can affect either cellular mechanisms and subsequent protein phosphorylation, or affect the size and migration characteristics of the extracted proteins. In this study, sperm proteins were extracted using non-reducing
conditions (SDS-PAGE; without β-mercaptoethanol) and separated with a 10% gel, while Tardif and co-workers (Tardif et al., 2001; 2003, Dube et al., 2005) used reducing conditions to extract proteins and separated them with 12% gel. Although the gel percent should only affect degree and speed of separation and not the interpretation derived by comparison against molecular mass standards run simultaneously, non-reducing conditions could allow proteins to retain more three-dimensional structure, affecting rate of movement through the gel, and thereby change apparent kDa. In addition, the importance of the effect of pH, clearly documented here for the first time to impact the actual nature of protein phosphorylation and not just apparent kDa, cannot be underestimated.

The major goal of the present study was to identify the role of ROS in regulation of protein tyrosine phosphorylation in boar spermatozoa under capacitating and non-capacitating conditions. Different studies on different mammalian sperm species, such as human (de Lamirande et al., 1997b) equine (Baumber et al., 2003), and bovine (Rivlin et al., 2004) showed that at physiological concentration, ROS induced sperm capacitation, acrosome reaction and tyrosine phosphorylation. Unexpectedly, and in partial congruence with, and partial contradiction of, the hypothesis, ROS had differential effects on tyrosine phosphorylation of different proteins in boar spermatozoa. The ROS generating system inhibited tyrosine phosphorylation of proteins with high kDa. The ROS hydrogen peroxide also inhibited tyrosine phosphorylation in bull spermatozoa incubated for four hours under capacitating conditions (Rivlin et al., 2004). In boar sperm studies (Guthrie and Welch, 2006; Chapter 3) H₂O₂ has been identified as the main ROS generated during
the capacitation of sperm previously exposed to xanthine/xanthine oxidase for 30 min.

H$_2$O$_2$ at controlled concentrations induces sperm capacitation, acrosome reaction and tyrosine phosphorylation (Ford, 2004; Rivlin et al., 2004) consequent to an increase in cAMP, activation of tyrosine kinase and/or inhibition of tyrosine phosphatase (Hechet and Zick, 1992; Nakamura et al., 1993; Ford, 2004). ROS often change and modulate their target function by oxidizing the sulfhydryl groups through chemical reactivity (Cooper et al., 2002). ROS modulate the redox of a sulfhydryl/disulfide (SH/SS) pair on proteins (reviewed in de Lamirande and O’Flaherty, 2008), which can break or form a protein disulfide bridge, converting cysteine into cystine and thereby modulate cell signaling. Oxidation by ROS can result in a disulfide bridge (cystine) between two adjacent thiols in one protein, or between different proteins, resulting in changes of protein structure and function (Cooper et al., 2002). This type of modification is reversible and specific and could affect the transduction elements that are involved in sperm capacitation (reviewed in de Lamirande and O’Flaherty, 2008), because a sulfhydryl/disulfide pair regulates the activity of tyrosine and Ser/Thr protein phosphatase, PKA, PKC, PTK and adenyl cyclase. Thus, H$_2$O$_2$ in the current study could be generated in sufficiently high concentrations to reverse the capacitation-associated inhibition or activation of tyrosine kinase or tyrosine phosphatase or both which in turn inhibited tyrosine phosphorylation of the high kDa proteins, particularly the 174 kDa. Thus, maybe H$_2$O$_2$ at this concentration inhibited the components of other pathways that associated directly or indirectly with tyrosine phosphorylation, such as the components of ERK family of MAPK signaling pathway. These components were found recently to be important participants in the human sperm capacitation and tyrosine phosphorylation (de
Lamirande and Gagnon, 2002; O'Flaherty et al., 2005). Since the tyrosine phosphorylation of proteins 93, 175, and 220/230 kDa is up regulated by cAMP (Kalab et al., 1998), H$_2$O$_2$ might inhibit the cAMP/PKA pathway and maybe other pathways such as ERK that regulate tyrosine phosphorylation of high kDa proteins.

In conclusion, boar sperm capacitation is accompanied by a set of tyrosine phosphorylated proteins with molecular weights of 23, 28, 35, 39, 48, 58, 94, 105, and 174 kDa, and we demonstrate for the first time there is a physiological role for ROS in regulating protein tyrosine phosphorylation of boar spermatozoa. The ROS generating system (xanthine/xanthine oxidase) inhibited tyrosine phosphorylation of proteins with high kDa (174 kDa), which could be regulated by different signaling pathways at the same time. We suggest that, in addition to cAMP-dependent PKA pathway, H$_2$O$_2$ possibly regulates protein tyrosine phosphorylation of boar spermatozoa by mediating other signaling pathways, such as the ERK pathway, which phosphorylates different proteins depending on their molecular mass. Thus, more studies are needed to investigate the physiological role of ROS in meditating signaling pathways that could regulate tyrosine phosphorylation of boar spermatozoa, such as the ERK family of MAPK pathway.
5. Extracellular signal-regulated kinases (ERKs) family of mitogen-activated protein kinase (MAPK) pathway and reactive oxygen species are involved in the regulation of tyrosine phosphorylation in boar spermatozoa.

5.1. Abstract

This study hypothesized that the extracellular signal-regulated kinases (ERKs) family of mitogen-activated protein kinase (MAPK) pathway are associated with capacitation and tyrosine phosphorylation of fresh boar spermatozoa, and that Reactive Oxygen Species (ROS) modulate the signal transduction of this pathway during sperm capacitation.

To detect the presence of the phosphorylated forms of the ERK pathway components Raf, MEK1/2, and ERK1/2 and to how determine ROS affect their phosphorylation, fresh boar spermatozoa were incubated for 30 min ± ROS generating system (1mM xanthine + 0.1 U xanthine oxidase per ml) at 38 °C, then incubated in BTS or capacitating medium (5×10⁶ spermatozoa/ml; 4hr) and the extracted proteins were immunoblotted using anti-phospho-Raf1, MEK1/2, ERK1/2 and anti-phosphotyrosine antibodies and quantified using Image Quant software. Phosphorylated forms of Raf-1, MEK1/2, and ERK1/2 were detected, and the protein profile differed with capacitation. The ROS generating system inhibited tyrosine phosphorylation of proteins with high molecular mass, and affected the phosphorylation of Raf-1, MEK1/2, and ERK1/2.

To determine how ROS and the ERK pathway are involved in capacitation-induced tyrosine phosphorylation of boar spermatozoa, freshly-ejaculated sperm were incubated for 30 min with inhibitors of specific elements of the ERK pathway, Raf (6 μM ZM
336372), MEK1 and 2 (1 µM U0126), or ERK1/2 (20 µM ERK Activation Inhibitor Peptide I, Cell-Permeable) in the presence or absence of an ROS generating system at 38°C, then either diluted in BTS or capacitating medium (5×10^6 spermatozoa/ml) and incubated for four hours. Proteins from 5×10^6 sperm were then extracted, immunoblotted using anti-phosphotyrosine antibody, and the intensity and kDa of detected proteins were quantified. Inhibiting ERK1/2 inhibited tyrosine phosphorylation of capacitated boar spermatozoa proteins of 172, 97 and 66 kDa (P<0.04), while the combination of ERK1/2 inhibitor and ROS generating system inhibited tyrosine phosphorylation of the same three proteins to a much greater extent (P<0.002) and also inhibited the tyrosine phosphorylation of the 111 kDa protein (P<0.028). Inhibiting MEK1/2 in the presence of the ROS generating system subsequently inhibited tyrosine phosphorylation of proteins of 187(P<0.01) and 112 kDa (P<0.04) after 4h incubation in capacitating medium compared to sperm incubated in capacitating medium alone or pre-exposed to ROS generating system and then incubated in capacitating medium for 4h respectively.

This study documents for the first time that the extracellular signal-regulated kinases (ERKs) of mitogen activated protein kinase pathway significantly regulate tyrosine phosphorylation in boar spermatozoa through its ERK1/2 component. The upstream regulators of ERK1/2, Raf-1 and MEK1/2 are possibly partially associated with the phosphorylation of Thr-Glu-Tyr motif of ERK1/2, pointing to the possibility that other kinases of different pathways and their crosstalk interaction, such as cAMP/PKA (cyclic AMP and Protein Kinase A) and Protein Kinase C (PKC), may have roles in phosphorylating the Thr-Glu-Tyr motif of ERK1/2 pathway. Finally, ROS have time
dependent effects on the phosphorylation the proteins of the Raf, MEK1/2 and ERK1/2 components of the ERK1/2 pathway of boar spermatozoa, suggesting that ROS have roles in regulating sperm capacitation and tyrosine phosphorylation through their association with ERK and other pathways elements.

These results also suggest that cross-talk interactions of other pathways, such as cAMP/PKA and PKC and ROS - ERK1/2 - tyrosine phosphorylation could influence the sequential events of boar sperm capacitation.

5.2. Introduction

To acquire fertilizing capacity, fresh spermatozoa need to undergo the process of capacitation to acquire the ability to reach and bind to the zona pellucida, undergo the acrosome reaction and fertilize an oocyte during their migration in the female genital tract (Yanagimachi, 1994). This process involves several components of signal transduction pathways (Yanagimachi, 1994; de Lamirande et al., 1997a). Although the mechanisms by which capacitation is controlled are not completely known, studies indicate that different signal transduction elements may regulate this process, such as protein kinase A (PKA), its substrates, and the cAMP/PKA-dependent tyrosine phosphorylation of fibrous sheath proteins (Visconti et al., 1995b; Leclerc et al., 1996; Visconti et al., 1997; Aitken et al., 1998; Harrison, 2004; O’Flaherty et al., 2004), protein kinase C (PKC) (Thundathil et al., 2002), protein tyrosine kinases (PTK) (Visconti et al., 1995b; Leclerc et al., 1996; Visconti et al., 1997; Aitken et al., 1998; Harrison, 2004; O’Flaherty et al., 2004), and components of the extracellular signal regulated kinase
(ERK) family of mitogen-activated protein kinase (MAPK) pathway (Thundathil et al., 2002; de Lamirande and Gagnon, 2002; O'Flaherty et al., 2005; O'Flaherty et al., 2006ab; de Lamirande and O'Flaherty, 2008). Regulation may be in association with Reactive Oxygen Species (ROS; de Lamirande and Gagnon, 2002; O'Flaherty et al., 2005; 2006ab; de Lamirande and O'Flaherty, 2008) such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO$^-$; de Lamirande and O'Flaherty, 2008).

The MAPK or ERKs are serine-threonine kinases involved in signal transduction of the extracellular stimuli (Luconi et al., 1998b) and connect cell-surface receptors to the regulatory targets within cells (Chang and Karin, 2001). MAPK exist in all eukaryotic cells and are activated by diverse stimuli ranging from cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adherence (Windmann et al., 1999). The basic assembly of MAPK pathways is a module of three kinases, conserved from yeast to humans, which are activated by sequential phosphorylation of each other. The three components of this module are MAPK kinase kinase (Raf), MAPK kinase (MEK), and MAPK (ERK1/2; Windmann et al., 1999; Kolch, 2000).

In mammalian cells, there are three MAPK families: the ERKs, e-Jun amino-terminal kinases (JNKs), and the p38 MAP kinases (p38) (Dimuthu et al., 1998; Windmann et al., 1999; Prowese et al., 2000) and each MAPK family has distinct biological functions (Windmann et al., 1999). The ERK pathway of the three MAP kinase pathways of mammalian cells is the best defined and is comprised of different isoforms (subfamilies) of Raf (A-Raf, B-Raf, and Raf1), MEK (many isoforms), and ERKs (5 isoforms; Segere
and Krebs, 1995; Cheng et al., 1996). However, ERK1 and ERK2 (ERK1/2; 44 and 42 kDa respectively) are the most extensively studied and MAPK is referred to as ERK (Windmann et al., 1999). The upstream cascade of the ERK pathway consists of the adaptor proteins of She and Grb2 (each of them has SH2 and SH3 domains that interact upon activation) and Sos (son of sevenless; a guanidine exchange factor) and the GTP-binding proteins of Ras or Rho family (Windmann et al., 1999; Kolch, 2000).

There are different receptor types that can activate the ERK pathway. The activation of receptor tyrosine kinase by growth factors, such as EGF, PDGF or Insulin (Segere and Krebs, 1995; Marais and Marshal, 1996) provides the binding site of the adapter protein Grb2 that in turn localizes Sos to the plasma membrane. Sos activates Ras by exchange of Ras-bound GTP with GDP, converting Ras into its activated conformation. Activated Ras in turn activates Raf1, the Serine-Threonine (Ser/Thr) MAPK kinase kinase, which then phosphorylates MEK, the dual-specificity Serine-Threonine-Tyrosine (Ser/Thr and Tyr) MAP kinase, and this dual-specificity MAP kinase activates ERK1/2, the Ser/Thr MAPK, by phosphorylating its two residues, Tyr and Thr that are located in the active site of the Thr-Glu-Tyr motif (Winddmann et al., 1999; Kolch, 2000). The activated Ser/Thr kinase ERK can phosphorylate over 80 substrates in the cytoplasm and the nucleus and either regulates gene expression directly by phosphorylating transcription factors such as Ets, Elk, and Myc, or indirectly by targeting substrates such as p90-RSK (ribosomal S6 kinase) family kinases, which can modify transcription factors and histones (Davie and Spencer, 2001). In addition, the cytoplasmic phospholipase A2 (cPLA2) is one of the major targets of the activated ERKs (Windmann et al., 1999; Kolch, 2000).
The other MAP kinase cascade (JNK/SAPK; c-jun kinase/stress activated protein kinase) and p38 are activated in response to different stimuli. The JNK cascade is activated following exposure to UV radiation, heat shock, or inflammatory cytokines while p38 is activated in response to inflammatory cytokine, endotoxins, and osmotic stress (Person et al., 2001).

Proteins extracted from capacitated and non-capacitated boar spermatozoa and immunoblotted with different anti-MAPK antibodies showed the presence of the MAPK family ERK5 in boar spermatozoa at Mr32,000 in capacitated spermatozoa only (Bailey et al., 2005). In addition, enzyme renaturation assays with myelin basic protein, revealed the presences of MAPK-like kinase of Mr 42,000 in capacitated sperm only, while the presence of MAPK like enzymes of Mr 42,000 and Mr 44,000 were active in capacitated and non capacitated boar spermatozoa, suggesting the association of the MAPK family with boar sperm capacitation (Bailey et al., 2005). Also, Berruti (1994) identified the 42 kDa boar sperm protein kinase as a dual- specificity protein kinase belonging to the MAPK family.

Human spermatozoa contain all the components of the ERK family of MAPK and its upstream regulators from Shc to ERK (de Lamirande and Gagnon, 2002), and are associated with sperm capacitation (Luconi et al., 1996; O'Flaherty et al., 2006ab; de Lamirande and O'Flaherty, 2008) and the acrosome reaction (Luconi et al., 1998b; Liguori et al., 2005; de Lamirande and O'Flaherty, 2008). Antiphospho-MEK antibody
detected three proteins in capacitated human sperm (55, 94, and 115 kDa) that were located along the flagellum; their presence was prevented by PD98059 and U0126, which are inhibitors of MEK (O’Flaherty and de Lamirande, 2005). The ERK pathway may aid in acquisition of motility in mouse sperm (Lu et al., 1999) and regulation of flagellar motility in fowl spermatozoa (Ashizawa et al., 1997).

Different ligands, such as cytokines, growth factors, and hormones, stimulate the generation of ROS in most mammalian cells (Meier et al., 1989; Lo and Cruz, 1995, Sundaresan et al., 1995). ROS are considered as injurious by-products of cellular metabolism and excessive amounts of ROS damage DNA (Lopes et al., 1998; Bennetts and Aitken, 2005), inhibit sperm-oocyte fusion (Aitken et al., 1989), and reduce sperm motility (human, de Lamirande and Gagnon, 1992; equine, Baumber et al., 2000). However, when produced in very low and controlled concentrations, ROS regulate cell signaling and functions (Ford, 2004).

Sperm capacitation is an oxidative process which is associated with producing small amounts of ROS such as O$_2^-$, H$_2$O$_2$, and NO' (O’Flaherty et al., 2006a) and promoting signal transduction pathways associated with capacitation. ROS at very low concentrations are known as regulators of protein tyrosine phosphorylation (Ford, 2004) through cAMP/PKA and the ERK pathways. These two signal mechanisms act independently and contribute to the modulation of protein tyrosine phosphorylation required for spermatozoa to achieve capacitation (O’Flaherty and de Lamirande, 2005).
In addition to the study of de Lamirande and Gagnon (2002), which reported that human sperm capacitation and tyrosine phosphorylation are regulated by $O_2^-$ in association with all ERK cascades, recent studies have demonstrated that generating ROS during sperm capacitation regulate most sperm capacitation pathways in human spermatozoa. Generating ROS during capacitation induces the increase of cAMP, activation of PKA and its substrate phosphorylation (at the arginine-X-X-serine/threonine motif), phosphorylation of MEK, then ERK1/2 (at the threonine-glutamate-tyrosine motif) and tyrosine phosphorylation of fibrous sheath proteins. In addition, the acquisition of hyperactivated motility and onset of the acrosome reaction in association with the different kinases such as PKC, PKA, PTK, and the ERK pathway kinases and their crosstalk interaction, support the concept that multiple parallel pathways contribute to the timely co-ordination of various sperm functions needed to fertilize an oocyte (reviewed in O'Flaherty et al., 2006a; de Lamirande and O'Flaherty, 2008).

ROS modulate the ERK pathway in many cell types (Thannickal and Fanburg, 2000). In Her14 fibroblasts cells, $H_2O_2$ induces phospholipase $A_2$ (PLA$_2$) activity through the Raf-MEK-p42/44 MAPK pathway and oxidizing lipids, thereby increasing the substrate availability for cytoplasmic PLA$_2$ (cPLA$_2$). $H_2O_2$ also induced time-dependent lipid peroxidation measured as degradation of arachidonate and linoleate and the formation of aldehydic degradation products. Finally, $H_2O_2$ induced translocation of cPLA$_2$ to the membrane fraction in a calcium-independent fashion, with a concomitant increase in cPLA$_2$ activity (van Rossum et al., 2004).
Capacitation of boar sperm is poorly understood in comparison with sperm capacitation in other species such as mouse and human (Bailey et al., 2005). Understanding the elements and the different pathways that control boar sperm capacitation and acrosome reaction will optimize boar sperm preservation and eventually aid the artificial insemination industry. Very little is known about the role of MAPKs or ERK pathway in boar sperm capacitation and tyrosine phosphorylation. Thus, the goals of this study were: 1) to detect the phosphorylated forms of ERK pathway components (Raf, MEK1/2, and ERK1/2) in boar spermatozoa using different anti-phospho-MAPKs antibodies, in the presence and absence of ROS; 2) to determine how exposure to an ROS generating system (xanthine/xanthine oxidase) would affect tyrosine phosphorylation and the ERK pathway of capacitated and non capacitated boar spermatozoa; 3) to investigate the role of the ERK pathway in capacitated and non capacitated boar sperm on tyrosine phosphorylation using anti-phosphotyrosine antibody and inhibitors of the ERK pathway components (Raf, MEK1/2, and ERK1/2) in the presence and absence of ROS.

5.3. Materials and methods

5.3.1. Semen handling

5.3.1.1. Semen collection: Boars of proven fertility were housed according to the requirement of the Canadian Council on Animal Care. The sperm rich fraction was collected using the gloved-hand method into a 35 °C Thermos®, immediately assessed for motility, and only ejaculates with motility of greater than 70% were used. Sperm concentration was determined by a calibrated spectrophotometer (Spectronic 20, Milton Roy, Rochester, New York) at an excitation of 550 nm.
5.3.1.2. **ROS generating system:** To generate $O_2^{-}$ and $H_2O_2$ in boar spermatozoa, an ROS generating system of xanthine and xanthine oxidase (Sigma, Oakville, ON, Canada) was used (Guthrie and Welch, 2006). Xanthine stock (20 mM in 1M NaOH) was diluted to 2 mM in water (pH 7.5) and 0.2 U of xanthine oxidase was added to 0.5 ml of 2mM xanthine. The pH was adjusted using Accument® pH Meter 925 (Fisher Scientific, ON, Canada) by adding drops of NaOH or HCl solution. This adjustment was carried out repeatedly over two hours while the medium was stirred, which resulted in the medium’s pH remaining stable; without this lengthy pH adjustment, the medium’s pH rose within 5 minutes.

5.3.2. **Experiment 1: The role of ERK signaling pathway and ROS on modulating tyrosine phosphorylation.**

5.3.2.1. **Treatments:**

Freshly-collected sperm (n=1 ejaculate from each of 4 boars) were mixed with BTS (final concentrations: $3 \times 10^7$ sperm/ml; 205.37mM Glucose (dextrose), 20.4 mM Sodium citrate dihydrate, 14.88 mM NaHCO$_3$, 3.36 mM EDTA, 10.01mM KCl, streptomycin containing 770 mg/g (activity); pH 7.4; Pursel and Johnson, 1975) ± ROS generating system and incubated for 30 min. After the 30 minutes, sperm were either processed immediately for protein extraction ($5 \times 10^6$ spermatozoa /ml) or diluted into BTS or capacitating medium (4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 95 mM NaCl, 5.55 mM Glucose (Dextrose), 25 mM NaHCO$_3$, 2mM CaCl$_2$, 2 mM Pyruvate 0.4% BSA (bovine serum albumen, fatty acid free, fraction V; Sigma, Oakville, ON, Canada; pH 7.4; Tardif et al., 2001; $5 \times 10^6$ spermatozoa/ml), and incubated for four hours at 36 °C, 5% CO$_2$ and 100%
humidity. The pH of the capacitating medium was also adjusted repeatedly over two hours while the medium was stirred, which resulted in the medium’s pH remaining stable; without this lengthy pH adjustment, the medium’s pH rose within 5 minutes.

5.3.2.2. Protein extraction, SDS-PAGE and western blotting:
At the appropriate incubation time, 1 ml containing $5 \times 10^6$ spermatozoa were taken for protein characterization. Proteins were extracted by adding 20 μl of 10 mM Na$_2$VO$_3$ (Fisher, Mississauga, ON, Canada) to the sperm aliquots, centrifugation (13000g, 10 min, room temperature), and sperm pellets re-suspended with 50 μl of 0.2 mM Na$_2$VO$_3$, 12.5 μl of 5x sample buffer [(containing 0.350 g dithiothreitol (DTT)], 0.5g SDS, 2.0 ml Tris (1M; pH 6.8), 2.5 ml of 50% glycerol, and 150 μl of 2.5% Bromophenol Blue) and boiled for 5 minutes. The boiled sperm preparation was centrifuged (13000g, 5 min, room temperature) and the supernatant kept on ice until used for SDS-PAGE.

The extracted proteins were subjected to SDS-PAGE on a 4% polyacrylamide stacking gel / 10% running gel for 15 minutes at 75V and then at 100V at room temperature for 150 minutes or until the dye front reached the bottom of the gel. The gel was equilibrated in transfer buffer (Bio-Rad; Mississauga, ON, Canada; containing 25 mM Tris, 192 mM Glycine and 20% (v/v) methanol; pH 8.3) for 30 minutes at room temperature and then transferred electrophoretically (100V) to Immobilon-PVDF-transfer membrane (Millipore, ON, Canada) for 60 minutes at 4 °C using transfer buffer. Non-specific binding was blocked by incubating membranes with 5% (w/v) skim milk powder in Tris.
(20 mM, pH 7.8)-buffered saline containing 1% Tween 20 (Fisher, Mississauga, ON, Canada; TTBS; 0.1% v/v) overnight at 4 °C with gentle agitation.

To detect the phosphorylated forms of ERK pathway components Raf, MEK1/2, and ERK 1/2, membranes were incubated with anti-phospho-Raf-1(Ser338/Tyr340; affinity purified rabbit polyclonal IgG; Upstate, NY 12946, USA; 1:1000 in TTBS supplemented with 1% Tween 20) or antiphospho-MEK1(Ser218/222)/MEK2(Ser222/226); rabbit monoclonal IgG (Upstate, NY 12946, USA; 1:2000 in TTBS supplemented with 1% Tween 20) or rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) affinity purified polyclonal antibody (Chemichon International) for 2 hours at room temperature with gentle agitation. Membranes were washed with TTBS (3×10 minutes) and then incubated with the anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (from donkey; NA934V, GE Healthcare UK Limited, Little Chalfont Buckinghamshire England; 1:2500 in TTBS supplemented with 1% Tween 20) for 60 minutes at room temperature with gentle agitation and washed with TTBS (3×10 minutes) and then twice with milliQ water.

To assess sperm capacitation status and the effect of ROS generating system on tyrosine phosphorylation for each membrane, the last two wells of each gel were loaded with the same amount of proteins from the same sperm aliquots that were either incubated with BTS only or with ROS generating system and then incubated for four hours in capacitating medium. After transferring, the last two lanes of each membrane were cut out using a scalpel and straight edge and then incubated with monoclonal anti-phosphotyrosine (4G10™), HRP conjugate antibody; Upstate, NY 12946, USA; 1:2000
in TTBS supplemented with 1% Tween 20) for two hours with gentle agitation and then washed with TTBS (3×10 minutes) and then twice with milliQ water.

Positive immunoreactive bands were detected using the enhanced chemiluminescence detection system (Luminol, 97% HPLC, Sigma; Kodak autoradiography films; Sigma-Aldrich) and their volume, area and kDa were measured using Image Quant TL.lnk software (Amersham Bioscience) and then bands’ intensities were calculated.

To quantify the amount of proteins that were loaded in each well, a membrane from one replicate of each experiment was stripped at 37 °C for 60 min using stripping buffer (restore western blot stripping buffer; PIERCE, Rockford, IL 61105 USA), washed with TTBS (1x), blocked with 5% (w/v) skim milk powder in Tris (20 mM, pH 7.8)-buffered saline containing 1% Tween 20 for 60 min at room temperature with gentle agitation. After 60 min blocking, the membranes were incubated with anti-bovine α-tubulin, mouse monoclonal antibodies (anti-bovine α-tubulin and biotinylated anti-bovine α-tubulin; Molecular Probes; 1:2000 in TTBS) for 2 hours at room temperature with gentle agitation, washed with TTBS (3×10 minutes) and then incubated with the polyclonal goat anti-mouse IgG, HRP antibody (Upstate, NY 12946, USA; 1:2500 in TTBS), washed with TTBS (3×10 minutes) and twice with milliQ water. Positive immunoreactive bands and their volume, area and kDa were measured and bands’ intensities were calculated as detailed above.
5.3.3. Experiment 2: The effect of inhibiting ERK signaling pathway components on boar sperm tyrosine phosphorylation

5.3.3.1. ERK pathway components inhibitors:

The inhibitors of ERK pathway components, C-Raf (ZM 336372; Calbiochem), MEK1 and 2 (U0126; Calbiochem), and ERK (ERK Activation Inhibitor Peptide I, Cell-Permeable; Calbiochem) were prepared in 1% DMSO as stock solutions of 2.568, 1.0, and 2.845 mM respectively, aliquotted and stored at -20 °C in the dark until used.

5.3.3.2. Treatments:

To investigate each of the 3 ERK pathway components inhibitors (Raf, MEK1/2, and ERK1/2), four freshly-collected ejaculates (n=1 ejaculate from each of 4 boars) were used. Sperm were diluted (final concentrations: $3 \times 10^7$ sperm/ml) in BTS ± ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml) ± an ERK pathway inhibitor [Raf, 6 µM ZM 336372; MEK 1/2 1µM U0126; ERK1/2, 20 µM ERK Activation Inhibitor Peptide I, Cell-Permeable] and incubated for 30 min at 38 °C.

Aliquots from each of the treatments were taken after the 30 minutes incubation and either diluted into BTS or capacitating media ($5 \times 10^6$ spermatozoa/ml), and incubated for four hours at 36 °C, 5% CO₂ and 100% humidity.

5.3.3.3. Protein extraction, SDS-PAGE and western blotting:

After the 4hr incubation, sperm proteins were extracted and subjected to SDS-PAGE as detailed above. To detect tyrosine phosphorylated proteins, membranes were incubated
with a monoclonal antibody (1:2000 in TTBS supplemented with 1% Tween 20) developed in mouse against phosphotyrosine proteins (Anti-Phosphotyrosine (4G10™), HRP conjugate; Upstate, NY 12946, USA) for 2 hours at room temperature with gentle agitation. Membranes were then washed with TTBS (3×10 minutes) and twice with milliQ water. Positive immunoreactive bands and their volume, area and kDa were measured and bands’ intensities were calculated as detailed above.

5.4. Statistical analysis

Data were analyzed using The Mixed Procedure in SAS 9.1 (SAS 9.1 for Windows TS Level 1M3, The SAS Institute Inc., Cary, NC, USA). Band intensities were analyzed as a split-plot in which the 4 boars represented the blocks and treatment (sperm preincubated with ROS generating system) versus control (sperm preincubated with BTS only) was the main plot factor. Image Quant band intensities after 30 minutes and after 4 hours incubation (Experiment 1) after 4 hours (Experiment 2) or either with capacitating medium or BTS (control and treatment), were the subplot factor. All data were log-transformed prior the analysis. The differences of Least Squares Means, Tukey-Kramer test were used to detect the differences among different treatments and controls.

To analyse the differences of Least Squares Means between tyrosine phosphorylated protein intensity of sperm incubated in capacitating medium (control) and sperm treated with ROS generating system and then incubated in capacitating medium(treatment), the General Linear Model (GLM) procedure in SAS 9.1 was used followed by Tukey test. Values of P<0.05 were considered to be statistically significant.
5.5. Results

5.5.1. Experiment 1: The role of ERK signaling pathway and ROS on modulating tyrosine phosphorylation.

To determine the effect of pre-incubation with ROS on protein tyrosine-phosphorylation after 4hr incubation, the kDa and intensity data of tyrosine phosphorylated protein bands of all trials were pooled. Incubating sperm for four hours under capacitating conditions induced tyrosine phosphorylation of proteins with molecular masses of 158.06±2.85 (~158), 109.76±1.84 (~110), 96.1±2.01 (~96), 75.92±1.84 (~76), 70.62 ±2.09 (~71), 60.76±0.46 (~61), 50.19±0.33 (~50), 46.56±0.42 (~47), 39.86±0.13 (~40), 36.13±0.12 (~36), 32.54±0.4 (~32), 27.77±0.28 (~28) and 24.51±0.18 (~25) kDa (Fig. 5.1). The presence of proteins of 71, 47 and 32 kDa was detectable in some trials but not all. Exposure to the ROS generating system before incubation in the capacitating conditions inhibited tyrosine phosphorylation of proteins 158 (P<0.005), 110 (P<0.02), 96 (P<0.03), 71 (when detectable, P<0.009), and 47 kDa (when detectable, P<0.049) and tended to reduce the 76 and 61 kDa proteins (P<0.14) compared to sperm incubated in capacitating medium without exposure to ROS (Fig. 5.1 A & B).
Figure 5.1. Effect of preincubating boar sperm ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent capacitation. Boar spermatozoa were treated either with ROS generating system (1 mM xanthine + 0.1U xanthine oxidase per ml) or BTS and incubated (30 min, 38 °C) and then diluted in capacitating medium and incubated for four hours. Proteins from $5 \times 10^6$ sperm were extracted and membranes immunoblotted using anti-phosphotyrosine antibody as described under Material and Methods. The molecular masses are of commercial protein standards indicated at the left side of the gel. A. Typical immunoblot B. kDa and band intensity quantified by Image Quant software. Values are means (SE: see appendix 5); n=12 ejaculates. (a, b) Within a kDa, values with no superscripts in common differ (P<0.05). Sperm incubated with capacitating medium for 4h (Cap-4h; control) sperm treated with ROS generating system for 30 min then incubated with capacitating medium for 4h hours (Cap-ROS-4h).
Raf-1 was present in boar sperm, as identified by the positive binding of anti-phospho-Raf-1 (Ser 338/Tyr340) antibody (Fig. 5.2). The kDa and amount of those Raf-1 proteins differed depending on incubation conditions and time (Fig. 5.3), and variously included proteins with kDAs of: 194.9±7.93 (~195), 96.5±3.2 (~96), 81.9±0.93 (~82), 57.3±0.9 (~57), 51.4±0.33 (~51), 35.4±0.16 (~35) and 24.4±0.42 (~24). Sperm incubated in BTS for 30 min contained almost solely proteins of 35 kDa, with notable but lesser amounts of 82 and 51 kDa proteins, minor amounts of 195 and 24 kDa, and no detectable other phospho-Raf proteins.

Capacitation affected phospho-Raf proteins. Sperm incubated for 4hr in BTS displayed different phospho-Raf profiles than those incubated in capacitation media (Fig.5.3). While the 24 kDa sperm protein disappeared after 4 hr incubation in either media, after 4hr all sperm, regardless of media or pretreatment, displayed a 96 kDa protein. Capacitating conditions induced the appearance of a 57 kDa phospho-Raf protein that was absent in sperm incubated in BTS, while sperm incubated for 4hr in BTS had more phospho-Raf 35 kDa protein than did those incubated in capacitation medium. Capacitating conditions also induced a significant decline in the phospho-35 kDa protein from the amount present at 30min.

Generation of ROS also significantly affected sperm phospho-Raf proteins, with the specific ROS-induced effects differing between incubation media (Fig.5.3). ROS generation immediately increased the 51 kDa phospho-protein compared to the 30 minute control (P<0.0059), and this level was sustained for 4hr incubation in BTS, but
significantly reduced in sperm incubated in capacitating conditions (P<0.0076). The amount of the 35 kDa protein declined significantly after 4hr incubation in capacitation medium, but there was no significant decline in the 35 kDa phospho-Raf protein in sperm exposed to ROS before incubation in capacitation medium (Fig. 5.3).

Figure 5.2. Effect of preincubating boar sperm ± ROS generating system on phospho-Raf1 proteins detected after subsequent incubation. Boar spermatozoa were treated either with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml) or BTS and then either diluted in BTS or capacitating medium and incubated for four hours. Proteins from 5x 10^6 sperm were extracted at the indicated times and membranes immunoblotted using anti-phospho-Raf-1 (Ser338/Tyr340) affinity purified rabbit polyclonal IgG antibody (primary antibody) and anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (secondary antibody). The molecular masses of commercial protein standards are indicated at the left side of the gel.
Figure 5.3. Effect of preincubating boar sperm ± ROS generating system on intensity of phospho-Raf proteins present during subsequent incubation. Proteins were extracted from boar sperm treated ±ROS generating system as detailed in figure 5.2 legend and incubated for four hours either in BTS or capacitating medium (Cap). Intensities of immunoblot bands were quantified by Image Quant software. Values are means (SE: see appendix 6); n=4 ejaculates. a, b, c: within a kDa, values with no superscripts in common differ (P<0.05). □ Sperm incubated with BTS for 30 min (BTS-30 min; control), ■ sperm treated with ROS generating system for 30 min (ROS-30 min), ▪ sperm incubated with BTS for 4h (BTS-4h; control), ■ sperm incubated with ROS generating system for 30 min then incubated with BTS for 4h (BTS-ROS-4h), ▪ sperm incubated with capacitation medium for four hours (Cap-4h; control), ■ sperm treated with ROS generating system for 30 min then incubated in capacitation medium for 4h (Cap-ROS-4h).
The antiphospho-MEK1 (Ser 218/222)/MEK2 (Ser 222/226); rabbit monoclonal antibody detected two major proteins of 60.4 ±1.06 (~ 60), and 41.8± 1.12 (~ 42) kDa in boar spermatozoa and three minor proteins of 50.2±1.94 (~50), 27.036±3.16 (~ 27 kDa), and 22.3 ±1.1 (~22) kDa (Fig. 5.4 and 5.5). Exposure of sperm to capacitating conditions did affect MEK1/2, as did exposure to ROS. The 60 kDa protein was only detected in sperm after 4hr incubation in capacitating medium, independent of ROS pre-exposure, while the 27 kDa protein disappeared under the same conditions. The amounts of the minor 50 and 22 kDa proteins did not change significantly with time or media or ROS exposure, although ROS pre-exposure tended to increase the amount of the 50 kDa phospho-MEK protein present after 4hr incubation in capacitating medium (P=0.063 vs. 4hr after no ROS exposure). The 42 kDa phospho-MEK1/2 decreased in sperm incubated under capacitating conditions, tending to significance in sperm not exposed to ROS (P=0.059 vs. 30 min), and significantly in those subjected to the ROS generating system compared to sperm subjected to ROS generating system and then incubated for 4hr in BTS (Fig. 5.5).
Figure 5.4. Effect of preincubating boar sperm ± ROS generating system on phospho-MEK1/2 proteins detected after subsequent incubation. Boar spermatozoa were treated either with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml; 30 min, 38 °C) or BTS and then either diluted in BTS or capacitating medium and incubated for four hours. Proteins from $5 \times 10^6$ sperm were extracted at the indicated times and membranes immunoblotted using anti-phospho MEK1(Ser218/222)/MEK2 (Ser222/226); rabbit monoclonal IgG antibody (primary antibody) and anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (secondary antibody). The molecular masses of commercial protein standards are indicated at the left side of the gel.
Figure 5.5. Effect of preincubating boar sperm ± ROS generating system on intensity of phospho-MEK1/2 proteins present during subsequent incubation. Proteins were extracted from boar sperm treated ± ROS generating system as detailed in figure 5.4 legend and incubated for 4h either in BTS or capacitating medium (Cap). Intensities of immunoblot bands were quantified by Image Quant software. Values are means (SE: see appendix 7); n=4 ejaculates. a, b, c: within a kDa, values with no superscripts in common differ (P<0.05). □ Sperm incubated with BTS for 30 min (BTS-30 min; control), ■ sperm treated with ROS generating system for 30 min (ROS-30 min), ● sperm incubated with BTS for 4h (BTS-4h; control), ▲ sperm incubated with ROS generating system for 30 min then incubated with BTS for 4h (BTS-ROS-4h), ■ sperm incubated with capacitation medium for four hours (Cap-4h; control), ▼ sperm treated with ROS generating system for 30 min then incubated in capacitation medium for 4h (Cap-ROS-4h).
The rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) affinity purified polyclonal antibody, detected 8 phosphorylated proteins in an incubation-dependent fashion (Fig. 5.6 and 5.7). These proteins were: 158.2 ± 2.65 (~158), 106.6 ± 4 (~107), 79.95 ± 0.72 (~80), 60.6 ± 0.91 (~61), 52.3 ± 0.17 (~52), 46.4 ± 0.2 (~46), 32.4 ± 0.21 (~32), and 23.8 ± 0.13 (~24) kDa. Proteins 158 and 61 kDa were only present in sperm incubated in capacitating medium for four hours (± ROS pre-exposure), proteins of 32 and 24 kDa were absent from sperm after 4hr capacitation, and proteins 107 and 46 kDa were only present in sperm at 30 min (± ROS). The proteins of 80 and 52 kDa were present in sperm in all media, and the amount of each was significantly increased after 30 min exposure to the ROS generating system. This higher level was apparently maintained over the 4hr incubations, but the difference was only statistically significant for the 52 kDa phospho-ERK after incubation in BTS for 4hr (Fig. 5.6 and 5.7). The ROS generator also tended to immediately increase the 24 kDa phospho-ERK (P=0.10).
Figure 5.6. Effect of preincubating boar sperm ± ROS generating system on phospho-ERK1/2 proteins detected after subsequent incubation. Boar spermatozoa were treated either with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml; 30 min, 38 °C) or BTS and then either diluted in BTS or capacitating medium and incubated for four hours. Proteins from $5 \times 10^6$ sperm were extracted at the indicated times and membranes immunoblotted using rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) affinity purified polyclonal antibody (primary antibody) and anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (secondary antibody). The molecular masses of commercial protein standards are indicated at the left side of the gel.
Figure 5.7. Effect of preincubating boar sperm ± ROS generating system on intensity of phospho-ERK1/2 proteins present during subsequent incubation. Proteins were extracted from boar sperm treated ± ROS generating system as detailed in figure 5.6 legend and incubated for 4h either in BTS or capacitating medium (Cap). Intensities of immunoblot bands were quantified by Image Quant software. Values are means (SE: see appendix 8); n=4 ejaculates. a, b, c: Within a kDa, values with no superscripts in common differ (P<0.05).

- □ Sperm incubated with BTS for 30 min (BTS-30 min; control), ■ sperm treated with ROS generating system for 30 min (ROS-30 min), □ sperm incubated with BTS for 4h (BTS-4h; control), ■ sperm incubated with ROS generating system for 30 min then incubated with BTS for 4h (BTS-ROS-4h), ■ sperm incubated with capacitation medium for four hours (Cap-4h; control), ■ sperm treated with ROS generating system for 30 min then incubated in capacitation medium for 4h (Cap-ROS-4h).
To ensure that equal amounts of proteins were loaded in each well, gels (N=3) were stripped and reprobed for alpha-tubulin (Fig.4.8), and when the intensities were analysed it was confirmed that there were no differences (P>0.05) in the amount of proteins that were loaded in the wells of the different treatments.

Figure 5.8. α-tubulin protein content during capacitation of boar sperm pre-incubated ± ROS generating system and then capacitated. Boar spermatozoa were treated either with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml; 30 min, 38 °C) or BTS and then either diluted in BTS or capacitating medium and incubated for four hours as indicated. Proteins from 5×10^6 sperm were extracted at the indicated times and membranes immunoblotted using anti-bovine α-tubulin, mouse monoclonal antibodies and polyclonal goat anti-mouse IgG, HRP antibody (primary antibody) and polyclonal goat anti-mouse IgG, HRP antibody (secondary antibody). The molecular mass of commercial protein standard is indicated at the left side of the gel.

5.5.2. Experiment 2: The effect of inhibiting ERK signaling pathway components on boar sperm tyrosine phosphorylation

5.5.2.1. Raf inhibitor (ZM336372): Boar spermatozoa incubated for four hours displayed tyrosine phosphorylation of several proteins with different molecular weights, including proteins of 194.8±7.66 (~195), 131.7±3.26 (~132), 97.2 ±1.75 (~ 97), 82.8±2.63 (~ 83), 63.6± 2.0 (~ 64), 53.2± 25 (~ 53), 46.98± 0.43 (~ 47), 37.3±0.26 (~ 37), and 33.2± 0.2 (~ 33) kDa (Fig. 5.9, lane 3 and Fig. 5.10).
Figure 5.9. Effect of preincubating boar sperm ± ZM336372 (C-Raf inhibitor; 6μM) ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent incubation. Boar spermatozoa were treated with BTS ± ZM336372 (6 μM) ± ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml; 30 min, 38 °C) and then either diluted in BTS or capacitating medium and incubated for 4 h. Proteins from $5 \times 10^8$ sperm were extracted and immunoblotted using the-anti-phosphotyrosine antibody as described under Material and Methods. Lane 1: sperm incubated for 4 h in BTS only (BTS; control), lane 2: sperm incubated with ZM336372 for 30 min and then in BTS for 4 h (BTS+ZM336372), lane 3: Control, sperm incubated for 4h in capacitating medium only (Cap), lane 4: Sperm incubated with ZM336372 for 30 min and then for 4h in capacitating medium (Cap+ZM336372), lane 5: Sperm incubated with ROS generating system for 30 min and then incubated in BTS for 4h (BTS+ROS), lane 6: Sperm incubated with ZM336372 and ROS generating system for 30 min and then in BTS for 4h (BTS+ROS+ZM336372), lane 7: Sperm incubated with ROS generating system for 30 min then in capacitating medium for 4h (Cap+ROS), lane 8: Sperm incubated with ZM336372 and ROS generating system for 30 min and then in capacitating medium for 4h (Cap+ROS+ZM336372). The kDa of the molecular mass standards are indicated at the left side of the gel.
Treating spermatozoa with 6 μM of ZM336372 for 30 minutes and then incubating for four hours in capacitating medium under capacitating conditions produced highly variable amounts of the 195, 132, 97, 83, 64, 53, 47, 37, and 33 kDa phospho-tyr proteins, so the apparent numerical declines due to ZM 336372, as with apparent ROS-induced declines in the 195, 132, 97, and 53 kDa, were not significant (P>0.05; Fig 5.9, lanes 4 and 7 and Fig 5.10). The 64kDa protein was only detected under capacitating conditions in the presence of ROS.

![Graph showing protein intensity](image)

Figure 5.10. Effect of preincubating boar sperm ± ZM336372 (C-Raf inibitor; 6μM) ± ROS generating system on intensity of tyrosine-phosphorylated proteins present during subsequent incubation. Boar sperm were pre-incubated for 30 minutes in BTS or ROS generating system ± ZM336372 as detailed in figure 5.9 legend and incubated for four hours in capacitating medium (Cap). Bands intensity was quantified by Image Quant software. Values are means (SE: see appendix 9); n=4 ejaculates. Within a protein, intensities did not differ among treatments. Sperm incubated in capacitation medium for four hours only, Sperm treated with ZM336372 for 30 min then incubated in capacitation medium for four hours, Sperm treated with ROS generating system for 30 min then incubated in capacitation medium for four hours.
5.5.2.2. MEK1/2 inhibitor (U0126): Capacitating medium induced tyrosine phosphorylation of proteins 298.2±12.7 (~298), 186.6±5.55 (~187), 102.9±1.43 (~103), 80.98±1.64 (~81), 70.5±0.57 (~71), 56.9±0.5 (~57), 49.7±0.34 (~50), 39.6±0.09 (~40), 35.4±0.12 (~35), 30.98±0.6 (~31), 27.3±0.16 (~27), and 23.99±0.28 (~24) kDa proteins. Of these proteins, only two (40 and 35 kDa) proteins were prominent in boar spermatozoa incubated with BTS buffer for four hours, although occasional slight evidence of phosphorylated proteins at 50 and 24 kDa, in addition to 112±2.4 kDa was detected in these non-capacitating conditions (Fig. 5.11, lanes 1 and 3 and Fig. 5.12). All other tyrosine-phosphorylated proteins were only detected in sperm incubated under capacitating conditions.
Figure 5.11. Effect of preincubating boar sperm ± U0126 (MEK1/2 inhibitor; 1µM) ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent incubation. Boar spermatozoa were treated with BTS ± U0126 (1µM) ± ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml; 30 min, 38 °C) and then either diluted in BTS or capacitating medium and incubated for 4h. Proteins from 5× 10⁶ sperm were extracted and immunoblotted using the anti-phosphotyrosine antibody as described under Material and Methods. Lane 1: sperm incubated for 4h in BTS only (BTS; control), lane 2: sperm incubated with U0126 for 30 min and then in BTS for 4h (BTS+ U0126), lane 3: Control, sperm incubated for 4h in capacitating medium only (Cap), lane 4: Sperm incubated with U0126 for 30 and then for 4h in capacitating medium (Cap+ U0126), lane 5: Sperm incubated with ROS generating system for 30 min and then incubated in BTS for 4h (BTS+ROS), lane 6: Sperm incubated with U0126 and ROS generating system for 30 min and then in BTS for 4h (BTS+ROS+ZM336372), lane 7: Sperm incubated with ROS generating system for 30 min then in capacitating medium for 4h (Cap+ROS), lane 8: Sperm incubated with U0126 and ROS generating system for 30 min and then in capacitating medium for 4h four (Cap+ROS+ZM336372). The kDa of the molecular mass standards are indicated at the left side of the gel.
Incubating spermatozoa with 1μM of U0126 inhibitor for 30 minutes and then for four hours either in capacitating medium or BTS, had no significant effect on inhibiting tyrosine phosphorylation, but numerically reduced tyrosine phosphorylation of proteins 81 and 31 kDa when spermatozoa were incubated in capacitating medium (Fig. 5.12).

Figure 5.12. Effect of preincubating boar sperm ± U0126 (MEK1/2 inhibitor; 1μM) ± ROS generating system on intensity of tyrosine phosphorylated proteins present during subsequent incubation. Boar sperm were incubated for 30 min in BTS or ROS generating system ± U0126 as detailed in figure 5.11 legend and incubated for four hours in either BTS or Cap. Bands intensity of immunoblotting was quantified by Image Quant software. Values are means (SE; see appendix 10); n=4 ejaculates. Within a protein, intensities did not differ among treatments. □ Sperm incubated in BTS only for 4h (BTS; control), ■ sperm treated with U0126 for 30 min and then incubated in BTS for 4h (U0126 in BTS), ▲ sperm incubated in capacitation medium for 4h only (Cap; control), ▼ sperm treated with U0126 for 30 min and then incubated in capacitation medium for 4h (U0126 in Cap).
Exposure to ROS for 30 minutes and subsequent incubation for four hours in capacitating medium reduced tyrosine phosphorylation of high kDa proteins (298, 187, 103 kDa; Fig. 5.13) numerically but not significantly, and stimulated tyrosine phosphorylation of a 112 kDa protein not seen in capacitation media alone. ROS pre-incubation eliminated appearance of three minor proteins (81, 31 and 24 kDa) present in capacitating medium-incubated sperm. Although pre-exposure to 1μM of U0126 inhibitor alone had no effect on tyrosine phosphorylation of proteins (P>0.05) from sperm subsequently incubated in capacitating medium, when the pre-incubation included both ROS generating system and U0126 inhibitor before the capacitating incubation there was significantly less 187 (P<0.01) proteins. There tended to be less 103 (P<0.15), and 71 (P<0.14) kDa proteins compared to sperm incubated in capacitating medium alone (control). Also there was significantly less 112 kDa proteins (P<0.04; Fig. 5.13) compared to sperm pre-exposed to ROS and then incubated in capacitating medium for four hours.
Figure 5.13. Effect of preincubating boar sperm ± U0126 (MEK1/2 inhibitor; 1µM) ± ROS generating system on intensity of tyrosine phosphorylated proteins present during subsequent incubation in capacitating medium. Boar sperm were pre-incubated for 30 minutes in BTS or ROS generating system ± U0126 as detailed in figure 5.11 legend and incubated for four hours in capacitation medium. Bands' intensity was quantified by Image Quant software. Values are means (SE; see appendix 10); n=4 ejaculates. a,b Values differ (P<0.05). ■ sperm incubated in capacitation medium only for 4h (Cap; control), ■ sperm treated with U0126 for 30 min and then incubated in capacitation medium for 4h (U0126 in Cap), ■ sperm incubated with ROS generating system for 30 min and then in capacitation medium for four hours (ROS+Cap), ■ sperm treated with U0126 and ROS generating system for 30 min and then incubated in capacitation medium for four hours (U0126 + ROS in Cap).
5.5.2.3. ERK 1/2 inhibitor (ERK Activation Inhibitor Peptide I, Cell-Permeable):

Eight tyrosine phosphorylated proteins with different molecular weights were present in sperm incubated in capacitation medium with kDa’s of: 172.01±8.49 (~172), 111.2±2.68 (~111), 97.2±2.87 (~97), 65.7±1.0 (~66), 53.4±0.6 (~53), 47.3±0.3 (~47), 38.7± (~39), and 34.5±0.25 (~35). Of these, only proteins 111, 39, and 35 kDa proteins were phosphorylated in BTS medium (Fig. 5.14, lanes 1, and 3, and Fig. 5.15).

Exposing boar spermatozoa to 20 µM of ERK inhibitor, or the ROS generating system, alone or in combination, followed by 4hr incubation in capacitation medium, inhibited tyrosine phosphorylation of different proteins (Fig. 5.14, lanes 4, 7 and Fig. 5.16).
Figure 5.14. Effect of preincubating boar sperm ± ERK inhibitor (20 μM) ± ROS generating system on tyrosine phosphorylation of sperm proteins during subsequent incubation. Boar sperm were preincubated in BTS ± ERK inhibitor (20 μM) ± ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml; 30 min, 38 °C) and then either diluted in BTS or capacitating medium and incubated for 4 h. Proteins from 5 × 10⁶ sperm were extracted and immunoblotted using the anti-phosphotyrosine antibody as described under Material and Methods. Lane 1: sperm incubated for hours in BTS only (BTS; control), lane 2: sperm incubated with ERK inhibitor for 30 min and then in BTS for four hours (BTS+ ERK inhibitor), lane 3: Control, sperm incubated for 4 h in capacitating medium only (Cap), lane 4: Sperm incubated with ERK inhibitor for 30 and then for 4 h in capacitating medium (Cap+ ERK inhibitor), lane 5: sperm incubated with ROS generating system for 30 min and then incubated in BTS for 4 h (BTS+ROS; treated), lane 6: Sperm incubated with ERK inhibitor and ROS generating system for 30 min and then in BTS for 4 h (BTS+ROS+ ERK inhibitor; treated), lane 7: Sperm incubated with ROS generating system for 30 min then in capacitating medium for four hours (Cap+ROS; treated), lane 8: Sperm incubated with ERK inhibitor and ROS generating system for 30 min and then in capacitating medium for four h (Cap+ROS+ ERK inhibitor; treated). The kDa of the molecular mass standards are indicated at the left side of the gel.
Sperm incubated with ERK inhibitor for 30 minutes and then for four hours in capacitation medium reduced the amount of 172 (P<0.03), 97 (P<0.014) and 66 (P<0.04) kDa phospho-tyr proteins present and had no effects on other proteins (P>0.05; Fig. 5.15) in comparison with sperm incubated in capacitation medium alone. Pre-incubation with ERK inhibitor, ROS generator or both had no effect on sperm subsequently incubated in BTS (Fig. 5.15).

Figure 5.15. Effect of preincubating boar sperm ± ERK inhibitor (20μM) ± ROS generating system on intensity of tyrosine-phosphorylated proteins present during subsequent incubation. Boar sperm were pre-incubated for 30 minutes in BTS or ROS generating system ± ERK inhibitor and then incubated for 4 hr in BTS or capacitating media. Bands intensity of immunobloting was quantified by Image Quant software. Values are means (SE: see appendix 11); n=4 ejaculates. a, b Values differ (P<0.05). □ Sperm incubated in BTS only for four hours (BTS; control), ■ sperm treated with ERK inhibitor for 30 min and then incubated in BTS for four hours (ERK inh. in BTS), □ sperm incubated in capacitation medium for four hours only (Cap; control), □ sperm treated with ERK inhibitor for 30 min and then incubated in capacitation medium for four hours (ERK inh in Cap).
The ROS generating system alone did not significantly reduce the amount of tyrosine phosphorylated proteins (although it tended to reduce the 97 kDa; P<0.11) of sperm incubated in capacitation medium, but exposure to the combined ERK inhibitor and ROS strongly inhibited tyrosine phosphorylation of various proteins [172 (P<0.0005), 111 (P<0.028), 97 (P<0.0008), and 66 (P<0.0022) kDa] when incubated in capacitating medium in comparison with sperm that were incubated with capacitating medium without pre-incubation (Fig. 5.16).

![Figure 5.16](image)

Figure 5.16. Effect of pre-incubating boar sperm ± ERK inhibitor (20μM) ± ROS generating system on intensity of tyrosine phosphorylated proteins present during subsequent incubation in capacitating medium. Boar sperm were pre-incubated for 30 minutes in BTS or ROS generating system ± ERK inhibitor as detailed in figure 5.14 legend and then incubated for 4h in capacitation medium. Intensity of immunoblot bands was quantified by Image Quant software. Values are means (SE; see appendix 11); n=4 ejaculates. a, b, c: Values with no superscripts in common differ (P<0.05). GSperm incubated in capacitation medium only for four hours (Cap; control), H sperm treated with ERK inhibitor for 30 min and then incubated in capacitation medium for 4h (ERK inh in Cap), [HT| sperm incubated with ROS generating system for 30 min and then in capacitation medium for 4 h (ROS+Cap), [HT| sperm treated with ERK inhibitor and ROS generating system for 30 min and then incubated in capacitation medium for four hours (ERK inh + ROS in Cap).
ERK inhibitor, ROS generating system, or the combination of ROS generating system and ERK inhibitor had no effect on amount of tyrosine phosphorylated proteins in sperm subsequently incubated in BTS.

5.6. Discussion:
This study elucidates the pathway by which extracellular signal-regulated kinases of mitogen-activated protein kinase regulate tyrosine phosphorylation in capacitating boar spermatozoa, and how the pathway(s) interact with ROS. The ERK1/2 component is the major regulatory component, although it might be partially regulated by the upstream Raf-1 or MEK1/2. Furthermore, generation of ROS inhibits tyrosine phosphorylation of proteins with high molecular mass, and affects phosphorylation of Raf-1, MEK1/2, and ERK1/2. These detailed investigations contribute significantly to our understanding of the signaling pathways involved in porcine sperm capacitation.

Tyrosine phosphorylated proteins were present in sperm in experiments 1 and 2 in this study, and the majority of these tyrosine phosphorylated proteins always were induced in sperm exposed to capacitating conditions and not in sperm incubated in the non-capacitating BTS medium. Tyrosine phosphorylation has long been associated with capacitation in boar sperm (Kalab et al., 1998; Flesch et al., 1999; Tardif et al., 2001; 2003; Bravo et al., 2005). Tyrosine phosphorylation is regulated by different specific signaling pathways during sperm capacitation including PKA, PKC (Thundathil et al., 2002), cAMP/PKA-dependent tyrosine phosphorylation of fibrous sheath protein and protein tyrosine kinase (Visconti et al., 1995b; Leclerc et al., 1996).
There was some small variability in the molecular mass of the tyrosine phosphorylated proteins detected among the individual trials within experiment 2 (section 5.5.2.1, 5.5.2.2 and 5.5.2.3), and when comparing experiments 1 (section 5.5.1) and 2. In examining the patterns of all the protein profiles, strong similarities exist, suggesting that thirteen proteins, or groups of proteins, can be readily identified. These are: 1] proteins with molecular mass ranging from 158 to 195, this range probably reflecting the lack of accuracy of the gel at separating proteins at this extreme mass; 2] a group of average kDa of approximately ~116 kDa (different trials reported as 110, 132, 112 and 111); 3] ~98 kDa (96, 97, 103, and 97); 4] ~80 kDa (76, 83 and 81); 5] ~71 kD (consistent when present); 6] ~62 kDa (61, 64, 57 and 66); 7] ~52 (50, 53, 50 and 53); 8] ~47 kDa (consistent); 9] ~39 (40, 37, 40 and 39); 10] ~35 (36, 33, 35 and 35); 11] ~32 kDa (31 and 32); 12] ~28 (27 and 28); 13] ~25 (24 and 25). This variability may be due to unique characteristics of the individual ejaculates, minor variations in the capacitation conditions on the day of the individual replicate, running gels, and to the accuracy of scanning the gels and using the Image Quant software by which all molecular masses were detected. An holistic examination of the results of the multiple replicates within the two experiments, all of which were carefully quantified in an unbiased fashion, identifies two major proteins (~39 and ~35 kDa) present in non-capacitating conditions, while capacitating medium induced tyrosine phosphorylation of all proteins mentioned above. The kDa of these capacitation-dependent phospho-tyr proteins identified here are very similar to those identified under the same capacitating conditions in Chapter 4, except Chapter 4 lacked the ~ 80, ~ 71, and 32 kDa proteins; the cause of these minor variations are suggested identified above could.
The kDas of these capacitation-dependent proteins are similar to the kDas of proteins identified by Kalab et al (1998) in capacitated boar spermatozoa, with the presence of a 39 kDa potentially encompassing three separate proteins of 36/38, 40 and 44 kDa (Berruti and Martegani, 1989; Kalab et al., 1998). The 32 kDa protein, occasionally seen here in sperm exposed to capacitating conditions, has been noted repeatedly in capacitated boar sperm (Tardif et al., 2001; 2003; Bravo et al., 2005; Dube et al., 2005), and its appearance is known to be calcium-dependent and not an absolute prerequisite for capacitation in boar sperm (Tardif et al., 2003). As the pH impacts the actual nature of protein phosphorylation (Chapter 4), considerable care was required to maintain the pH in a range of 7.3-7.4. Other authors have not reported, and may not have considered, this important factor.

ROS inhibited the capacitation-dependent tyrosine phosphorylation of various proteins, including those with high kDas, and those of ~110, 96, 71, and 47 kDa. This inhibition was similar to the results documented in Chapter 4, in which ROS pre-incubation inhibited tyrosine phosphorylation of proteins with high kDa. This may well be an action of H$_2$O$_2$, which is the major ROS species produced by the XA/XO ROS generating system in boar spermatozoa (Guthrie and Welch, 2006; Chapter 3 of this study). H$_2$O$_2$ may inhibit the cAMP/PKA pathway since this pathway has been involved in the up-regulation of tyrosine phosphorylation of proteins with high molecular mass in boar sperm (Kalab et al., 1998; Bravo et al., 2005); H$_2$O$_2$ may perhaps modulate tyrosine kinase or phosphatase activity (Ford, 2004).
The components of the capacitation medium, particularly bicarbonate and calcium, plays an important role in the capacitation process of pig sperm (Tardif et al., 2003), affecting the induction of signaling in the cAMP/PKA/PTK pathway (Kalab et al., 1998; Flesch et al., 1999; and Bravo et al., 2005)

This study demonstrates, for the first time, that ERK pathway components (Raf, MEK, and ERK1/2) exist in boar spermatozoa and are activated in their association with capacitation and tyrosine phosphorylation signaling.

The presence of the MAP Kinases of the ERK pathway was demonstrated first by detecting their presence with specific antibodies for each component, and secondly by observing the tyr-phosphorylated, capacitation-induced, proteins present after specifically inhibiting each ERK-pathway component. The three anti-phospho MAPKs antibodies (anti-phospho Raf-1, anti-phospho MEK1/2, and anti-phospho ERK1/2) each recognized a number of unique phosphorylated proteins, although they all recognized one protein with a very similar kDa. This protein was 57, 60, and 61 kDa detected by anti-phospho Raf-1, MEK1/2, and ERK1/2 antibodies respectively, and 61 kDa detected by anti-phosphotyrosine antibody. This approximately 60 kDa protein is possibly a dual specificity (Ser/Thr and Tyr) phosphorylated protein since its phosphorylation was detected on Ser338/Tyr340, Thr202/Tyr204, Ser218/222 and Ser222/226 residues by antiphospho-Raf-1, ERK1/2 and MEK1 and MEK2 antibodies respectively. Thus, it is possible that this protein has Ser/Tyr residues in its motif, and upon its capacitation-dependent activation, anti-phospho-Raf and anti-phospho-ERK1/2 antibodies detected its
phosphorylation on Tyr residues, while anti-phospho-MEK 1/2 antibody detected its phosphorylation on Ser residues.

Other proteins detected by the different anti-phospho MAPKs antibodies were unique to the particular antibody, supporting the specificity of binding. Unfortunately, no commercially verified positive controls were available to detect and confirm the specific molecular weights of Raf, MEK1/2 and ERK1/2 proteins.

Fresh boar sperm in BTS demonstrated Raf-like proteins primarily of 35, 51, and 82 kDa. Incubation of sperm under capacitating conditions for 4hr reduced the amount of the 35 kDa protein and maintained amounts of the other proteins (Fig. 5.3). While capacitation introduced significant amounts of a 57 kDa phospho-protein, this was the protein detected by all the antibodies, and so cannot be assumed to truly be Raf. When Raf action was inhibited by pre-incubating sperm with ZM336372, a potent and specific inhibitor of the protein kinase c-Raf (IC50= 70 nm; Hall-Jackson et al., 1999), capacitation-associated tyrosine phosphorylation was not affected (Fig. 5.10), which may mean that Raf has no direct control over capacitation, but could also mean that the inhibition of the Raf pathway is compensable, presumably through cross-talk with other pathways. The rapid increase of the 51 kDa phospho-Raf when sperm were exposed to ROS generation (Fig. 5.3) indicates Raf's sensitivity to reactive oxygen species, and may have been achieved by the cAMP/PKA pathway (Ford, 2004; de Lamirande and O’Flaherty, 2008). That the ROS-induced high level of phospho-51 Raf was maintained in sperm subsequently incubated for 4hr incubation in BTS, but was completely reduced to control
levels in sperm subsequently incubated in capacitating medium, indicates that unique and complex signaling pathways are operative in capacitation. ROS exposure also affected the high molecular mass phospho-Raf proteins. Changing the high kDa phospho-Tyr proteins is a late-capacitation event, occurring downstream of many parallel pathways (O’Flaherty et al., 2005; O’Flaherty et al., 2006ab; de Lamirande and O’Flaherty, 2008), further supporting the contention that Raf’s role in capacitation signaling is compensable due to pathway crosstalk.

It is also possible that the ZM336372 inhibitor of Raf-1 failed to inhibit tyrosine phosphorylation because it cut off a negative feedback mechanism involved in Raf-1 signaling. Raf is commonly activated by its upstream activator, Ras, and MEK is the only commonly accepted downstream substrate for Raf (Morrison and Cutler, 1997; Schaeffer and Weber, 1999). Raf activates MEK on its two serine residues in the activation loop and the Raf inhibitors ZM336372 and SB203580 failed to block the activation of MEK and ERK by growth factors and phorbol esters and actually activated Raf-1 when administered to cells (Hall-Jackson et al., 1999). It was suggested that Raf-1 activity is controlled by negative feedback usually initiated by Raf-1 itself, so Raf inhibitors, by cutting off the negative feedback, can thereby activate Raf-1 (Hall-Jackson et al., 1999). If negative feedback were crucial for Raf-1 action in sperm, then ZM336372 would not effectively inhibit Raf-1, and the results obtained could not be reliably interpreted.

Alternatively, assuming that Raf induces capacitation-dependent tyrosine phosphorylation through the classic Raf-MEK-ERK pathway, then capacitation could
proceed due to Raf's inhibition if another path existed that could phosphorylate MEK and/or ERK. In fact, many other kinases such as MEK-1, mos or Tpl-2 are associated with phosphorylating the same serine on the activation loop of MEK that Raf kinase phosphorylates (Windmann et al., 1999). Thus, even if ZM336372 inhibited Raf-1 in this study, MEK1/2 could still have been phosphorylated through crosstalk interaction with these other kinases. Raf itself could be activated by crosstalk with other pathways such as PKC (Van Der Hoeven et al., 2000), while cAMP/PKA crosstalk can regulate ERK1/2 phosphorylation (Bornfeldt and Krebs, 1999; Rice et al., 2001).

After Raf, the next component of the typical ERK signalling pathway is MEK. This study identified MEK's existence in boar sperm, in a capacitation-associated fashion, for the first time. The monoclonal anti-phospho-MEK1(Ser218/222)/MEK2 (Ser222/226), which recognizes MEK1/MEK2 (45 kDa), detected two major phospho-proteins in boar spermatozoa, at 60 and 42 kDa, and other minor phospho-proteins including 50, 27 and 22 kDa proteins (Fig. 5.4 & 5.5). Several of these appear to be unique to the boar, since a very similar antibody (anti-phospho-MEK1/2 antibody Ser 217/Ser221), recognized three phosphorylated proteins with molecular masses of 55, 94 and 115 kDa in capacitating human sperm (O'Flaherty et al., 2005), and the higher kDa proteins were not detected in boar sperm under either capacitating or non-capacitating conditions. The 60 kDa protein present in capacitated sperm cannot be specifically identified as MEK, but the 42 kDa phospho-protein is of considerable interest. This may be the dual-specificity protein kinase that has long been acknowledged to be present in the MAPK family of boar spermatozoa but has not been identified (Berruti, 1994). This 42 kDa phospho-MEK is a
reasonable candidate, because it is recognized by the MEK1/2 antibody, and because MEK1/2 elements are known as dual specificity proteins (Windmann et al., 1999).

This 42 kDa phospho-MEK appears to be involved in capacitation. Incubating sperm in capacitating conditions, but not BTS, reduced the amount of phospho-42-MEK protein, and prior exposure to ROS generation enhanced this decrease. This decrease, coupled with the complete disappearance of the 27 kDa phospho-MEK protein, subsequent to 4h incubation in capacitating medium (± ROS), could indicate that MEK proteins have a role in regulating capacitation-dependent tyrosine phosphorylation. The loss of phospho-42 MEK protein occurred simultaneously with the significant inhibitory effect of ROS generating system on tyrosine phosphorylation of proteins with high molecular masses subsequent to 4h incubation in capacitation medium.

However, using U0126, a potent and specific inhibitor of MEK1/2 (IC50 = 72 nM for MEK1 and 58 for MEK2; Favata et al., 1998) could not significantly inhibit tyrosine phosphorylation of boar spermatozoa (Fig. 5.12), suggesting that, like Raf, MEK1/2 may either not be directly involved, or its actions may be supplemented by crosstalk pathways. Tyrosine can be phosphorylated through many parallel pathways such as PKA, its substrates, and the cAMP/PKA-dependent tyrosine phosphorylation known to affect such sperm components as fibrous sheath proteins (Visconti et al., 1995b; Leclerc et al., 1996; Visconti et al., 1997; Aitken et al., 1998; Harrison, 2004; O’Flaherty et al., 2004), PKC (Thundathil et al., 2002), PTK (Visconti et al., 1995b; Leclerc et al., 1996; Visconti et al., 1997; Aitken et al., 1998; Harrison, 2004; O’Flaherty et al., 2004), and in addition to
The active site of ERK1/2 associated with capacitation and tyrosine phosphorylation (O'Flaherty et al., 2006ab) is a Thr-Glu-Tyr motif. Both Raf and MEK1/2 are elements of the ERK pathway existing as upstream regulators of this motif which is only partially associated with tyrosine phosphorylation, so inhibiting their activity might not affect tyrosine phosphorylation detectably. Also, the Thr-Glu-Tyr motif can be phosphorylated by other tyrosine kinases such as PKA, PTK and PKC, directly or through their crosstalk interaction (O'Flaherty et al., 2005; 2006ab; de Lamirande and O'Flaherty, 2008).

The next step in the signaling pathway, ERK, appeared to play a vital role in boar sperm capacitation. The polyclonal antiphospho-ERK1/2 (Thr202/Tyr204), which recognizes the activated forms of ERK1 and ERK2 (p44 and p42 kDa), detected a variety of phospho-ERK proteins, the exact nature of which was dependent on capacitation status and ROS exposure.

Two proteins, of 158 and 61 kDa, only appeared in sperm after capacitation incubation. The ubiquitous 60 kDa phospho-protein cannot be confirmed as ERK, having been bound by all other MAPK and Tyr-P antibodies. The 158 kDa protein, on the other hand, was only identified by the ERK1/2 and the phospho-tyrosine antibodies, suggesting that this protein may therefore be a capacitation-dependent, tyrosine phosphorylated-ERK1/2 protein. Two low molecular mass proteins, of 32 and 24 kDa, disappeared when incubated for 4h in capacitating medium, but remained evident in BTS incubation.
The ROS-induced phospho-52 ERK1/2 protein matches the kDa of the phospho-51 Raf-1 protein, which was also stimulated by the ROS generating system, and so they possibly are the same protein. To be detected by the two antibodies suggests that this protein possesses Tyr 340 and Tyr204 residues that are detected by anti-phosph-Raf1 and anti-phospho-ERK1/2 upon activation and have similar antigenic sequences. ROS also stimulated an immediate significant increase of phospho-80 kDa ERK, the magnitude of which declined after the sperm were incubated for 4hr in either BTS or capacitation media, in a similar manner to that of the 52kDa protein. Therefore these two phospho-ERK proteins appear to be rapidly induced in the presence of ROS, but are not maintained in the absence of continual exposure to ROS. And the increase in these phospho-ERKs does not appear to be related to capacitation.

That ERK1/2 has a crucial role in boar sperm capacitation was demonstrated for the first time here, when inhibiting ERK significantly reduced, or virtually eliminated, the capacitation-dependent induction of phospho-tyrosine proteins of 172, 111, 97, and 66 kDa (Fig.5.16). This indicates for the first time the importance of the ERK1/2 component of the ERK pathway in capacitation and tyrosine phosphorylation of boar spermatozoa.

Preincubating sperm with the ERK inhibitor decreased the amount of tyrosine-phosphorylated proteins induced by capacitating conditions (Fig.5.16) and pre-exposing sperm to a combination of ROS and the ERK inhibitor enhanced that decline (Fig.5.16); a similar although less global effect was seen when the MEK1/2 inhibitor was combined with ROS in the pre-incubation (Fig.5.13). This ROS generating system (1 mM xanthine
+ 0.1 U xanthine oxidase per ml) inhibits tyrosine phosphorylation of high molecular mass proteins from boar spermatozoa (Chapter 4) whose phosphorylation are regulated by cAMP-dependent pathway (Kalab et al., 1998; Bravo et al., 2005). Thus, the combination of ROS and MEK inhibition may possibly act as a complex inhibitor, with ROS inhibiting the cAMP/PKA pathway simultaneously with MEK1/2 inhibition. Their crosstalk interaction could effectively block signal transduction of cAMP/PKA and ERK pathways, resulting in the greater inhibition of tyrosine phosphorylation of proteins 187 and 112 kDa seen with the combined ROS/MEK inhibitor than with either treatment individually. The ERK inhibitor would block phosphorylation of Thr-Glu-Tyr motif in the active site of ERK1/2, and when coupled with ROS inhibition of the cAMP/PKA pathway, would produce a coincident highly negative effect on both signaling pathways, producing the concomitant strong reduction of tyrosine phosphorylation of the 172, 111, 97, and 66 kDa proteins.

For the first time, the presence of ERK pathway elements, Raf, MEK1/2, and ERK1/2 have been identified in boar spermatozoa and its ERK1/2 component significantly associated with the regulation of tyrosine phosphorylation and capacitation. Also ERK1/2 may be at least partially regulated by its upstream ERK pathway Raf and MEK1/2, suggesting that other kinases of different pathways and their crosstalk interaction, such as cAMP/PKA and PKC, may have roles in phosphorylating the Thr-Glu-Tyr motif of ERK1/2 pathway.
ROS, in addition to its inhibitory effect on tyrosine phosphorylation of proteins with high molecular mass, have time-dependent effects on the phosphorylation proteins of Raf, MEK1/2 and ERK1/2 components of ERK1/2 pathway of boar spermatozoa. Through such associations with ERK and other pathway elements, ROS may play a role in regulating boar sperm capacitation and tyrosine phosphorylation.

These results also suggest that cross-talk interactions of other pathways, such as cAMP/PKA and PKC and ROS - ERK1/2 - tyrosine phosphorylation could influence the sequential events of boar sperm capacitation.
6. General discussion

The results of this study suggest that ROS in association with cAMP/PKA and ERK pathways act as signal transduction elements playing physiological roles in regulating boar sperm functions and tyrosine phosphorylation. The large body of evidence generated indicates that H$_2$O$_2$ is the major intracellular product of the ROS generating system in boar spermatozoa, which, in association with inhibiting cAMP/PKA pathway, inhibits sperm motility and tyrosine phosphorylation of proteins with high molecular mass, while inducing lipid peroxidation. The membrane lipid peroxidation caused by high intracellular levels of H$_2$O$_2$ activates PLA in dead sperm, particularly PLA$_2$, and increases acrosomal membrane lysis. The ERK pathway elements, particularly ERK1/2, appear to work in association with the cAMP/PKA pathway to regulate tyrosine phosphorylation of boar spermatozoa. Furthermore, ROS-mediated PKA/ERK1/2 elements inhibit tyrosine phosphorylation. ERK pathway elements (Raf, MEK and ERK1/2) are regulated by different crosstalk kinases and ROS phosphorylate ERK pathway elements in a time-dependent manner. Finally, cryopreservation apparently decreased sperm intracellular level of O$_2^{•−}$ and had no effect on H$_2$O$_2$ even though frozen-thawed sperm had significantly poorer sperm motility and viability.

6.1. Sperm intracellular levels of ROS and response to an ROS generating system

Signaling and subsequent sperm functions during sperm capacitation and the acrosome reaction are regulated by different species-specific pathways such as cAMP/PKA, PTK, PKC and components of the ERK family of MAPK pathway (Visconti et al., 1995b,
1997; Leclerc et al., 1996; Aitken et al., 1998; Thundathil et al., 2002; de Lamirande and Gagnon, 2002; Harrison, 2004; O’Flaherty et al., 2005, 2006ab; de Lamirande and O’Flaherty, 2008). Several pathways are associated with such ROS as $O_2^{-}$, $H_2O_2$ and NO$^-$; de Lamirande and Gagnon, 2002; Thundathil et al., 2002; O’Flaherty et al., 2005; 2006ab (de Lamirande and O’Flaherty, 2008). The detailed signaling pathways of capacitation are not completely elucidated, particularly in boar sperm, while the nature of the ROS involvement in the signaling is poorly understood in sperm of virtually all species. The findings detailed here contribute greatly to our understanding in these areas.

The presence of ROS in boar sperm, documented by Guthrie and Welch (2006), was confirmed here using flow cytometry. Flow cytometry used Hydroethidine (HE) and 2', 7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) to identify sperm intracellular levels of $O_2^{-}$ and $H_2O_2$ respectively, and compared their amounts in live and dead sperm by combining these dyes with YOPRO-1 and PI (Fig. 3.8 and 3.11 respectively). Dead fresh sperm had significantly less intracellular $O_2^{-}$ than live fresh sperm, while there was no significant difference in their intracellular level of $H_2O_2$. These flow cytometric measurements can be considered reliable, even though ROS have short-lived intermediates and are highly unstable, making direct in vivo measurement difficult (Kobayashi et al., 2001). Certainly, older methods such as electron spin resonance, spin trapping and chemiluminescence had many shortcomings, being complicated, of poor sensitivity, ineffective in living cells, and/or inaccurate (Aitken et al, 1992; Sharma and Agarwal, 1996; de Zwart et al., 1999; Ochsendorf, 1999). Flow cytometry offers several advantages, being able to both quantitatively examine characteristics of large numbers of
individual cells of a particular sub-population as well as the total population (Amer et al., 2003), and by using H<sub>2</sub>DCFDA and HE can provide accurate measurement of intracellular ROS in live cells. These dyes are permeable to the cell plasma membrane and upon crossing the membrane, H<sub>2</sub>DCFDA undergoes deacetylation by intracellular esterases, producing the non-fluorescent DCFH, which is trapped inside the cells. Any intracellular H<sub>2</sub>O<sub>2</sub> and peroxides oxidize DCFH to fluorescent 2', 7'-dichlorofluorescein and the intensity of the green (530 nm) fluorescence is proportional to the H<sub>2</sub>O<sub>2</sub> produced (Bass et al., 1983; Bucana et al., 1986). The non-fluorescent HE is directly oxidized to ethidium by intracellular superoxide anion (Rothe and Valet, 1990), fluorescing at 610 nm when excited at 488 nm, producing an accurate and fast cellular assay (Bass et al., 1983) for O<sub>2</sub><sup>-</sup>.

Flow cytometric assays employed here clearly demonstrated that fresh untreated boar sperm contain ROS. ROS can be generated intracellularly when extracellular ligands bind to cell membrane receptors and stimulate intracellular responses that regulate different cell functions (Meier et al., 1989; Lo and Cruz, 1995, Sundaresan et al., 1995). The exposure of boar sperm to the ROS generating system significantly increased sperm intracellular level of H<sub>2</sub>O<sub>2</sub> in the overall average sperm population and the live, but not dead, sperm populations (Fig. 3.11). In comparison, the ROS generating system had no significant effect on live, moribund, dead, or overall average sperm intracellular level of O<sub>2</sub><sup>-</sup> (Fig. 3.8). This ROS generating system uses xanthine oxidase to act aerobically upon xanthine to generate O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> (Link and Riley, 1988), and has been shown to be a simple and reliable method to study the effect of controlled concentrations of O<sub>2</sub><sup>-</sup>.
and H₂O₂ on biological systems (Beckman et al., 1988). Incubating sperm in this system for 30 min at 38 °C is known to increase intracellular H₂O₂ of porcine sperm (Guthrie and Welch, 2006) and also in sperm from other species such as human (Aitken et al., 1993) and equine (Baumber et al., 2000). Guthrie and Welch (2006) suggested that H₂O₂ is the major ROS generated by XA/XO in boar spermatozoa, since they saw no increase in O₂⁻⁻ levels, which was confirmed here. The ROS could cause oxidative damage because boar sperm have a high intracellular activity of SOD (Mennella and Jones, 1980) which dismutates O₂⁻⁻ to H₂O₂ (McCord and Fridovich, 1969), and because boar sperm also have an extremely low content of CAT (Foote, 1962) and possibly low glutathione peroxidase (GPx), so they are unable to sufficiently convert H₂O₂ to H₂O and O₂. Therefore, boar spermatozoa have a very low resistance for H₂O₂ in comparison with sperm from other species such as rabbit, which have high CAT content and can rapidly decompose H₂O₂ (Wales et al., 1959).

Therefore the results here have confirmed others’ findings in boar sperm, demonstrated that methods employed here effectively assess intracellular ROS levels in boar sperm, and have confirmed that the ROS generating system increases intracellular H₂O₂. Interestingly, while ROS generating system had no effect on the percent of sperm that were alive, fresh dead sperm had less O₂⁻⁻ than live fresh sperm, and there was no significant difference in their intracellular levels of H₂O₂.
6.2. ROS generating system affects sperm functions

The significant increase of sperm intracellular level of H$_2$O$_2$ occurred concurrently with a 
decline in the % of motile sperm (Fig. 3.5), an increase in the % of acrosome-reacted sperm (Fig. 3.12), more lipid peroxidation in live and dead sperm (Fig. 3.13) and greater 
PLA activity in dead sperm (Fig. 3.14). Therefore, as expected, high levels of H$_2$O$_2$ are 
deleterious to boar sperm functions.

6.2.1. Motility

The H$_2$O$_2$-associated inhibition of sperm motility (detected by CASA, Fig. 3.5) is similar 
to the effect this system induced in spermatozoa from humans (de Lamirande and 
Gagnon, 1992) where sperm motility was completely inhibited. Possibly the high 
intracellular level of H$_2$O$_2$ oxidizes intracellular sulphhydryl moieties, depressing 
glycolytic flux and decreasing ATP levels (Baumber et al., 2000). Spermatozoal ability to 
generate ATP may also be limited by H$_2$O$_2$ inhibiting one or more enzymes of oxidative 
phosphorylation and/or glycolysis (de Lamirand and Gagnon, 1992), thereby decreasing 
phosphorylation of axonemal proteins. This corresponds well with the significant 
inhibition of tyrosine phosphorylation of the high kDa proteins in sperm exposed to the 
ROS generating system (Fig. 4.2 and 5.1). These results suggest that boar sperm motility 
is regulated by tyrosine phosphorylation of proteins with high molecular mass, potentially 
proteins of the cAMP/PKA pathway, since this pathway is known to regulate tyrosine 
phosphorylation of high kDa proteins (Kalab et al., 1998; Bravo et al., 2005). 
Alternatively, the ERK1/2 element of the ERK pathway might impact sperm motility,
since it also significantly regulated tyrosine phosphorylation of high kDa proteins (Chapter 5).

High concentrations of ROS also increase lipid peroxidation (Aitken, 1994). The significant increase in lipid peroxidation products documented here (Fig. 3.13) confirms recent findings (Guthrie and Welch, 2007) and could contribute to the loss of sperm motility. Peroxidation could release PUFAs from sperm plasma membrane to alter membrane fluidity, permeability and cellular capacity to regulate the intracellular ions that control sperm motility as was speculated in equine sperm (Baumber et al., 2000).

In this study, it is likely that both decreasing phosphorylation of axonemal proteins and inducing lipid peroxidation products by H$_2$O$_2$ were significantly associated with decreasing sperm motility.

6.2.2. Acrosome reaction

Microscopic assessment of acrosomal integrity showed for the first time an ROS-induced time-dependent significant increase in the percent of capacitated sperm when compared to sperm not pre-exposed to the ROS generating system. This finding, however, came in contrast with the inhibitory effect of ROS on protein tyrosine phosphorylation (Chapter 4 and 5), and possibly reflects the nature of the tyrosine phosphorylation associated with inducing the acrosome reaction in boar spermatozoa (Bravo et al., 2005). Low concentrations of ROS have been associated with increasing tyrosine phosphorylation and acrosome reaction in different species (human, de Lamirande et al., 1993ab; 1997b;
equine, Baumber et al., 2003; bovine, Rivlin et al., 2004). Therefore, the increased % of acrosome reacted sperm in this study could be due to ROS-induced membrane lipid peroxidation of live and dead spermatozoa, and not physiological capacitation. Exogenous lipid peroxides result in the selective destabilization and loss of the acrosomal cap in ram spermatozoa (Jones and Mann, 1977). ROS significantly increased sperm membrane phospholipid peroxidation and PLA activity in dead sperm (Fig. 3.13 and 3.14 respectively), seen here for the first time. This could disorder membrane phospholipid structure (Sevanian et al., 1988), change membrane fluidity and elevate intracellular Ca\(^{2+}\) resulting from increased Ca\(^{2+}\) permeability. Thus, the coupled action of lipid peroxidation and Ca\(^{2+}\) on membrane phospholipids may activate PLA\(_2\) (Martinez and Moreno, 2001) that can create cis-unsaturated free fatty acids and lysophospholipids, induce membrane instability and fusion (Karnovsky et al., 1982) such as in fertilization (Fleming and Yanagimachi, 1981) and the acrosome reaction of hamster sperm (Meizel and Turner, 1983).

6.2.3. Lipid peroxidation

The common methods that have been used to detect lipid peroxidation in cells are based on the detection malondialdehyde (MDA) as a secondary reaction product. These methods are poorly sensitive and not sufficiently validated (Ramsay and Singer, 1992). However, the use of the novel assay for peroxyl radicals based on lipid derivatives conjugated to BODIPY dyes, such as BODIPY 581/591 hexadecanoic acid, permit accurate measurements of lipid peroxidation in live cells (Esposti, 2002). This probe, whose fluorescence emission spectrum changes irreversibly from red to bright green
upon exposure to ROS, has been used to assess lipid peroxidation and oxidizability of living cells, including sperm from stallion (Ball and Vo, 2002), bull (Brouwers and Gadella, 2003) and boar (Guthrie and Welch, 2007).

In the current study, BODIPY 581/591 was combined with PI to differentiate membrane lipid peroxidation in living and non-living sperm, using flow cytometry. Lipid peroxidation of membrane phospholipids was significantly higher in all sperm exposed to the XA/XO-induced ROS generation, as it was for boar sperm incubated with FeSO₄/Na ascorbate ROS-generating system (Guthrie and Welch, 2007). The resultant loss of membrane PUFAs and production of cytotoxic aldehydes such as malondialdehyde and 4HN could inhibit anaerobic glycolysis, in addition to altering membrane architecture (Comporti, 1989).

During lipid peroxidation ROS attack the polyunsaturated acyl chains of phospholipids and extract a hydrogen atom from a bisallylicmethylene (-CH₂-) group (Girotti, 1985; Aruoma et al., 1989). This leaves a carbon carrying an unpaired electron (-C'H; alkylradical) which readily reacts with molecular oxygen to form a -ROO⁻ radical. The -ROO⁻ radical can propagate the oxidation by abstracting a hydrogen atom (thus becoming a lipid hydroperoxide, -ROOH-) from a neighbouring unsaturated fatty acid, which in turn, becomes an alkyl radical. The presence of a hydroperoxy group disrupts hydrophilic lipid:lipid and lipid:protein interactions, causing structural alteration of biomembranes and lipoproteins (Girotti, 1985; Aruoma et al., 1989).
Changes in plasma membrane properties induced by lipid peroxidation are responsible for the impairment of sperm-oocyte fusion through the reduction of plasma membrane fluidity (Ohyashiki et al., 1988), changes in membrane-bound enzyme and alteration of ion channels (Slater, 1984). Damage to the plasma membrane by ROS-induced lipid peroxidation could increase intracellular \( \text{Ca}^{2+} \), supporting more PLA activity such as seen in dead sperm. Sperm that were susceptible to such extensive activation of PLA\(_2\) could undergo membrane lysis and cell death (Shier, 1979), implying there are subpopulations of sperm with differing susceptibilities. These events could be time dependent as noticed in murine macrophages exposed to \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) (Martinez and Moreno, 2001). Depending on which sperm membranes are affected, these changes could be responsible for damage to plasma and acrosomal membranes, alteration of membrane fluidity and permeability to ions and solutes, and changes in sperm motility.

### 6.2.4. Phospholipase A activity

This study measured PLA activity for the first time by direct incubation of spermatozoa with bis-BODIPY FL C11 fluorescent probe, and combined it with PI to measure sperm PLA activity linked to cell viability (Appendix 3). Endogenous PLA\(_1\) or PLA\(_2\) cleaves the BODIPY group off the bis-BODIPY FL C11 probe, thereby increasing fluorescence emission at 530 nm in proportion to PLA activity without differentiating between cleavage of acyl chains at the 1 and 2 positions (Meshulam et al., 1992).

PLA\(_2\) activity is regulated by, and involved in, signal transductions in animal cells (Liscovitch and Cantly, 1994) and has been implicated in sperm function (guinea pig,
Singleton and Killan, 1983; human, Thakkar et al., 1984; Ram, Roldan and Fragio, 1993). Activation of cPLA₂ in Her 14 cells was partially achieved by its phosphorylation through the Raf-MEK-ERK1/2 pathway (van Rossum et al., 2004), and can also be activated by G proteins or Ca²⁺ (Axelrod, 1990; Liscovitch and Cantley, 1994). PLA₂ hydrolyses membrane phospholipids to lysophospholipids and free fatty acids, preferentially targeting phosphatidylcholine with arachidonic acid at the sn2 position. Therefore the most common PUFA released is arachidonic acid (Smith et al., 1991; Martinez and Moreno, 2001). The high levels of H₂O₂ documented to have been produced would stimulate oxidative stress and lipid peroxidation, increasing intracellular calcium (Golconda et al., 1993; van Rossum et al., 2004) to a level that would support PLA activation and help translocate it from the cytoplasm to the membrane (Schalkwijk et al., 1996). Alternatively cPLA₂ activity could increase by increasing its substrate availability. High cPLA₂ activity is implicated in cell death (Hornfelt et al., 1999; van Rossum et al., 2004), and certainly sperm with excessive levels of PLA were dead.

6.3. Tyrosine phosphorylation signal transduction

A variety of tyrosine-phosphorylated proteins appeared in boar spermatozoa only after incubation in capacitating conditions (Chapters 4 and 5). There was some variation between experiments in the molecular masses of the proteins, (particularly in high kDa proteins), however all the experiments demonstrated the same proteins pattern and variations were minimal. Many factors can contribute to such discrepancies, including minor variations in protocol (gel, running time) and gels analysis with the Image Quant software used to detect all molecular masses. Detection of the high kDa proteins would
be expected to be most affected, as those high kDa standards have limited separation, reducing the precision of mass determination. The great similarity among experiments provides confidence that the results reported are reliable and repeatable. Therefore, it can be concluded that the capacitating conditions used here induced tyrosine phosphorylation of proteins with kDa's of: 1) 158 to 195; 2) ~114 (different trials reported as 105, 110, 132, 112 and 111); 3) ~97 (94,96, 97,103, and 97); 4) ~ 80 (76, 83 and 81); 5) ~71 (71 and 71); 6) ~61 (58,61,64,57 and 66); 7) ~52 (50, 53,50 and 53); 8) ~ 47 (48,47,47 and 47); 9) ~ 39 (39,40, 37, 40 and 39); 10) ~35 (35,36, 33,35 and 35); 11) ~ 32 (31 and 32); 12) ~ 28 (28,27 and 28); 13) ~ 24 (23, 24 and 25). These kDas of are similar to those identified by others (Kalab et al, 1998; Tardif et al., 2001; 2003; Bravo et al, 2005; Dube et al., 2005) in capacitated boar spermatozoa, further supporting that the capacitation conditions used here were functional.

The significant inhibitory effect of ROS generating system on tyrosine phosphorylation of high kDa proteins (Chapters 4, 5) could be due to the actions of high concentration of intracellular H₂O₂, since H₂O₂ was the major ROS detected by boar sperm exposed to the ROS generating system (Chapter 3). However, O₂⁻ could also be active, because the rapid conversion of O₂⁻ to H₂O₂ (McCord and Fridovich, 1969) means it could be present for long enough to affect intracellular reactions but unstable enough to be undetectable by the methods used. The exogenous ROS (O₂⁻, H₂O₂, or both) could act on non-RTK or RTK since it is known that ROS induce cellular tyrosine phosphorylation in association with either type of tyrosine kinases (Natarajan et al., 1998) and at controlled concentrations induce sperm capacitation, acrosome reaction and tyrosine
phosphorylation (Ford, 2004; Rivlin et al., 2004) consequent to an increase in cAMP, activation of tyrosine kinase and/or inhibition of tyrosine phosphatase (Hechet and Zick, 1992; Nakamura et al., 1993; Ford, 2004). Since tyrosine phosphorylation of high kDa proteins was strongly affected, it seems most likely that H2O2 acted through the Ac/cAMP/PKA pathway to reduce tyrosine phosphorylation (through inhibiting tyrosine kinase and/or activating tyrosine phosphatases) because cAMP/PKA pathway regulates tyrosine phosphorylation of proteins with high molecular weights in boar spermatozoa (Kalab et al., 1998; Bravo et al., 2005).

The elements Raf and MEK1/2 of the ERK pathway appear to have minimal effect on tyrosine phosphorylation, since sperm incubated with their specific inhibitors showed little change in tyr-phosphorylation patterns. In contrast, inhibiting ERK1/2 was associated with a significant reduction in protein tyrosine phosphorylation, suggesting that the Thr-Glu-Tyr motif in the ERK1/2 significantly mediates tyrosine phosphorylation. It also strongly suggests that the crosstalk of other kinases are regulating Thr-Glu-Tyr motif, pointing particularly toward PKA and PKC. This is because blocking ERK1/2 upstream pathway components (Raf1 and MEK1/2) did not affect tyrosine phosphorylation, while inhibiting ERK1/2, significantly inhibited tyrosine phosphorylation. This evidence supports the concept that other kinases, such as PKA and PKC are associated with the activation of the ERK1/2 component of boar spermatozoa. In addition, in human sperm, PKA and PKC kinases have been suggested to be possible kinases associated with the phosphorylation of ERK pathway elements (O'Flaherty et al., 2006ab, de Lamirande and O'Flaherty, 2008).
6.4. ERK pathway and tyrosine phosphorylation signaling

One very important objective of this research was to test the hypothesis that the ERK family of the MAPK pathway influences the capacitation and protein tyrosine phosphorylation of fresh boar spermatozoa, and ROS modulate the signal transduction of this pathway during sperm capacitation. This pathway plays an important role in regulating tyrosine phosphorylation in human spermatozoa (de Lamirande and Gagnon, 2002; O'Flaherty et al., 2005, 2006ab; de Lamirande and O'Flaherty, 2008).

The presence of the phosphorylated (activated) forms of ERK pathway elements (Raf, MEK1/2 and ERK1/2) in boar spermatozoa, and their protein profiles, differed with capacitation. Pre-exposing sperm to the XA/XO ROS generating system stimulated time-dependent effects on this capacitation-associated phosphorylation of Raf, MEK1/2 and ERK1/2. Although this suggests that all these ERK elements may regulate sperm capacitation and tyrosine phosphorylation, when their actions were individually inhibited by specific inhibitors, only the ERK1/2 Activation Inhibitor significantly inhibited the tyrosine phosphorylation of high kDa proteins that is associated with capacitation. This is the first evidence that this ERK pathway is associated directly with capacitation-associated protein tyrosine phosphorylation. Furthermore, this can be interpreted as a late event of sperm capacitation of boar spermatozoa, because tyrosine phosphorylated proteins are present after incubation in capacitating conditions and are associated with hyperactivation and the acrosome reaction, all of which occur late in capacitation.
Interestingly, sperm exposed to the ROS generating system and subsequent ERK1/2 inhibition had an additive effect to ROS followed by MEK inhibition (Chapters 4, 5). ROS plus MEK1/2 inhibition significantly inhibited tyrosine phosphorylation of proteins 187 and 112 kDa, while ROS followed by ERK inhibition significantly reduced tyrosine phosphorylation on presumably the same two proteins (172 and 111 kDa) and two other proteins of 97 and 66 kDa. ROS alone inhibits tyrosine phosphorylation of high kDa proteins (Chapter 4), which is regulated by cAMP/PKA pathway (Kalab et al., 1998; Bravo et al., 2005). Thus it is most likely that the combination of ROS and MEK inhibitor or ROS and ERK1/2 inhibitor act as a complex, or sequential, inhibitor. In this mechanism, ROS would inhibit the phosphorylation/activation of the cAMP/PKA pathway, while the other inhibitors would, simultaneously, prevent activation of either MEK1/2 or ERK1/2. The combination would affect the signal transduction of cAMP/PKA and ERK pathways and their crosstalk interaction with other pathways, resulting in inhibition of tyrosine phosphorylation of these proteins through inhibiting PTK and/or activating PTP.

These results therefore suggest the existence of crosstalk of different pathways such as cAMP/PKA, its substrates, PKC, and ERK components to regulate tyrosine phosphorylation as a late event associated with sperm capacitation, hyperactivation and acrosome reaction (O’Flaherty et al., 2005, 2006ab; de Lamirande and O’Flaherty, 2008). Also these results suggest that the phosphorylation of Thr-Glu-Tyr motif in the activation site of ERK1/2 elements of the ERK pathway of boar spermatozoa may be regulated by crosstalk of other kinases such as PKA and PKC (Fig. 6.1).
Figure 6.1. Hypothetical mechanism by which ROS generating system (XA/XO) impacts membrane lipid peroxidation, motility, PLA activity, acrosome reaction and modulating signal transduction of tyrosine phosphorylation. H₂O₂ is the major intracellular ROS generated, causing membrane lipid peroxidation (LP) by oxidizing PUFAs. This then increases membrane fluidity and permeability, raising intracellular Ca²⁺ that then promotes translocation of cPLA2 to the plasma and acrosomal membrane, further enhancing membrane lysis. The attendant release of toxic products inhibits anaerobic glycolysis, and all actions combined alter membrane architecture and inhibit sperm motility. H₂O₂ inhibits tyrosine phosphorylation by acting on adenyl cyclase (Ac) and mediating cAMP/PKA to inhibit non receptor tyrosine kinase (TK) or increasing tyrosine phosphatase (TP). H₂O₂ may also possibly inhibit the phosphorylation of ERK1/2 by crosstalk with PKA. The inhibition of tyrosine phosphorylation by H₂O₂ is associated with inhibiting sperm motility.
6.5. ROS and sperm cryopreservation

A final objective of this research was investigating the hypothesis that the process of sperm cryopreservation induces ROS generation in boar spermatozoa. Unexpectedly, flow cytometric measurement of sperm $O_2^{-}$ and $H_2O_2$ showed fresh sperm had significantly higher intracellular level of $O_2^{-}$ than live frozen-thawed boar spermatozoa and no significant differences in their intracellular level of $H_2O_2$, in seeming contradiction of fresh sperm's significantly better motility and viability. These results do concur with Guthrie and Welch (2006), who reported similar levels of $H_2O_2$ and no significant difference in ROS concentrations between frozen-thawed and fresh boar sperm. However, cryopreservation of sperm of other species increases ROS production (murine, Alvares and Storey, 1992; bovine, Bilodeau et al., 2000; Chatterjee and Gagnon, 2001; equine, Ball et al., 2001). Reducing sperm intracellular level of $O_2^{-}$ in boar spermatozoa by the process of sperm cryopreservation could be related to the collapse of the mitochondrial oxidative phosphorylation, which led to decrease the endogenous ROS production in comparison to fresh sperm.

6.6. Summary and conclusions

The present research employed different recent methodology and techniques, such as fluorescent probes, dyes, ROS generating system, flow cytometer, western blotting, inhibitors and anti-phospho-protein antibodies, to investigate the hypothesis that ROS are associated with different physiological aspects of sperm functions in terms of motility, viability, acrosome reaction, membrane lipid peroxidation, PLA activity, tyrosine
phosphorylation and ERK pathway signal transductions. The results of this study can be summarized as following:

1. H$_2$O$_2$ is the major intracellular ROS generated during exposing boar spermatozoa to the XA/XO generating system.

2. ROS generating system completely inhibited sperm motility, significantly increased membrane lipid peroxidation of live and dead sperm, induced acrosome reaction, increased PLA activity of dead sperm and inhibited tyrosine phosphorylation of proteins with high molecular weights.

3. ERK pathway components (Raf, MEK1/2, and ERK1/2) exist in boar sperm and their protein profiles differed with capacitation.

4. ROS generating system has time dependent effects on the phosphorylation of ERK pathway components (Raf, MEK1/2 and ERK1/2).

5. The ERK pathway significantly regulates tyrosine phosphorylation in boar spermatozoa through its ERK1/2 component.

6. The process of freezing and thawing significantly decreased boar spermatozoa intracellular level of O$_2^-$.

The results of this study suggest that boar sperm motility, acrosome integrity and lipid peroxidation are more sensitive indicators of oxidative stress than viability and PLA activity. Lipid peroxidation caused by H$_2$O$_2$, possibly through increasing intracellular Ca$^{2+}$ concentration, was associated with inhibiting sperm motility and increasing sperm plasma and acrosomal membrane lysis. Tyrosine phosphorylation of boar spermatozoa is regulated by the signal transductions of cAMP/PKA and ERK pathways and their
crosstalk kinases and possibly PKC. Physiological control of capacitation is also exerted through ROS, possibly acting through the increased $\text{H}_2\text{O}_2$, to inhibit tyrosine phosphorylation of high kDa proteins affecting the cAMP-dependent PKA pathway and possibly ERK1/2. This inhibitory effect could be associated with impaired sperm motility.

This deeper understanding of the physiological roles of ROS in regulating different pathways that control boar sperm capacitation and acrosome reaction will definitely enhance comprehension of the events preceding fertilization and may find application in optimizing boar sperm preservation and the artificial insemination industry.

Different concentrations of ROS may be associated with the regulation of different pathways and their cross-talk interaction during sperm capacitation, tyrosine phosphorylation and acrosome reaction and those events could be associated with sperm cryopreservation and sperm fertility.


Martinez, J. and Moreno, J.J. 2001. Role of Ca\textsuperscript{2+} independent phospholipase A\textsubscript{2} on arachidonic acid releases induced by reactive oxygen species. Arch Biochem Biophys 392: 257-262


Appendices

Appendix 1: Measuring sperm Motility by CASA

Sperm motility was determined using Computer Assisted Sperm Analysis (CASA; IVOS System Software, Hamilton Thorne Biosciences, Massachusetts, USA). Fresh semen was diluted to the concentration of $1 \times 10^7$ spermatozoa/ml with BTS:BF5 fraction-A extenders (3:1; vol: vol). Samples were kept warm at 37 C°. About 8 μl of diluted sperm were loaded into pre-warmed chamber of a 20 micron 4 chamber standard count analysis slide (Leja products B.V. Luzernestraat 10, 2153 GN Nieuw-Vennep, The Netherlands). Samples then were analyzed at 37 C° using the boar spermatozoa instrument (CASA) setting and as following: frames acquired 100 at 30 Hz, minimum contrast 50 and minimum cell size 7 pixels, non-motile head size 11 pixels, non-motile head intensity 118, medium average path velocity 45μm/sec, low path velocity 20μm/sec, and threshold straightness 45μm/sec.

200 sperm cells were recorded from different fields of the slide of the sample. The % of motile, progressive, rapid, medium, slow sperm were recorded in addition to the sperm parameters of path velocity (VAP), progressive velocity (VSL), track speed (VCL), amplitude of lateral head displacement (ALH), straightness (STR), linearity (LIN) were also recorded.
Appendix 2: Measuring sperm viability

Sperm viability was assessed using Live: Dead Sperm Viability Kit 200-1000 assay (cat. no. L-7011, Molecular Probes, Inc., Eugene, OR) as following:

Sperm were diluted with BTS (at 37 C°) to the concentration of $1 \times 10^7$ spermatozoa/ml. 0.5 ml of diluted sperm were mixed with 5 µl of 20 µM SYBR-14 (component A of the kit) in Dimethyl Sulfoxide (DMSO) and 5 µl of 2.4 mM Propidium iodide in water (component-B of the kit) and incubated in dark at 37°C for at least 15-30 min. Two drops of 10 µl were taken of the incubated and stained sperm and placed on the two ends of a glass slide and covered by separated cover slips and sperm viability visually determined by a fluorescence microscope (Leitz, Laborlu S, Germany); blue filter; at 450-490 nm excitation wavelength; at 400X magnification. One hundred spermatozoa were counted from each drop of the two drops as live sperm (live sperm emits green fluorescence; SYBR-14) or dead sperm (dead sperm emits red fluorescence; Pi). Then the percentages of live or dead sperm were calculated as a percentage from the total. If the counts of each drop differed by more than 5%, then a third drop was counted and the three reading of the three drops were averaged.
Appendix 3: Measuring bis-BODIPY FL C11 Uptake by Boar Sperm
(PLA activity- Preliminary Experiment)

Objective

Measuring the impact of an ROS generating system on boar sperm function necessitated measuring intracellular PLA activity in boar sperm accurately, reliably and repeatably. The bis-BODIPY FL C11 fluorescent probe has been used to measure PLA activity in somatic cells by incorporating this probe into liposomes for delivery to the target cells (Meshulam et al., 1992). Since incorporating liposomes can affect sperm membranes (He et al., 2001) which could adversely affect interpretation of any results, the current experiment was designed to investigate the hypothesis that this probe will be taken up by sperm in the absence of a liposome delivery vehicle.

Material and Methods:

The sperm rich fraction (n=2) was collected using the gloved-hand method into a 35°C Thermos®, immediately assessed for motility, and only ejaculates with motility of greater than 70% were used. Sperm concentration was determined by a calibrated spectrophotometer (Spectronic 20, Milton Roy, Rochester, New York) at an excitation of 550 nm.

Sperm were diluted with BTS to the concentration of $2 \times 10^7$ spermatozoa/ml. A 1 mM stock solution of bis-BODIPY FL C11 fluorescent probe was prepared in absolute ethanol as recommended by the manufacturer (B-7701, Molecular Probes, Inc., Eugene, OR)
sperm received BODIPY and PI (8 and 24 μM final concentration) and were incubated for 20 minutes at 37°C. The PLA activity of sperm samples were analyzed by flow cytometer (excitation 488 nm, emission with 650 LP filter detected PI fluorescence, and a separate 530/30nm filter detected the fluorescence of BODIPY (PLA activity).

Sperm (2×10⁷ spermatozoa/ml) were either diluted with BTS and 8μl ethanol as a control, or diluted in BTS and then 8μl of 1 mM bis-BODIPY FL C11 in absolute ethanol. 24 μM (final concentration) of PI were added for both treatment and control and incubated for 20 minutes at 37°C. The bis-BODIPY FL C11 and PI fluorescence in each of a total of 10000 individual sperm-sized events were analyzed by flow cytometer (excitation 488 nm, emission with 650 LP filter detected PI fluorescence, and a separate 530/30nm filter detected the fluorescence of bis-BODIPY FL C11). The sperm populations were subsequently gated to live and dead populations based on low and high PI; and the mean fluorescence intensity and number of sperm in each population identified by bis-BODIPY FL C11 were calculated in the statistical region of FACS.

Statistical analysis

Fluorescence intensity of bis-BODIPY FL C11 and PI for each sperm were analyzed using the SAS 9.1 (SAS 9.1 for windows TS Level 1M3, The SAS Institute Inc., Cary, NC, USA). General Linear Model Procedure (GLM) and Multiple Comparison Tukey’s Test were used to determine the differences of Least Squares Means between treatment and control. Differences with values of P<0.05 were considered to be statistically significant.
Results and conclusions:

Incubating boar spermatozoa with the bis-BODIPY FL C11 fluorescent probe significantly increased fluorescence of sperm at the BODIPY wavelength above that shown by control sperm (Fig A3.1), indicating that the probe was incorporated into sperm. Both live (P< 0.04) and dead (P<0.015) sperm incorporated the probe, with fluorescence intensity of the bis-BODIPY FL C11 fluorescent probe being higher in dead sperm than viable sperm (P< 0.02; Fig.A3.1) There was no significant difference in the % of viable sperm between the treatment and the control of (91.4 ±2.36 vs. 90.83 ± 1.28, control and treatment respectively; means ± SE). The fluorescence intensity of sperm exposed to this probe indicated that this probe was taken up by both live and dead sperm. The presence of endogenous PLA₁ or PLA₂ cleaves off the BODIPY group and increases fluorescence emission at 530 nm (Meshulam et al., 1992).

The significant increase in PLA in dead sperm versus live sperm, as detected by the higher fluorescence intensity of dead sperm, is indicating either that dead sperm have high activity of PLA or that dead sperm take up more of the probe, perhaps because of high permeability of damaged plasma membrane of dead sperm to this fluorescent probe.
Figure A3.1. Measuring bis-BODIBY-FL C11 incorporation into boar spermatozoa. Sperm from fresh ejaculates of boar spermatozoa were incubated with PI ± 8 µM of bis-BODIPY-FL-C11 and incubated for 20 minutes at 37°C. The bis-BODIBY-FL C11 fluorescence intensity (means± SE; n=2 ejaculates) of viable (low PI intensity) and dead (high PI intensity) sperm were analyzed by flow cytometer. a, b, c: bars with no superscripts in common differ (P<0.05).
Appendix 4: Band intensity (mean± S.E) of tyrosine phosphorylated proteins extracted from boar sperm preincubated ± ROS generating system and subsequently incubation in BTS or capacitating medium (Chapter 4; section 4.5). Boar spermatozoa were treated either with ROS generating system (1 mM xanthine + 0.1 U xanthine oxidase per ml) or BTS and incubated (30 min, 38°C) and then either diluted in BTS or capacitating medium and incubated for four hours. Proteins from $5 \times 10^6$ sperm were extracted by sample buffer, loaded in each well, electrophoresed, electrotransferred, and immunoblotted using the-anti-phosphotyrosine antibody for four hours. Intensities of immunoblot bands were quantified by Image Quant software. ND: not detectable.

<table>
<thead>
<tr>
<th>MW Average kDa</th>
<th>BTS 30min ±S.E</th>
<th>ROS 30min ±S.E</th>
<th>BTS 4h ±S.E</th>
<th>BTS-ROS 4h ±S.E</th>
<th>Cap-4h ±S.E</th>
<th>Cap-ROS 4h ±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>174</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>33.57</td>
<td>1.61</td>
</tr>
<tr>
<td>105</td>
<td>6.42</td>
<td>3.67</td>
<td>7.87</td>
<td>4.14</td>
<td>32.3</td>
<td>5.04</td>
</tr>
<tr>
<td>94</td>
<td>0.90</td>
<td>0.90</td>
<td>1.87</td>
<td>2.93</td>
<td>44.48</td>
<td>7.78</td>
</tr>
<tr>
<td>58</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>47.91</td>
<td>36.30</td>
</tr>
<tr>
<td>48</td>
<td>5.35</td>
<td>2.39</td>
<td>8.91</td>
<td>8.45</td>
<td>6.57</td>
<td>5.83</td>
</tr>
<tr>
<td>39</td>
<td>64.36</td>
<td>9.64</td>
<td>64.25</td>
<td>68.83</td>
<td>57.91</td>
<td>66.58</td>
</tr>
<tr>
<td>35</td>
<td>51.66</td>
<td>13.35</td>
<td>51.91</td>
<td>46.76</td>
<td>33.90</td>
<td>46.59</td>
</tr>
<tr>
<td>31</td>
<td>8.78</td>
<td>5.51</td>
<td>2.34</td>
<td>7.42</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>28</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.13</td>
<td>0.76</td>
</tr>
<tr>
<td>23</td>
<td>1.75</td>
<td>1.75</td>
<td>3.25</td>
<td>ND</td>
<td>0.86</td>
<td>1.98</td>
</tr>
</tbody>
</table>
Appendix 5: Band intensity (mean± S.E) of tyrosine phosphorylated protein extracted from boar sperm incubated ± ROS generating system and then diluted in capacitating medium (CAP) and incubated for four hours (Chapter 5; section 5.5.1). Proteins from $5 \times 10^6$ sperm were extracted at the indicated times and membranes immunoblotted using anti-phosphotyrosine antibody. Bands intensity of immunoblots was quantified by Image Quant software. Band intensities data of 3 different experiments (3x4 replicates) were pooled according to there molecular mass.

<table>
<thead>
<tr>
<th>MW Average /kDa</th>
<th>Cap-4h</th>
<th>±S.E</th>
<th>Cap-ROS</th>
<th>±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>158</td>
<td>27.87</td>
<td>7.61</td>
<td>3.07</td>
<td>1.58</td>
</tr>
<tr>
<td>110</td>
<td>24.53</td>
<td>6.45</td>
<td>6.29</td>
<td>2.22</td>
</tr>
<tr>
<td>96</td>
<td>3.85</td>
<td>7.71</td>
<td>13.84</td>
<td>4.18</td>
</tr>
<tr>
<td>76</td>
<td>40.71</td>
<td>11.64</td>
<td>18.11</td>
<td>8.23</td>
</tr>
<tr>
<td>71</td>
<td>59.99</td>
<td>5.47</td>
<td>15.85</td>
<td>10.33</td>
</tr>
<tr>
<td>61</td>
<td>49.54</td>
<td>6.67</td>
<td>36.03</td>
<td>5.75</td>
</tr>
<tr>
<td>50</td>
<td>22.09</td>
<td>6.47</td>
<td>13.70</td>
<td>3.36</td>
</tr>
<tr>
<td>47</td>
<td>20.04</td>
<td>8.63</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>40</td>
<td>60.77</td>
<td>4.18</td>
<td>61.52</td>
<td>3.85</td>
</tr>
<tr>
<td>36</td>
<td>49.61</td>
<td>2.57</td>
<td>51.75</td>
<td>3.01</td>
</tr>
<tr>
<td>32</td>
<td>6.60</td>
<td>1.08</td>
<td>7.15</td>
<td>2.66</td>
</tr>
<tr>
<td>28</td>
<td>7.56</td>
<td>2.18</td>
<td>14.30</td>
<td>6.27</td>
</tr>
<tr>
<td>25</td>
<td>4.15</td>
<td>1.01</td>
<td>4.41</td>
<td>1.65</td>
</tr>
</tbody>
</table>
Appendix 6: Band intensity (mean± S.E) of phospho-Raf proteins extracted from boar sperm incubated ± ROS generating system and then either diluted in BTS or capacitating medium (CAP) and incubated for four hours as indicated (Chapter 5, section 5.5.1). Proteins from 5× 10⁶ sperm were extracted at the indicated times and membranes immunoblotted using anti-phospho-Raf-1 (Ser 338/Tyr340) affinity purified rabbit polyclonal IgG antibody (primary antibody) and anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (secondary antibody). Intensities of immunoblot bands were quantified by Image Quant software. ND: not detectable.

<table>
<thead>
<tr>
<th>Average MW/kDa</th>
<th>BTS 30min</th>
<th>±S.E</th>
<th>ROS 30min</th>
<th>±S.E</th>
<th>BTS 4h</th>
<th>±S.E</th>
<th>BTS-ROS 4h</th>
<th>±S.E</th>
<th>Cap 4h</th>
<th>±S.E</th>
<th>Cap-ROS 4h</th>
<th>±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>2.11</td>
<td>0.63</td>
<td>0.0</td>
<td>0.0</td>
<td>3.18</td>
<td>1.84</td>
<td>0.0</td>
<td>0.0</td>
<td>0.95</td>
<td>0.51</td>
<td>2.32</td>
<td>0.73</td>
</tr>
<tr>
<td>96</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>2.36</td>
<td>2.35</td>
<td>1.80</td>
<td>1.80</td>
<td>2.47</td>
<td>2.47</td>
<td>4.07</td>
<td>1.16</td>
</tr>
<tr>
<td>82</td>
<td>4.89</td>
<td>0.79</td>
<td>4.97</td>
<td>1.21</td>
<td>4.40</td>
<td>2.25</td>
<td>1.51</td>
<td>1.00</td>
<td>4.50</td>
<td>1.44</td>
<td>3.94</td>
<td>1.06</td>
</tr>
<tr>
<td>57</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>16.59</td>
<td>6.59</td>
<td>13.32</td>
<td>2.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>6.89</td>
<td>2.45</td>
<td>31.37</td>
<td>7.15</td>
<td>8.50</td>
<td>2.82</td>
<td>28.68</td>
<td>8.34</td>
<td>6.95</td>
<td>2.01</td>
<td>10.69</td>
<td>4.07</td>
</tr>
<tr>
<td>35</td>
<td>19.46</td>
<td>6.34</td>
<td>19.21</td>
<td>6.57</td>
<td>9.58</td>
<td>3.47</td>
<td>7.38</td>
<td>4.84</td>
<td>1.91</td>
<td>1.17</td>
<td>4.54</td>
<td>1.76</td>
</tr>
<tr>
<td>24</td>
<td>2.26</td>
<td>0.93</td>
<td>1.60</td>
<td>0.12</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
Appendix 7. Band intensity (mean ± S.E) of phospho MEK1/2 protein extracted from boar sperm incubated ± ROS generating system and then either diluted in BTS or capacitating medium (CAP) and incubated for four hours as indicated (Chapter 5; section 5.5.1). Proteins from $5 \times 10^6$ sperm were extracted at the indicated times and membranes immunoblotted using anti-phospho-MEK1 (Ser 218/222)/MEK2 (Ser 222/226); rabbit monoclonal IgG antibody (primary antibody) and anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (secondary antibody). Intensities of immunoblot bands were quantified by Image Quant software. ND: not detectable.

<table>
<thead>
<tr>
<th>Average MW/kDa</th>
<th>BTS 30min</th>
<th>±S.E</th>
<th>ROS 30min</th>
<th>±S.E</th>
<th>BTS 4h</th>
<th>±S.E</th>
<th>BTS-ROS 4h</th>
<th>±S.E</th>
<th>Cap 4h</th>
<th>±S.E</th>
<th>Cap-ROS 4h</th>
<th>±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>47.61</td>
<td>8.07</td>
<td>47.73</td>
<td>11.66</td>
</tr>
<tr>
<td>50</td>
<td>5.02</td>
<td>0.46</td>
<td>8.11</td>
<td>1.46</td>
<td>5.02</td>
<td>0.37</td>
<td>7.19</td>
<td>0.48</td>
<td>4.49</td>
<td>0.84</td>
<td>8.47</td>
<td>1.40</td>
</tr>
<tr>
<td>27</td>
<td>3.08</td>
<td>3.08</td>
<td>2.55</td>
<td>0.10</td>
<td>1.94</td>
<td>1.94</td>
<td>3.03</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>3.49</td>
<td>-</td>
<td>3.42</td>
<td>0.61</td>
<td>3.81</td>
<td>1.47</td>
<td>2.91</td>
<td>0.48</td>
<td>2.55</td>
<td>0.14</td>
<td>3.70</td>
<td>1.92</td>
</tr>
</tbody>
</table>
**Appendix 8.** Band intensity (mean± S.E) of phospho ERK1/2 protein extracted from boar sperm incubated ± ROS generating system and then either diluted in BTS or capacitating medium (CAP) and incubated for four hours as indicated (Chapter 5; section 5.5.1). Proteins from $5 \times 10^6$ sperm were extracted at the indicated times and membranes immunoblotted using rabbit anti-phospho-ERK1/2(Thr202/Tyr204) affinity purified polyclonal antibody (primary antibody) and anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (secondary antibody). Intensities of immunoblot bands were quantified by Image Quant software. ND: not detectable.

<table>
<thead>
<tr>
<th>Average MW/kDa</th>
<th>BTS 30min</th>
<th>±S.E</th>
<th>ROS 30min</th>
<th>±S.E</th>
<th>BTS 4h</th>
<th>±S.E</th>
<th>BTS-ROS 4h</th>
<th>±S.E</th>
<th>Cap-4h</th>
<th>±S.E</th>
<th>Cap-ROS 4h</th>
<th>±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>158</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>9.29</td>
<td>8.58</td>
<td>19.85</td>
<td>12.95</td>
</tr>
<tr>
<td>107</td>
<td>0.0</td>
<td>0.0</td>
<td>10.04</td>
<td>8.71</td>
<td>ND</td>
<td>2.40</td>
<td>8.91</td>
<td>4.19</td>
<td>4.68</td>
<td>4.03</td>
<td>8.24</td>
<td>5.31</td>
</tr>
<tr>
<td>80</td>
<td>6.32</td>
<td>5.04</td>
<td>17.82</td>
<td>10.24</td>
<td>3.48</td>
<td>2.40</td>
<td>8.91</td>
<td>4.19</td>
<td>4.68</td>
<td>4.03</td>
<td>8.24</td>
<td>5.31</td>
</tr>
<tr>
<td>61</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>45.27</td>
<td>5.09</td>
<td>35.44</td>
<td>14.87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>7.50</td>
<td>2.16</td>
<td>18.50</td>
<td>5.48</td>
<td>6.32</td>
<td>2.46</td>
<td>15.04</td>
<td>2.62</td>
<td>6.49</td>
<td>2.81</td>
<td>9.62</td>
<td>3.70</td>
</tr>
<tr>
<td>46</td>
<td>0.0</td>
<td>0.0</td>
<td>3.76</td>
<td>2.23</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>32</td>
<td>2.15</td>
<td>2.15</td>
<td>15.82</td>
<td>13.21</td>
<td>1.07</td>
<td>1.07</td>
<td>9.54</td>
<td>8.37</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>0.0</td>
<td>0.0</td>
<td>8.50</td>
<td>-</td>
<td>0.81</td>
<td>0.81</td>
<td>1.66</td>
<td>0.22</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>
**Appendix 9**: Band intensity (mean± S.E) of tyrosine phosphorylated protein extracted from boar sperm following exposure to media ± ZM336372 (6μM) ± ROS generating system and then either diluted in BTS or capacitating medium and incubated for 4 h (Chapter 5; section 5.5.2.1). Proteins from 5× 10⁶ sperm were extracted and immunoblotted using the anti-phosphotyrosine antibody as described under Material and Methods. Intensities of immunoblot bands were quantified by Image Quant software. ND: not detectable.

<table>
<thead>
<tr>
<th>Average MW/kDa</th>
<th>BTS ±S.E</th>
<th>BTS + ZM336372 ±S.E</th>
<th>BTS + ROS ±S.E</th>
<th>BTS + ZM336372 + ROS ±S.E</th>
<th>Cap ±S.E</th>
<th>Cap + ZM336372 ±S.E</th>
<th>Cap + ROS ±S.E</th>
<th>Cap + ZM336372 + ROS ±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>4.29</td>
<td>4.85</td>
<td>1.86</td>
<td>ND</td>
<td>-</td>
<td>5.19</td>
<td>1.65</td>
<td>1.13</td>
</tr>
<tr>
<td>132</td>
<td>1.26</td>
<td>4.63</td>
<td>0.06</td>
<td>ND</td>
<td>-</td>
<td>9.94</td>
<td>3.96</td>
<td>5.99</td>
</tr>
<tr>
<td>97</td>
<td>5.03</td>
<td>6.45</td>
<td>2.09</td>
<td>2.86</td>
<td>1.08</td>
<td>2.34</td>
<td>0.65</td>
<td>6.45</td>
</tr>
<tr>
<td>83</td>
<td>2.99</td>
<td>4.85</td>
<td>4.85</td>
<td>2.99</td>
<td>0.01</td>
<td>0.0</td>
<td>5.28</td>
<td>2.83</td>
</tr>
<tr>
<td>64</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>47</td>
<td>3.73</td>
<td>3.65</td>
<td>1.72</td>
<td>4.56</td>
<td>2.21</td>
<td>2.89</td>
<td>0.46</td>
<td>5.31</td>
</tr>
<tr>
<td>37</td>
<td>46.01</td>
<td>42.32</td>
<td>8.40</td>
<td>42.04</td>
<td>3.98</td>
<td>37.98</td>
<td>5.71</td>
<td>35.55</td>
</tr>
<tr>
<td>33</td>
<td>34.05</td>
<td>30.46</td>
<td>9.08</td>
<td>30.30</td>
<td>5.10</td>
<td>26.14</td>
<td>6.29</td>
<td>24.60</td>
</tr>
</tbody>
</table>
### Appendix 10. Band intensity (mean± S.E) of tyrosine phosphorylated protein extracted from boar sperm following exposure to media ± U0126 (1μM) ± ROS generating system and then either diluted in BTS or capacitating medium and incubated for 4h (Chapter 5; section 5.5.2.2. Proteins from 5× 10⁶ sperm were extracted and immunoblotted using the-anti-phosphotyrosine antibody as described under Material and Methods. Intensities of immunoblot bands were quantified by Image Quant software. ND: not detectable.

<table>
<thead>
<tr>
<th>Average MW/kDa</th>
<th>BTS</th>
<th>±S.E</th>
<th>BTS+U0126</th>
<th>±S.E</th>
<th>BTS+ROS</th>
<th>±S.E</th>
<th>BTS+U0126+ROS</th>
<th>±S.E</th>
<th>Cap</th>
<th>±S.E</th>
<th>Cap+U0126</th>
<th>±S.E</th>
<th>Cap+ROS</th>
<th>±S.E</th>
<th>Cap+ROS+U0126</th>
<th>±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>298</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>7.08</td>
<td>0.13</td>
<td>9.36</td>
<td>1.15</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>187</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>17.58</td>
<td>7.06</td>
<td>17.46</td>
<td>4.31</td>
<td>5.21</td>
<td>1.47</td>
<td>1.67</td>
<td>1.676</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>112</td>
<td>1.24</td>
<td>1.24</td>
<td>6.86</td>
<td>0.73</td>
<td>6.16</td>
<td>1.81</td>
<td>4.60</td>
<td>1.20</td>
<td>ND</td>
<td>-</td>
<td>4.04</td>
<td>2.44</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>103</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>13.91</td>
<td>7.18</td>
<td>12.76</td>
<td>6.07</td>
<td>7.45</td>
<td>3.56</td>
<td>2.15</td>
<td>0.69</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>81</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>6.35</td>
<td>3.71</td>
<td>1.22</td>
<td>1.18</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>71</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>16.46</td>
<td>9.31</td>
<td>17.12</td>
<td>6.12</td>
<td>17.70</td>
<td>7.25</td>
<td>0.98</td>
<td>0.98</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>57</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>29.49</td>
<td>5.89</td>
<td>31.50</td>
<td>6.12</td>
<td>31.26</td>
<td>9.01</td>
<td>26.0</td>
<td>8.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>38.27</td>
<td>6.94</td>
<td>37.57</td>
<td>8.33</td>
<td>39.94</td>
<td>6.64</td>
<td>40.61</td>
<td>7.80</td>
<td>37.24</td>
<td>6.08</td>
<td>33.71</td>
<td>7.27</td>
<td>43.24</td>
<td>6.53</td>
<td>42.71</td>
<td>4.34</td>
</tr>
<tr>
<td>31</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>3.12</td>
<td>1.08</td>
<td>1.23</td>
<td>1.23</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>3.59</td>
<td>2.08</td>
<td>0.0</td>
<td>0.0</td>
<td>1.90</td>
<td>1.90</td>
<td>4.54</td>
<td>0.10</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>ND</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>5.87</td>
<td>2.43</td>
<td>3.59</td>
<td>3.59</td>
<td>1.77</td>
<td>1.77</td>
<td>4.59</td>
<td>0.27</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
**Appendix 11.** Band intensity (mean± S.E) of tyrosine phosphorylated protein extracted from boar sperm following exposure to media ± ERK1/2 inhibitor (20µM) ± ROS generating system and then either diluted in BTS or capacitating medium and incubated for 4h (Chapter 5; section 5.5.2.3). Proteins from $5 \times 10^6$ sperm were extracted and immunoblotted using the-anti-phosphotyrosine antibody as described under Material and Methods. Intensities of immunoblot bands were quantified by Image Quant software. ND: not detectable.

<table>
<thead>
<tr>
<th>Average MW/kDa</th>
<th>BTS ±S.E</th>
<th>BTS+ ERK1/2 Inhibitor ±S.E</th>
<th>BTS+ ROS ±S.E</th>
<th>BTS+ ROS+ ERK1/2 Inhibitor ±S.E</th>
<th>Cap ±S.E</th>
<th>Cap+ ERK1/2 inhibitor ±S.E</th>
<th>Cap+ ROS ±S.E</th>
<th>Cap+ ROS+ ERK1/2 inhibitor ±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>172</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>63.1</td>
<td>16.65</td>
<td>30.39</td>
<td>15.96</td>
</tr>
<tr>
<td>111</td>
<td>7.20</td>
<td>10.91</td>
<td>8.31</td>
<td>4.54</td>
<td>1.36</td>
<td>13.26</td>
<td>29.60</td>
<td>21.20</td>
</tr>
<tr>
<td>97</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>70.66</td>
<td>15.18</td>
<td>18.33</td>
<td>7.91</td>
</tr>
<tr>
<td>66</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>80.58</td>
<td>25.91</td>
<td>41.10</td>
<td>18.2</td>
</tr>
<tr>
<td>53</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5.78</td>
<td>1.27</td>
<td>0.0</td>
<td>3.63</td>
</tr>
<tr>
<td>47</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>8.27</td>
<td>1.66</td>
<td>1.69</td>
<td>1.93</td>
</tr>
<tr>
<td>39</td>
<td>24.0</td>
<td>18.67</td>
<td>4.84</td>
<td>28.93</td>
<td>27.37</td>
<td>14.86</td>
<td>34.88</td>
<td>12.66</td>
</tr>
<tr>
<td>35</td>
<td>16.6</td>
<td>15.30</td>
<td>2.10</td>
<td>14.54</td>
<td>17.99</td>
<td>14.98</td>
<td>20.64</td>
<td>7.21</td>
</tr>
</tbody>
</table>