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The Ability of Bull and Stallion Thawed Spermatozoa Refrozen without Cryoprotectants to Activate Intra- and Interspecies Oocytes

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THE ABILITY OF BULL AND STALLION SPERMATOZOA REFROZEN
WITHOUT CRYOPROTECTANTS TO ACTIVATE INTRA- AND
INTERSPECIES OOCYTES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

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The Department of Veterinary Clinical Sciences

by

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ABBREVIATIONS USED IN THE DISSERTATION

ADP: adenosine diphosphate
AO: acridine orange
APC: anaphase promoting complex
ART: assisted reproductive technique
ATP: adenosine triphosphate
Ca: calcium
Cal: calcium ionophore A23187
CaMKII: calmodulin-dependent protein kinase II
CC: cumulus cells
CDK1: cyclin-dependent kinase 1
CFDA: 6-carboxylfluorescein diacetate
CHX: cycloheximide
COC: cumulus oocyte complex
Cp: compacted cumulus cells
CPAs: cryoprotectants
CSF: cyostatic factor
DAG: diacylglycerol
DMAP: 6-dimethylaminopurine
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
DTT: dithiothreitol
Ex: expanded cumulus cells
EY: egg yolk
FCS: fetal calf serum
FPN: female pronucleus
FSH: follicle stimulating hormone
GLY: glycerol
GSH: glutathione
GV: germinal vesicle
HA: hyaluronic acid
HBP: hexosamine biosynthesis
HK: hexokinase
H-SOF: hepes-buffered synthetic oviductal fluid
H-TCM199: hepes-buffered TCM199
IBMX: 3-isobutyl-1-methylxanthine
ICSI: intracytoplasmic sperm injection
I_o: ionomycin
IP3: inositol 1,4,5-trisphosphate
IP3R-1: IP3 receptors type 1
IVF: in vitro fertilization
IVM: in vitro maturation

IVP: in vitro production
LH: luteinizing hormone
MII: metaphase II
MPF: maturation promoting factor
MPN: male pronucleus
PB: polar body
PBS: phosphate-buffered saline
PHE: penicillamine, hypothaurine and epinephrine
PI: phosphoinositide
PI: propidium iodide
PIP2: phosphatidylinositol 4,5-bisphosphate
PLC: phospholipase C
PLC ζ : phospholipase C zeta
PM: plasma membrane
PN: pronucleus
PPP: pentose phosphate pathway
PT: perinuclear theca
PTK: protein tyrosine kinase
PVP: polyvinylpyrrolidone
ROS: reactive oxygen species
SCD: sperm chromatin dispersion
SCSA: sperm chromatin structure assay
SOF: synthetic oviductal fluid
TCM199: tissue culture medium 199
TEM: transmission electron microscopy
TUGA: transvaginal ultrasound-guided aspiration
TZP: transzonal cytoplasmic processes
ZP: zona pellucida

ABSTRACT

Semen cryopreservation has allowed the establishment of genome banks and the large scale propagation of species. The development of simple techniques to cryopreserve semen or alternatives to efficiently use cryopreserved semen from males of valuable genetics that have become infertile will permit continuous propagation of the genetics from these males and may serve as a model for preservation and propagation of endangered species. Sperm cryopreservation without cryoprotectants is a simple process, and offspring have been produced following intracytoplasmic sperm injection (ICSI); however the ability of frozen-thawed sperm refrozen without the addition of cryoprotectants to activate oocytes following ICSI was unknown. In the series of experiments performed, bull and stallion frozen-thawed sperm refrozen without the addition of cryoprotectants was used to activate intra- and interspecies oocyte following ICSI. Additionally, equine cumulus-oocyte complexes (COCs) glucose metabolism during *in vitro* maturation was evaluated. The first experiment demonstrated that bull and stallion frozen-thawed sperm refrozen without the addition of cryoprotectants had their plasma membrane damaged; however the DNA was unaffected. The second experiment demonstrated that bull and stallion frozen-thawed sperm refrozen without the addition of cryoprotectants had the ability to activate bovine oocytes following intracytoplasmic sperm injection; although at a lower rate compared to frozen-thawed sperm. The third experiment demonstrated that frozen-thawed stallion sperm refrozen without the addition of cryoprotectants was unable to activate equine oocytes. The exact reason for this failure could not be explained from the experiment; however COC metabolism during *in vitro* maturation impacts embryo activation/development and

required further investigation. The fourth experiment demonstrated that equine COCs consume and metabolize glucose through glycolysis during *in vitro* maturation; however, results from this experiment were unable to explain the failure of refrozen stallion sperm to activate equine oocytes. To our knowledge, this is the first report of the use of bull or stallion frozen-thawed sperm refrozen without the addition of cryoprotectants to activate oocytes following ICSI. Furthermore, this is also the first report of equine COCs glucose metabolism during *in vitro* maturation.

CHAPTER 1 INTRODUCTION

Cryopreservation of semen from valuable breeding animals as well as from endangered species has allowed large-scale propagation and preservation of species. Loss of fertility due to injury, disease or death of males with high genetic merit or from endangered species may be devastating for breeders and for the preservation of species. Consequently, efficient use of the cryopreserved material comes to be extremely important.

The use assisted reproductive techniques (ART's) such as artificial insemination (AI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been used for decades to overcome male infertility, improve reproductive efficiency, spread valuable genetics and study the events of fertilization and early embryonic development. In regards to efficient use of cryopreserved sperm, ICSI and IVF have an advantage over AI, and resides in the low numbers of sperm needed to produce an offspring. Moreover, ICSI can be considered the most efficient technique in regards to sperm usage, as only one sperm is required to produce an offspring.

In ICSI, the sperm is injected into the oocyte, thus the sperm-oocyte membranes interaction is bypassed, and sperm motility and plasma membrane integrity are not required (Wakayama et al., 1998; Ward et al., 2003; Choi et al., 2006). This fact has lead researchers to investigate the use of alternative methods of sperm preservation, where sperm motility and sperm plasma membrane integrity are not conserved, to activate oocytes and produce offspring via ICSI. Methods investigated include freeze-drying (Keskin-tepe et al., 2002; Kwon et al., 2004; Kusakabe et al., 2008; Choi et al.,

2011), freezing without cryoprotectants (Wakayama et al., 1998; Ward et al., 2003; Li et al., 2010) and refreezing frozen-thawed semen (McCue et al., 2004; Choi et al., 2006).

The use of sperm frozen without cryoprotectants to successfully activate oocytes has been reported in laboratory animals (Wakayama et al., 1998; Lacham-Kaplan et al., 2003; Li et al., 2010); however the ability of large animals (e.g. bovine and equine) sperm cryopreserved without cryoprotectants to activate oocytes is unknown. Freezing or refreezing bull and/or stallion sperm without the addition of cryoprotectants and direct immersion into liquid nitrogen is a very quick procedure that saves time, inexpensive, does not require specialized semen freezing equipment and potentially very useful in busy veterinary clinics and/or semen processing centers. In addition some cryoprotectants used have animal origin that can pose restrictions for international distribution. The objectives of these studies were to evaluate the ability of bull and stallion frozen-thawed and then refrozen without further addition of cryoprotectants to activate intra- and inter-species oocytes as well as to investigate the effect of COCs metabolism during IVM on oocyte activation/developmental competence.

After we determined that bull and equine frozen-thawed and then refrozen without cryoprotectants sperm have the ability to activate oocytes, recommendations on the efficient use of cryopreserved semen from males of high genetic merit or endangered species or on semen cryopreservation methods may be addressed. In addition, results from these studies will serve as the platform for future research to improve COCs IVM and embryo production in large animals and endangered species.

CHAPTER 2 LITERATURE REVIEW

Oocyte Activation during Fertilization

In most mammals (except canine), ovulated oocytes are arrested at the MII stage and finalize meiosis after fertilization. The term oocyte activation encompasses the events involved in oocyte liberation from MII arrest, resumption of meiosis, pronuclear formation and initiation of cell division (Ducibella et al., 2002). Oocytes arrested in MII are characterized for having high activity of the MPF (Kubiak et al., 1993), an heterodimer composed of the CDK1/cdc2, a regulatory cyclin B1 and stabilized by the CSF (Hyslop et al., 2004; Jones, 2004). Exit from the MII is stimulated by intracellular rises of Ca^{2+} which reduces the MPF activity. The reduction of MPF comes from polyubiquitination of cyclin B1 by the anaphase promoting complex (APC) and destruction by the 26S proteasome (Nixon et al., 2002; Peters, 2002; Hyslop et al., 2004).

In mammals, interaction between the sperm and oocyte during fertilization induce oscillatory waves of intracellular Ca^{2+} (Fissore et al., 1992; Kline and Kline, 1992; Vitullo and Ozil, 1992; He et al., 1997; Ducibella et al., 2002; Knott et al., 2003; Jones, 2005) that last for several hours (Miyazaki et al., 1993; Nakada and Mizuno, 1998) and release the oocyte from the MII arrest. How this sperm-egg interaction initiates the Ca^{2+} oscillations has been a topic for debate and three hypotheses explaining the possible mechanism have been postulated (Jaffe, 1983; Whitaker and Swann, 1993; Alberio et al., 2001; Malcuit et al., 2006a). The first or “sperm conduit hypothesis” suggests that the sperm-oocyte fusion causes oocyte plasma membrane de-polarization, allowing

extracellular Ca^{2+} to flow into the oocyte starting at the point of fusion and initiating the Ca^{2+} waves (Jaffe, 1983). However, this hypothesis was disproved after finding that extracellular Ca^{2+} was not necessary to initiate Ca^{2+} oscillations (Stricker, 1996) and changes in Ca^{2+} concentrations were not observed near the site of sperm-oocyte fusion (Jones et al., 1998b). The second or “receptor hypothesis” states that at sperm-oocyte contact a receptor-ligand interaction mediated by G-protein or PTK, signals the release of Ca^{2+} (Jaffe, 1990; Shilling et al., 1994). G-protein activates phospholipase β isoforms (Miyazaki, 1988; Carroll et al., 1997) and/or PTK activates the src homology 2 (SH2) domains of PLC γ (Carroll et al., 1997) that hydrolyze PIP_2 into DAG and IP_3 (Miyazaki, 1988; Berridge, 1993; Miyazaki et al., 1993; Carroll et al., 1997; Alberio et al., 2001) to initiate Ca^{2+} release from the endoplasmic reticulum (Berridge, 1993). Nevertheless; research indicating that G-proteins blockage has no effect on the fertilization-induced Ca^{2+} oscillations (Swann, 1990; Williams et al., 1998) and SH2 receptors inhibition at fertilization did not prevent Ca^{2+} oscillations in the mouse (Mehlmann et al., 1998; Mehlmann and Jaffe, 2005) argues against this hypothesis.

The third and perhaps the current accepted theory is “the fusion hypothesis”. This hypothesis propose that at fertilization the sperm carries and introduces a sperm factor into the oocyte which activates the PI pathway (Swann, 1990; Parrington et al., 1996; Wu et al., 2001). Supporting this hypothesis are the experiments demonstrating the ability of injected hamster (Swann, 1990), mouse (Swann, 1994), human (Homa and Swann, 1994), pig (Machty et al., 2000), cattle (Malcuit et al., 2006b) and horse (Bedford et al., 2003) oocytes with sperm factors derived from their respective sperm, to stimulate Ca^{2+} oscillations and activation. In addition, the fact that oocyte activation and

embryo development occurs after intracytoplasmic sperm injection (ICSI) in different species (Palermo et al., 1992; Tesarik et al., 1994; Kimura and Yanagimachi, 1995a; Choi et al., 2002; Wei and Fukui, 2002) reinforces the proposal that the sperm is the carrier of the factor(s) stimulating the cascade of events involved in oocyte activation.

The identity of the sperm factor was unknown and thought to be a ~ 33kDa protein (Parrington et al., 1996). Using sea urchin eggs homogenates, Jones et al. (1998a) showed the involvement of PLC to induce Ca^{2+} rises through IP_3 production. Further, Parrington et al. (1999) demonstrated that the same PLC that causes Ca^{2+} rises in the sea urchin homogenates also induced Ca^{2+} oscillations when injected into mouse oocytes. This finding suggests that the sperm factor is able to cause Ca^{2+} oscillations across species and supporting this are studies showing oocyte Ca^{2+} oscillation after cross species injection of sperm extracts (Homa and Swann, 1994; Wu et al., 1997; Choi et al., 2002; Bedford-Guaus et al., 2008). In mammals, there are eleven PLC isoforms divided into β , γ , δ and ϵ subfamilies (Rhee, 2001); however the sperm PLC isoform appears to be unique. Tissue extracts from brain, kidney and liver or recombinant PLC β 1, PLC γ 1 and PLC δ 1 were unable to stimulate Ca^{2+} release after addition to sea urchin eggs homogenates (Jones et al., 2000). Moreover, although PLC subfamilies β , γ and δ are present in sperm (Walensky and Snyder, 1995; Mehlmann et al., 1998; Parrington et al., 2002), none was able to stimulate Ca^{2+} rises in sea urchin homogenates or mouse oocytes (Parrington et al., 2002). So, if the sperm factor is really a PLC, why were tissue extracts, recombinant PLC β , γ , δ or PLCs present in sperm not able to stimulate Ca^{2+} rises? Saunders et al. (2002), using PLC-related sequences from mouse testis, immunoblot and chromatography revealed a novel PLC

isoform, the ~70 kDa sperm-specific PLC ζ . To prove this novel PLC has the sperm factor activity, PLC ζ complementary RNA was injected into MII mouse oocytes. All injected oocytes displayed Ca²⁺ oscillations similar to the observed after injection of sperm extracts and during fertilization (Kline and Kline, 1992). In support of the role of PLC ζ as the sperm factor, injection of recombinant PLC ζ or PLC ζ into mouse (Cox et al., 2002; Fujimoto et al., 2004), cattle (Ross et al., 2008), human (Rogers et al., 2004) and mare (Bedford-Guaus et al., 2008) oocytes caused Ca²⁺ oscillations and parthenogenetic activation.

Following the release of the sperm PLC ζ within the ooplasm, a not well characterized cascade of events are activated to initiate the oscillatory Ca²⁺ waves, exit from MII arrest and embryo development (Figure 1). In most cells Ca²⁺ response to different stimuli is mediated by the production of IP₃ from the hydrolysis of PIP₂ by a PLC (Berridge, 1993). In oocytes, PLC ζ hydrolyzes PIP₂ into IP₃ and mediates the Ca²⁺ oscillations (Miyazaki, 1988; Fissore et al., 1995). The IP₃ binds to IP₃R-1 (Miyazaki et al., 1993) at the endoplasmic reticulum to release Ca²⁺. To sustain long-lasting Ca²⁺ oscillations, replenishment of Ca²⁺ stores appears to be mediated by the activation of Ca²⁺ channels through DAG and downstream by PKC (Halet et al., 2004). Then, Ca²⁺ oscillations through CaMKII activates APC resulting in MPF degradation, exit from MII arrest and cell cycle resumption (Lorca et al., 1993; Nixon et al., 2002).

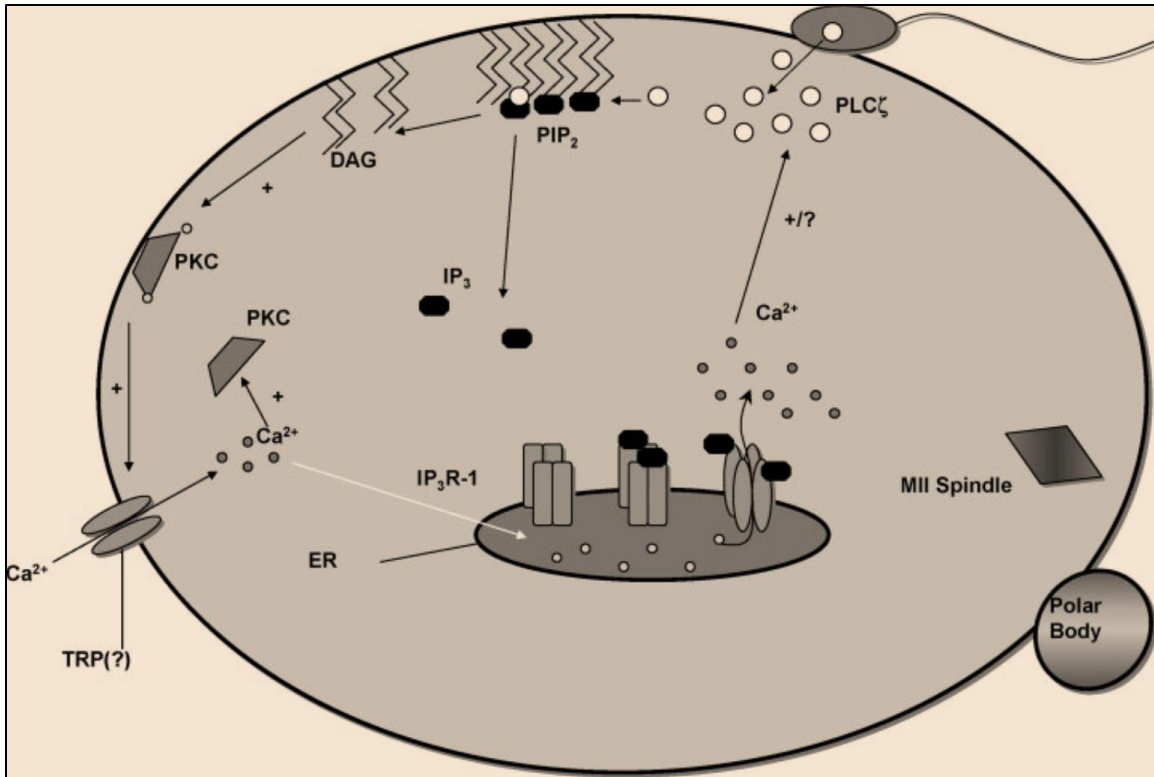


Figure 1: Proposed mechanism of Ca^{2+} oscillation during mammalian fertilization. Upon gamete fusion, the fertilizing sperm delivers into the ooplasm a soluble substance, presently thought to be $\text{PLC}\zeta$ capable of hydrolyzing PIP_2 into two signaling molecules, IP_3 and DAG. IP_3 is involved in intracellular Ca^{2+} release by binding and gating its receptor, the $\text{IP}_3\text{R}-1$, located in the ER, the Ca^{2+} store of the cell, while DAG may play an integral role in signaling events at the plasma membrane. DAG together with the elevations in Ca^{2+} may further target and activate PKC to the plasma membrane, where PKC may regulate Ca^{2+} influx to refill the intracellular stores making possible the persistence of oscillations. (From: Calcium Oscillations and Mammalian Egg Activation; Malcuit et al., 2006).

Sperm Preservation Methods

Freezing sperm with cryoprotectants (conventional freezing) has been extremely beneficial for the propagation and preservation of animals with valuable genetics as well as for the preservation of endangered species. Sperm cryopreservation causes cell damage and frozen-thawed sperm has lower fertility compared to fresh sperm (Watson, 2000). Therefore, in conventional freezing, the main objective is to preserve sperm

motility, plasma membrane and DNA integrity, characteristics that enable a sperm to fertilize an oocyte under normal fertilization conditions.

When a cell (in this case sperm) is slow-frozen at an optimal cooling rate to the final storage temperature of -196°C , several physical events take place. During cooling from 37°C to subzero temperatures, the plasma membrane undergoes rearrangement of the lipids bilayer (Quinn, 1985). At -5°C the cell and surrounding medium remain unfrozen and super cooled due to the solutes present. Between -5°C to -15°C , extracellular ice formation begins. To maintain the system in osmotic equilibrium, the cells will dehydrate as a response of the increase extracellular osmolality (Mazur, 1977). As extracellular ice formation continues, more intracellular water flows out and the cells will be trapped in the hypertonic unfrozen channels between ice plates until the eutectic point is reached (Chenier et al., 1998). In addition, cryopreservation cause the production of superoxide radicals that cause DNA defragmentation (Chatterjee and Gagnon, 2001; Baumber et al., 2003). During thawing, the physical events listed are reversed and the osmolality and cell volume will return to the pre-freezing state (Mazur and Cole, 1989).

Conventional Freezing

With the goal of protecting sperm from suffering cryodamage, CPAs are added to the medium in which the sperm are suspended before freezing. Depending on their ability to permeate through the plasma membrane, cryoprotectants are divided into non-permeating and permeating.

To prevent “cold shock”, combinations of skim milk-egg yolk (Cochran et al., 1984; Graham and Foote, 1987) or purified milk fractions-egg yolk (Vidament et al.,

2000) and sugars (Loomis et al., 1983; Chen et al., 1993) are common in commercially available semen freezing extenders. The percentage of egg yolk varies from 2 – 20% (Graham et al., 1978; Martin et al., 1979; Vidament, 2005). In addition to skim milk and egg yolk to prevent cold shock, recent reports have loaded sperm cells plasma membrane with cholesterol before mixing with the freezing extenders showing promising results (Purdy and Graham, 2004; Moore et al., 2005).

The proposed mechanism of protection from cold shock resides in the ability of the low-density lipoprotein fraction in the egg yolk to prevent lipid efflux from the sperm plasma membrane (Bergeron et al., 2004). Unfortunately egg yolk contains multiple undefined components that may cause beneficial and/or deleterious effects.

The mechanism by which milk based extenders protect the sperm plasma membrane against cold shock still unclear; however, it is suggested that their protective mechanism is based on their antioxidant properties (Aurich, 2005). Similar to egg yolk, skim-milk based extenders contain undefined components. Semen extenders with purified milk fractions (native phosphocaseinate and β -lactoglobulin) have been developed and shown to be effective protecting equine sperm against cold shock (Batellier et al., 1997).

The protective properties of sugars when added to freezing medium are not fully elucidated. The mechanism may be related to their colligative properties, reducing the electrolyte concentration (Lovelock, 1953) or increasing the size of the channels where the sperm cells are trapped within the unfrozen fraction (Mazur and Cole, 1989; Woelders et al., 1997). In addition to these theories of protective mechanism, sugars will

interact with the sperm membrane phospholipids and stabilize the membrane during freezing (Anchordoguy et al., 1987).

The ideal permeating cryoprotectant must have a low molecular weight, high water solubility and minimal toxicity (Fahy et al., 1990). The cryoprotective mechanisms of permeating CPAs are not completely elucidated. Permeating CPAs may exert their protection against cryodamage through their colligative property (Storey, 1997). In this regard, their action is multifactorial; first they will increase the osmolality of the extracellular compartment and therefore cell dehydration. Second, the melting point of the solution is depressed. Third, they may decrease the electrolytes concentration in the unfrozen fraction (Mazur and Cole, 1989). Cryoprotection by these compounds can also be attributed to their interaction with membrane phospholipids (Anchordoguy et al., 1987). Unfortunately, permeating CPAs exert deleterious effects to sperm and are considered “toxic” (Fahy et al., 1990). The deleterious effects might be related to enzymatic inactivation or protein denaturation; however, the mechanism of toxicity remains an enigma. The plasma membrane permeability of these compounds is different due to difference in their molecular weights; compounds with greater molecular weight may cause damage to the plasma as they cross in and out of the cell during freezing/thawing (Ball and Vo, 2001).

Glycerol (GLY) was the first compound utilized to successfully cryopreserve sperm (Smith and Polge, 1950), and has been the most common compound utilized. In addition to the previously mentioned protective mechanisms, GLY also has the property of binding hydrogen from the water molecules, inhibiting ice formation (Smith and Polge, 1950). Other permeating CPAs that have been used to freeze sperm include ethylene

glycol (Martins-Bessa et al., 2006; Yildiz et al., 2007; Taşdemir et al., 2013), DMSO (Sztejn et al., 2001; Taşdemir et al., 2013) and amides (Alvarenga et al., 2005; Futino et al., 2010).

Sperm frozen by the conventional method is commonly packed into 0.25 – 0.5 mL straws and tenth to hundreds of millions per straw. It is a time consuming procedure that commonly take 1 – 3 hours, depending on the freezing extenders protocols. In addition some of the cryoprotectants are animal derived products (e.g. egg yolk), which pose a risk for disease transmission and limits the international distribution of frozen semen.

Although sperm motility and an intact plasma membrane are necessary under normal fertilization conditions, these characteristics are not essential in ARTs such as ICSI (Wakayama et al., 1998), where fertilization is assisted. This fact has permitted researchers the examination of alternative sperm preservation techniques such as freeze-dried (Keskintepe et al., 2002; Ward et al., 2003; Kwon et al., 2004; Choi et al., 2011) and freezing without CPAs (Wakayama et al., 1998; Lacham-Kaplan et al., 2003; Ward et al., 2003; Li et al., 2010) for the production of embryos *in vitro* and normal offspring.

Freeze-dried Sperm Preservation

Freeze-drying (lyophilization) is a method utilize to preserve biological material by dehydration through sublimation (Sherman, 1957). Sperm preserved using this method was first reported in the fowl (Polge et al., 1949) and later in human and bull (Sherman, 1957).

Freeze-dried sperm are packed in glass ampules with $0.5 - 1 \times 10^6$ sperm, and can be stored at room temperature for periods of > 1 year (Keskinetepe et al., 2002; Ward et al., 2003); however sperm motility is poorly recovered (Sherman, 1957) and the plasma membrane damaged (Martins et al., 2007) upon sperm pellet reconstitution. While the sperm motility and plasma membrane integrity are compromised with freeze-drying, slight negative effects are observed in DNA integrity (Kusakabe et al., 2001; Martins et al., 2007).

Loss of sperm motility and damage to the plasma membrane render freeze-dried sperm unable to undergo normal fertilization; however, fertilization can be obtained assisted by ICSI. Oocyte activation (Kwon et al., 2004), development to blastocyst (Keskinetepe et al., 2002; Kwon et al., 2004; Riel et al., 2007) and live offspring (Ward et al., 2003; Choi et al., 2011) have been reported following ICSI with freeze-dried sperm. Drawbacks from this sperm preservation method is that chromosomal abnormalities in embryos produced are increased (Ward et al., 2003) and special equipment to process the sperm and fertilize oocytes is required.

Freezing Sperm without CPAs

The mouse is extensively use for research purposes and storing sperm from different strains insure the possibility of combining genetics to produce offspring. Freezing mouse sperm by the conventional method has been problematic and was not until 1990 (Tada et al., 1990) when a successful cryopreservation protocol using a combination of skim-milk, raffinose and GLY was devised. Nevertheless, this cryopreservation protocol is effective preserving sperm motility for some mouse strains but not others (Nakagata, 2000).

The fact that the sperm nucleus is very stable (Ward and Coffey, 1991) and the discovery that immotile mouse sperm with damaged plasma membrane had the ability to produce offspring after ICSI (Kuretake et al., 1996), prompted the investigation of sperm cryopreservation methods where sperm motility and plasma integrity were not conserved. Wakayama et al. (1998), reported that mouse sperm without CPAs, packed in vials (1 mL) and directly plunged into liquid nitrogen were highly fertile and able to produce live offspring after ICSI. Thereafter, more embryos and offspring using sperm frozen without CPAs after ICSI have been reported in the mouse (Lacham-Kaplan et al., 2003; Ward et al., 2003) and rabbit (Li et al., 2010).

Another method used to cryopreserve sperm is vitrification (Isachenko et al., 2003). A small volume of sperm suspension (~ 20 μ L) on cooper loops is directly plunged into liquid nitrogen. The ultra-rapid cooling rate (160 – 270 $^{\circ}$ C/min) avoids the formation of ice crystals; therefore circumvents the addition of CPAs to the sperm suspension (Isachenko et al., 2004). This characteristic also permits the retention of sperm motility and plasma membrane integrity upon thawing. Human sperm has successfully been vitrified without CPAs (Isachenko et al., 2004) and birth of two healthy babies reported (Isachenko et al., 2012). Vitrification without CPAs of goat sperm has recently been reported (Katanbafzadeh et al., 2014); however instead of cooper loops, 0.25 mL straws were used to pack the semen.

Freezing/vitrification sperm without CPAs offer some advantages over the conventional freezing. The process is shorter and less stringent, the volume and sperm numbers frozen per vial, straw or loop may be considerably reduced, allowing storage of greater number of possible samples for the production of embryos. CPA free sperm

freezing also allows the preservation of males with valuable genetics but unable to withstand conventional freezing.

Although cryopreservation without CPAs offer advantages over the conventional freezing method, it also carries some disadvantages. Due to the low sperm numbers, samples cannot be used for intrauterine artificial insemination and sperm frozen without CPAs (not vitrified) can only fertilize oocytes through ICSI, a technique that requires specialized equipment.

Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic sperm injection is a technique for fertilizing a MII oocyte by micro-injecting a single sperm into the ooplasm. Application of ICSI has proven to be a powerful technique towards the study of mechanisms involving fertilization and early embryo development, and in the field of ARTs for embryo IVP. The first successful ICSI and pronuclear formation in mammals was reported 40 year ago (Uehara and Yanagimachi, 1976). In human medicine, the use of ICSI was first reported by Palermo et al. in 1992 (Palermo et al., 1992) to overcome male infertility. In mammals other than humans, ICSI-derived offspring have been reported in cattle (Katayose et al., 1999; Horiuchi et al., 2002), horses (Cochran et al., 1998; McKinnon et al., 2000), sheep (Gómez et al., 1998), mice (Kimura and Yanagimachi, 1995a), rabbit (Bhullar et al., 2001) and cats (Gomez et al., 2000).

Intracytoplasmic Sperm Injection (ICSI) in Cattle

Fertilization of bovine oocytes following ICSI was first reported by Keefer et al. (1990). In this study, *in vitro* capacitated sperm were injected into the ooplasm of MII oocytes and two percent (2%) of injected oocytes cleaved without exogenous activation;

however only 21% of the injected oocyte contained a sperm within the ooplasm. Exogenous activation with Cal increased the number of cleavage (28%) to the two- to four-cell stage of development after 48 h; however, non-injected oocytes but activated with Cal cleaved at a similar rate (18%). Oocytes not incubated with Cal showed partenogenetic activation of 4% and 9% in the sham-injected and non-injected respectively. The researchers concluded that the stimulus from microinjection was insufficient to activate bovine oocytes but may undergo normal activation and cleavage upon exogenous activation.

Goto et al. (1990), was the first to report the cleavage, development to blastocyst and birth of normal calves after MII bovine oocytes were injected with an immobilized sperm. Epididymal frozen-thawed bull sperm was *in vitro* capacitated and immobilized (killed) by freezing (-20°C) and thawing twice in the absence of cryoprotectants. Metaphase II oocytes were injected with or without (sham-injection) sperm. Activation of injected oocytes was stimulated with 50 or 100µM of Cal and then co-cultured for 6 days in a conditioned cumulus cells monolayer medium. Of the injected and stimulated oocytes (50 µM), 30/507 (5.9%) and 9/115 (7.8%) developed to the morula and blastocyst stage respectively. Sham-injected oocytes cleaved to the two- to four- cell stage but none develop past the six- cell stage. At the end of culture, two to four morula/blastocyst were transferred to each recipient cow. Sixty six percent of recipients became pregnant and 50% delivered a live calf.

Calcium plays an important role in triggering oocyte activation (Fissore et al., 1992; Carroll, 2001), and to determine whether oocyte activation was caused by the microinjection procedure or by exogenous Ca²⁺ stimuli, an experiment in humans

measured intra-oocyte changes in Ca^{2+} (Tesarik et al., 1994). Results from this experiment showed that microinjection causes an immediate rise in Ca^{2+} ; however this rise was not responsible for oocyte activation. Instead, oocyte activation was due to a sperm dependent rise in Ca^{2+} observed 4 – 12 h after microinjection. Catt and Rhodes (1995), performed a study to determine if oocyte activation/fertilization after ICSI between domestic species was different. Cow, sheep and sow oocytes were microinjected with an immobilized sperm. After overnight culture, fertilization (as per the presence of MPN and FPN, and a sperm tail within the oocyte) was assessed. Oocyte fertilization was 63% in sheep, 25% in cattle and 60% in pigs. Oocyte activation after sham-injection was 55% in sheep, and less than 20% in cattle and pigs. Results from this study highlighted the oocyte activation differences between species and suggested that similar to humans, bull and boar sperm may release a sperm factor responsible for oocyte activation. The authors also suggested that the release of the sperm factor may have been facilitated by swiping the sperm during immobilization prior to injection. This suggestion was later confirmed in human ICSI (Dozortsev et al., 1997). Damage to the sperm plasma membrane during immobilization is necessary prior to ICSI to facilitate the release of the sperm activating factor, PLC ζ (Saunders et al., 2002).

Same as interspecies differences in oocyte activation (Catt and Rhodes, 1995), there are differences in the ability of the sperm nuclei to de-condense after ICSI (Perreault et al., 1988). In this regard, bull and rat sperm nuclei de-condensed and formed a MPN at a lower rate three hours after microinjection into hamster oocytes compared to human, hamster, mouse and chinchilla sperm. Strength and number of disulfide bonds in the bull and rat confers greater nuclear stability delaying nuclear de-

condensation (Perreault et al., 1988). The effect of different sperm treatments prior to ICSI and exogenous oocyte activation stimulus after ICSI on embryonic development was evaluated by Chen and Seidel Jr (1997) and Rho et al. (1998). In the study by Chen and Seidel Jr (1997), sham-injected as well as injected oocytes with non-treated sperm, sperm incubated with heparin (5 U/mL) for two hours or capacitated with heparin and then incubated with 0.1 μ M Cal for 1 min were either non-activated (control) or exogenously activated with 50 μ M Cal or 7% ethanol after ICSI. Oocytes were denuded and centrifuged before injection to allow visualization of the polar body and sperm within the ooplasm after injection. Sham-injected oocytes were partenogenetically activated by Cal (55%) or ethanol (52%) but did not develop to blastocyst. Conversely, sham-injection alone induced partenogenetic activation in only 4% of oocytes. Sperm-injected oocytes cleaved at a higher rate when treated with Cal (47%) and ethanol (50%) compared to control (25%); however, development to the blastocyst stage was not different between Cal (33%) and control (24%). In this study, sperm treatment prior to injection showed no beneficial effect on oocyte activation/cleavage or development to the blastocyst stage.

Rho et al. (1998), did a series of experiments in which sperm was treated prior to injection with 5 mM DTT, to reduce disulphide bonds and promote nuclear decondensation (Sutovsky and Schatten, 1997). Following ICSI the oocytes were activated with I_o or I_o followed by DMAP three hours later (I_o + DMAP). The researchers assessed the effect of sperm treatment and exogenous activation stimulus on oocyte activation, pronuclear formation, cleavage and development to blastocyst and chromosomal constitution of blastocysts; that the blastocysts were from fertilization and

the viability of blastocysts after transfer. Oocytes were considered activated if one PN was observed. After maturation, oocytes were denuded and centrifuged to facilitate polar body visualization and proper delivery of the sperm into the ooplasm. From the successfully injected oocytes, activation was higher in DTT-treated sperm (59.6%) compared to untreated (23.1%) sperm. Cleavage and development to blastocyst after ICSI with DTT-treated sperm were 3.1% and 0% without exogenous activation, 16.5% and 3.8% with I_o, and 61.2% and 23.9% with I_o + DMAP. Sham-injected oocytes cleavage and development to blastocyst without exogenous activation was lower (4.5% and 0% respectively) compared to oocytes activated with I_o + DMAP (42% and 10.8% respectively). Diploid blastocysts between DTT-treated and untreated sperm was not different (60.8% and 47.1% respectively); however the percentage of mixoploid was (21.6% and 29.4%, respectively) and polyploid (17.6% and 23.5%, respectively) blastocyst for both groups. Transfer of two embryos into each of 16 heifers resulted in 6 (38%) pregnancies; however none was carried to term. This study concluded that sperm pre-treatment with DTT and exogenous activation with I_o + DMAP promote sperm nucleus de-condensation and PN formation; however embryos with chromosomal abnormalities were frequent.

Gender selection is important in the management and production efficiency in both the dairy and beef cattle industry. Sex sorted semen using high-speed flow cytometry (Johnson and Welch, 1999) offers the advantage of gender pre-selection before conception; however the sex sorting process is still inefficient with low numbers of viable sperm surviving and those surviving apparently having lower fertility (Maxwell et al., 2004). The use of one sperm to fertilize an oocyte makes ICSI an attractive

technique when limited numbers of sperm are available, such as in sex sorted semen. Production of normal calves after ICSI with sex sorted bull sperm heads was evaluated by Hamano et al. (1999). IVM cow oocytes were injected with a single sex sorted bull sperm head and activated with 7% ethanol before and after ICSI. Confirmed sperm heads having a Y chromosome in the sorted semen was 82% and used to perform ICSI. The percentage of oocytes matured *in vitro* for 22, 24 and 26 h before ICSI developing to the blastocyst stage were 4.8%, 6.9% and 3.5% respectively. A single embryo was transferred to each of 48 cows, 10 (20%) became pregnant and delivered a calf (8 males and 2 females). From the study, the authors concluded that accurate gender pre-selection is obtained with the flow cytometric/cell sorter. The reason for the low fertilization rate and embryonic development was not known, but the authors suggested that the sorting procedure may have affected the sperm fertilizing ability.

The Piezo-driven sperm injection technique was introduced to perform ICSI in the mouse by Kimura and Yanagimachi (1995a), and reported increased numbers of fertilized oocytes (78% vs 8%) and doubled the number of injected oocytes that develop to the blastocyst stage compared to the conventional method (68% vs 33%). Furthermore, 80% of oocytes survived microinjection with the Piezo technique and 16% with the conventional technique. The Piezo-driven sperm injection is based on the piezoelectric effect, where the microinjection pipette is advanced a very short distance ($\geq 0.1 \mu\text{m}$) at very high speed ($\leq 40 \mu\text{m S}^{-1}$), stabbing the ZP and the oolema precisely without damage (Yoshida and Perry, 2007).

Efficacy of the Piezo-driven sperm injection in cattle was first evaluated by Katayose et al. (1999) and later by Suttner et al. (2000). Katayose et al. (1999),

compared oocyte activation between the Piezo-driven and conventional ICSI technique with or without exogenous activation with Cal. Results demonstrated that fertilization with the Piezo-driven ICSI was superior to the conventional technique as oocyte fertilization (presence of two PN) with Piezo-driven ICSI was 72% with or without exogenous activation and higher than 19% and 0% with the conventional technique, respectively. The researchers concluded that the conventional technique was not effective delivering the sperm, as sperm outside the ooplasm was frequently observed. The high rates of fertilization in the study compared to previous cattle ICSI studies was a result of effective delivery of sperm within the ooplasm and damage of the sperm plasma membrane allowing the release of the sperm PLC ζ .

Suttner et al. (2000), also compared the Piezo-driven against the conventional ICSI technique, as well as the effect of sperm pre-treatment and exogenous activation. Sperm pre-treatment consisted in incubation with 10 μ g/mL heparin (capacitated) or DTT. Protocols used for exogenous activation were incubation with 5 μ M Cal for 10 min, incubation with Cal followed by CHX for five h (Cal + CHX) or incubation with 5 μ M Io for 4 min followed by 1.9 mM DMAP three hours later (Io + DMAP). Oocytes were considered activated if they extruded a second PB and contained one PN. Oocytes were considered fertilized if two PN, a second polar body and a sperm tail were observed within the ooplasm. Using conventional injection with capacitated sperm and exogenous activation with Io + DMAP resulted in the highest activation rate (73.3%). Fertilization rates however, were not different. The highest cleavage rates were obtained with capacitated sperm, conventional injection and exogenous activation with either Io + DMAP (79.6%) and Cal + CHX (77.6%). The highest development to the

blastocyst stage was obtained with capacitated sperm, conventional injection and exogenous activation with I_o + DMAP (28.2%) compared to the rest with the exception of DTT sperm, conventional injection and I_o + DMAP (18.5%), and DTT sperm, Piezo injection and I_o + DMAP (17.2%). This study was the first to report the use of CHX to induce oocyte activation; therefore the blastocysts chromosomal content of the CHX activate oocytes was evaluated. Ninety percent (90%) of the CHX-activated blastocysts were diploid. This study concluded that bovine oocytes required exogenous activation and that the sperm itself may contribute in the activation process. Pre-treatment with DTT may be detrimental to sperm DNA affecting early embryogenesis.

Results from these studies did not clarify the requirement for exogenous oocyte activation or the potential use of the Piezo-driven injection technique in cattle. Further evaluation of the Piezo-driven ICSI potential for use in embryo IVP was evaluated by Wei and Fukui (2000). This study evaluated the effect of ooplasm clarification, the amount of fluid injected into the oocyte and the concentration of PVP in the sperm media on the survival and PN formation after Piezo-driven ICSI without exogenous activation. The ooplasm was clarified by centrifugation (Chen and Seidel Jr, 1997; Rho et al., 1998) to visualize sperm delivery. The sperm tail was cut at the midpiece with a glass needle (20 µm in diameter) to reduce the amount of fluid injected. PVP was previously shown to stabilize the plasma membrane and interferes with human sperm de-condensation (Dozortsev et al., 1995), thus the effect on sperm nuclear de-condensation of media with different PVP concentrations (10%, 4% and 0%) were evaluated. Survival and pronuclear formation were higher when oocytes ooplasm was clarified by centrifugation compared to non-centrifuged (92% vs. 67.7% and 87.6% vs.

57.8%, respectively). Centrifugation also improved delivery of a sperm into the ooplasm (94.5%) compared to non-centrifuged (79%) oocytes and also permitted visualization of the amount of fluid injected. Survival and pronuclear formation were also improved when the sperm tail was cut compared to injection of an entire sperm (94.8% vs 77.3% and 86.6% vs. 67.7%, respectively). Survival decreased as PVP concentration was reduced from 10% to 4% and 0% (95% vs. 89.3% vs. 74.6%, respectively); conversely, pronuclear formation was improved (74.5% vs. 84.9% vs. 88.6%). The authors concluded that clarifying the ooplasm, cutting the sperm tail to reduce the amount of medium injected and reducing the PVP concentration significantly improve the Piezo-driven injection technique in cattle.

Horiuchi et al. (2002), evaluated the effect of sperm treatment and exogenous activation on cleavage, embryonic development and birth of calves after transfer. Sperm was immobilized by scoring the tail against the bottom of the dish or killed by repeat freezing/thawing in liquid nitrogen without cryoprotectants. Four hours after ICSI, oocytes were exogenously activated by incubating for 5 min in 7% ethanol. An oocyte was considered activated if a de-condensed sperm nucleus was observed after 4 h and fertilized if two PBs and PNs were observed after 21 h of injection. No difference in activation was observed between immobilized and killed sperm injection (70.1% and 67.3%, respectively). Fertilization of previously activated oocytes with immobilized and killed sperm increased after treatment with 7% ethanol to 88.2% and 89.6%, respectively. Development to blastocyst of oocytes injected with immobilized sperm was higher with ethanol (33.9%) compared to without ethanol (13.8%) activation. Although activation and fertilization was high when oocytes were injected with killed sperm,

development to blastocyst was extremely low with or without treatment with ethanol (3.6% and 0%). After transfer of one blastocyst to each of 10 cows, 50% became pregnant and delivered a normal calf. This study concluded that live offspring could be efficiently produced after Piezo-driven ICSI. Injection with the conventional ICSI technique may cause oocyte degeneration after the oolema is broken by aspiration. Nuclear de-condensation after injection into the ooplasm is efficient without sperm chemical treatment. Activation and fertilization are efficient after Piezo-driven ICSI, but some substance or signal may be missing or not sufficient, or perhaps some sperm components (acrosome or plasma membrane) may prevent development to blastocyst. Therefore, treatment with agents that stimulate activation may provide the substances or signal, or may remove the factors preventing embryo development. The inability of killed sperm to support embryo development may have been due to the time lapse between plasma membrane disruption and injection, altering the integrity of the sperm nucleus.

Wei and Fukui (2002) reported the birth of normal calves following the Piezo-driven ICSI protocol they previously described (Wei and Fukui, 2000). In this study they assessed activation (oocytes with one or more PNs) in sperm injected, sham-injected and non-injected oocytes. Also embryonic development, blastocysts cell number, chromosomal number and pregnancies after embryo transfer were investigated. Of the sperm injected oocytes, 86.3% were activated compared to 23.8% and 2.2% of the sham-injected and non-injected, respectively. From sperm injected oocytes, 22.7% developed to the blastocyst stage and 82% were diploid. After embryo transfer, 57.1% of cows become pregnant. This study concluded that exogenous oocyte activation is not

a requirement for oocyte activation and embryo development after Piezo-driven ICSI. The percentage of blastocyst with normal chromosomal constitution (82%) was superior compared to the normal blastocysts obtained with exogenous activation $\leq 60\%$ (Rho et al., 1998).

Despite improvement of the ICSI technique, development to the blastocyst stage without or with exogenous activation was still low (22% - 33%) (Horiuchi et al., 2002; Wei and Fukui, 2002). In an attempt to improve the development to the blastocyst stage after Piezo-driven ICSI, Galli et al. (2003) performed a series of experiments. In the first experiment, injected oocytes were either activated with I_o or in combination with CHX (I_o + CHX) or not (control). In experiment 2, sperm was incubated for 10 min or 4 h with heparin in combination with PHE before injection. In experiment 3, sperm were either not-treated or treated with DTT prior to injection. In experiment 4, injected oocytes were either non-treated or treated with DTT before activation. The effect of the different treatments on the rate of oocyte activation (extrusion of second PB three hours after ICSI), cleavage (48 h after ICSI) and development to the blastocyst stage (day 7 and 8 after ICSI) was evaluated. In experiment 1, they found no difference in oocyte activation, cleavage or development to the blastocyst stage between treatment groups. In experiment 2, incubation of sperm for 4 h cause a reduction in cleavage rate compared to incubation for 10 min (48.4% and 59.7% respectively); however the proportion of embryos reaching the blastocyst stage was not different at either day 7 (7.4% and 6.6% respectively) or day 8 (7.4% and 8.5% respectively). In experiment 3, treatment with DTT showed no improvement on cleavage rate (66.4%) or development to the blastocyst stage (24.3%) compared to non-treated sperm (67.2% and 21.9%

respectively). Experiment 4 results showed that treatment of injected oocytes with DTT improved the development to the blastocyst stage when oocytes were not activated following ICSI compared to non-treated oocytes (20% and 8.1% respectively); however this improvement was not observed when oocytes were activated after ICSI (23.8% and 22% respectively). In summary, embryo development to the blastocyst stage (22 – 24%) was not improved by exogenous oocyte activation or treatment with DTT. The authors suggested that the low developmental capacity of ICSI-derived embryo cannot be attributed to insufficient activation as the sperm has all the components to do so and it is unlikely that exogenous activation will enhance the development to the blastocyst stage; however, exogenous activation is likely to increase the parthenogenetic development (Li et al., 1999). An interesting finding from the study was the increased development to the blastocyst stage from day 7 to day 8. This finding may suggest that the low developmental capacity of ICSI-derived embryo can be in part explained by an asynchronous or retarded PN formation.

Sperm preservation in a lyophilized state (freeze-dried) has been used as an alternative to liquid nitrogen cryopreservation (Polge et al., 1949). Performing ICSI with freeze-dried sperm was initially reported in mice strains with sperm unable to sustain conventional cryopreservation (Kusakabe et al., 2001). Subsequent studies demonstrated that oocytes injected with freeze-dried sperm were activated, cleaved (Ward et al., 2003; Kwon et al., 2004) and able to produce offspring (Ward et al., 2003). Keskinetepe et al. (2002), reported the first ICSI with freeze dried sperm in cattle. The objectives of the study were a) to evaluate the effect on cleavage and embryo development of different oocyte activation treatments (5 or 10 μ M I_o only for 5 min or

further incubation with 1.9 mM DMAP for 4 h after injection with freeze-dried sperm, b) to compare oocyte activation (presence of 2 PN) and development to blastocyst after injection with freeze-dried, frozen-thawed or sham injection sperm and activation (10 μ M Ionomycin only for 5 min or further incubation with 1.9 mM DMAP for 4 h), and c) to compare the chromosomal composition of produced embryos. Cleavage and development to blastocyst was superior when freeze-dried sperm injected oocytes were activated with the combination of Ionomycin + DMAP (5 μ M: 64.7 and 23.3%; 10 μ M: 66.7 and 30.7%, respectively) compared to activation with Ionomycin alone (5 μ M: 45.7 and 12.1%; 10 μ M: 52.1 and 10.7%, respectively). Pronuclear formation after injection with either frozen-thawed or freeze-dried sperm and activation was not different (66% and 56%, respectively); however, pronuclear formation was higher after injection with frozen-thawed sperm (82%) and activated with Ionomycin + DMAP compared to oocytes injected with freeze-dried sperm (74%). Although PN formation was higher, development to the blastocyst stage was not different (34.2% and 29.6%, respectively). Parthenogenetic activation and development was higher in sham-injected oocytes after treatment with Ionomycin + DMAP (35.8% and 15.3%, respectively) compared to Ionomycin alone (12.6% and 0%, respectively). Cytogenetic analysis revealed that 80% of the frozen-thawed, 73.3% of the freeze-dried and 16% of the sham-injection blastocysts produced were diploid. This study concluded that long-term stored lyophilized bull sperm has the ability to produce embryos after injection into a cow oocyte.

As with any sperm preservation method, cellular damage and loss of the fertilizing ability is of great concern. The process of sperm lyophilization causes complete loss of sperm motility (Polge et al., 1949; Kusakabe et al., 2001) and damage

to the plasma membrane (Kusakabe et al., 2001); however the sperm chromatin appear to be unharmed (Kusakabe et al., 2001; Kusakabe et al., 2008). Martins et al. (2007), investigated the cellular damage of lyophilized bull sperm and its effect on the fertilizing ability after ICSI. The objective of the study was to test the ability of different freeze-drying solutions to protect the sperm against damage. Before and after freeze-drying, the sperm plasma membrane, mitochondria, acrosome and DNA integrity were assessed. Then MII oocytes were injected with the freeze-dried sperm, activated with I_o + DMAP and cultured for 7 days to evaluate the development to blastocyst. Regardless of the freeze-drying solution, all sperm lost motility and had a damaged plasma membrane as previously reported (Polge et al., 1949; Kusakabe et al., 2001). Conversely, the acrosome and mitochondria integrity was unaffected. DNA fragmentation was lower when the freeze-drying solution was supplemented with ethylene glycol-bis (EGTA) (2%) compared to freeze-drying solution supplemented with FCS and trehalose (5%), and the highest was in FCS only (14%). After ICSI and activation, cleavage was higher in freeze-drying solution supplemented with EGTA (57.7%); however development to blastocyst was not different compared to solutions supplemented with FCS and trehalose (19.4% and 18.3%, respectively). The authors concluded that any of the tested solutions were able to preserve the whole sperm integrity during freeze-drying. However; solutions supplemented with FCS and trehalose or EGTA better preserve the DNA integrity and therefore the fertilizing ability.

These results suggest that exogenous activation does not improve embryo development and that the sperm itself contains all the components to stimulate oocyte activation and further development to blastocyst. Then, why or what is causing the high

oocyte activation but low development to blastocysts reported in cattle ICSI studies? As previously mentioned (section 2.1), sperm induced Ca^{2+} oscillations are responsible for oocyte activation and initiation of embryonic development. Malcuit et al. (2006b), investigated the effect of different bull sperm treatments on Ca^{2+} oscillations and embryo development after ICSI and IVF. Sperm treatments were: a) decapitation, b) incubation in a mild detergent (Triton-X) to permeabilize the sperm membrane, c) incubation with DTT or d) capacitation with heparin, dibutyryl cAMP and IBMX. Results showed that ICSI with an intact, decapitated, capacitated or permeabilized bull sperm fail to stimulate Ca^{2+} oscillations after injection into cattle oocytes; however, decapitated bull sperm did stimulate oscillations after injection into mouse oocytes and were comparable to oscillations displayed after injection of a whole bull sperm into mouse oocytes (Knott et al., 2003). Conversely, mouse sperm injected into cattle oocytes were unable to stimulate oscillations required for oocyte activation. Bull sperm treated with DTT increased the number of oocytes showing a rise in Ca^{2+} (58%) but inconsistently stimulate oscillations (18%). Although ICSI derived zygotes reached the blastocyst stage at very low rates (<10 %) compared to IVF (30%), the rate of cleaved embryos developing to blastocysts was not different between ICSI (40%) and IVF (30%). This study clearly indicates that the major limitation of ICSI in cattle resides in the inability to stimulate consistent and sustained Ca^{2+} oscillations. Reasons for this may include failure to completely release, compromised function within the ooplasm and/or oocyte cortex reorganization during sperm-oocyte membrane, which is necessary to activate downstream steps for PLC ζ to become fully active.

Intracytoplasmic Sperm Injection (ICSI) in the Horse

Thus far, embryo IVP in the horse relies on ICSI. Only one IVF derived foal has been produced (Bezard et al., 1989; Palmer et al., 1991) and further attempts have been unsuccessful producing a foal (Li et al., 1995; Dell'Aquila et al., 1996; Choi et al., 2001; Hinrichs et al., 2002). The poor results of equine IVF have been credited to inadequate stallion sperm *in vitro* capacitation (Zhang et al., 1990; Varner et al., 1993) and/or zona pellucida hardening after IVM (Dell'Aquila et al., 1999). Nevertheless, in a recent study on equine IVF, fertilization of mare IVM COCs reached 60.7% after improvements on stallion sperm *in vitro* capacitation (McPartlin et al., 2009).

The first pregnancy after ICSI was reported by Squires et al. (1996). Cumulus-oocyte complexes were matured *in vitro* and four MII oocytes injected with a single sperm. From the injected oocytes, one developed to the 10 – 12 cells stage, surgically transferred into a mare's oviduct and established a pregnancy.

Fertilization and development of IVM equine oocytes between ICSI and IVF was compared by Dell'Aquila et al. (1997a). Cumulus-oocyte complexes aspirated from abattoir-derived ovaries were divided into Cp or Ex cumulus, matured and either subjected to ICSI or IVF. They considered an oocyte to be normally fertilized when two PB's with two PN's or cleavage was observed after 24 h of culture. Embryo development was assessed after 72 h of culture in either a) media with granulosa cell monolayers, b) mare oviductal epithelial cells or 3) Vero cell monolayer. The percentage of oocytes surviving after ICSI was 82.5%. Fertilization after ICSI was higher in oocytes with Ex (52.2%) compared to Cp (14.7%) cumulus. No difference in fertilization after IVF was observed between oocytes with Ex and Cp cumulus (17.1% and 4.4%,

respectively). When fertilization between ICSI and IVF was compared, only oocytes with Ex cumulus were fertilized at a higher rate. Embryo development was higher after ICSI (12.7%) compared to IVF (0%). Within the ICSI groups, embryo development was higher in oocytes with Ex (31%) compared to Cp (5%) cumulus. The authors concluded that ICSI is an attractive technique for the equine industry particularly for stallions with valuable semen. A drawback of ICSI is that it is time-consuming and the number of oocytes injected is less compared to IVF insemination. The horse oocyte has a very dark ooplasm and makes sperm delivery difficult to visualize.

The sequence of events during oocyte activation, pronuclear formation and cleavage after ICSI in the horse were studied by Grondahl et al. (1997). COCs aspirated were culture for 36 h, denuded and oocytes with an extruded PB selected for ICSI. After injection, oocytes were placed in culture and then divided into different experiments. In the first experiment two groups of oocytes (12 each) were cultured for 10 h (group 1) or 20 h (group 2) and then fixed for histological (light microscopy) and ultrastructure (transmission electron microscopy) evaluation. Ten sham-injected oocytes were also place in culture and fixed after 20 h of culture. In the second experiment oocytes were cultured *in vitro* for 72 h, or cultured for 4 – 5 h and then transferred into mice and/or mare oviducts to assess embryonic developmental capacity. On the first experiment, histologic evaluation at 10 h of culture showed that 50% of the oocytes had a metaphase plate and one PB, 17% had two PB's and 33% were degenerated. At 20 h of culture, 50% had two PN's, 17% had one PN and 33% were degenerated. Of the sham-injected oocytes, 40% were at MII and 60% were degenerated. On ultrastructure evaluation at 10 h, 50% showed metaphase plates and spindles. The lipid droplets

localization gave the ooplasm a polarized appearance and the cortical granules were in the periphery, close to the oolema. At 20 h the nuclear envelope was spherical and the cortical granules had almost disappeared. In the second experiment, 16% of injected oocytes have cleaved. None and one of the embryos transferred into mice and/or mare oviducts cleaved, respectively. The study concluded the following: a) pronuclear formation takes place between 10 to 20 h after ICSI, b) equine oocytes may be incompletely activated after sperm injection and c) sham-injection did not activate equine oocytes.

In the horse, the effect of exogenous oocyte activation and embryonic development after ICSI has been evaluated (Kato et al., 1997; Li et al., 2000). Kato et al. (1997), incubated sperm injected oocytes for 5 min in medium supplemented with 10 μ M Cal. Cleavage and embryo development were assessed after two and four days of culture, respectively. Cleaved embryos were transferred into recipient mares and pregnancy diagnoses performed 15 days after transfer. A higher cleavage rate was observed in injected oocytes treated with Cal compared non-treated oocytes (21% vs 5%, respectively). The percentage of embryos reaching the 6- to 8-cell stage was 80% for the treated with Cal and 40% for the non-treated. A total of seven embryos were transferred into recipient mares but failed to establish a pregnancy. The authors concluded that sperm injection may have stimulated oocyte activation in non-treated oocytes. The reason for the failure of transferred embryos to establish a pregnancy was unknown; possibly the embryos developed parthenogenetically. Li et al. (2000), evaluated oocyte activation and normal fertilization after treatment with different compounds. Following ICSI, oocytes were cultured in medium supplemented with a) 10

μmol I_o for 10 min, b) 7% (v/v) ethanol for 7 min, c) 100 μmol thimerosal for 10 min, d) injection of 250 μmol of IP₃ into the oocyte cytoplasm and e) control (no treatment). An oocyte was considered activated if the oocyte nucleus progressed from MII to anaphase-telophase, a female PN was formed and/or if the oocyte has cleaved. Oocytes were normally fertilized if a cleaved embryo had a single nucleus in each of the blastomeres. Treatment with thimerosal resulted in the highest activation and normal fertilization (79% and 64%) compared to I_o (59% and 29%), ethanol (56% and 40%), IP₃ (56% and 0%) and control (0% and 0%). Exogenous activation resulted in a high proportion of oocytes with parthenogenetic development, 71%, 40% and 21% for I_o, ethanol and thimerosal respectively. It was concluded that thimerosal is more effective inducing oocyte activation and normal fertilization after ICSI than I_o, ethanol and IP₃. The possible explanation for this is the ability of thimerosal to induce repeated increases in intracellular Ca²⁺ and may resemble the Ca²⁺ oscillation pattern observed during normal fertilization.

Activation of injected oocytes with Cal (Kato et al., 1997) or culture in Vero cells (Dell'Aquila et al., 1997a) have shown to improve cleavage rates and embryo development after ICSI. Guignot et al. (1998), evaluated the effect of oocyte activation with Cal and then culture in Vero cells monolayers on embryo cleavage and development after ICSI within the same study. Injected oocytes were cultured in Cal for 10 min and then cultured in TCM199 with Vero cells monolayers. After two days of culture, presumptive embryos were evaluated for fertilization and if cleavage was observed then culture continued for another 5 days. From the successfully injected oocytes, 84.2% were fertilized and of those 46% have cleaved. After culture on Vero

cells, 77%, 14% and 0.08% developed to the 2- to 8-cells, 9- to 16-cells and > 16 cells, respectively. The investigators concluded that oocytes activation with Cal and culture on Vero cells have a beneficial effect on equine embryo cleavage and development; however, there was no control (non-activated and culture without Vero cells) group to compare these findings.

Birth of foals following oocyte aspiration from pregnant mares was reported by Cochran et al. (1998). This study evaluated the efficacy of ICSI, effects of culture in glucose-free and phosphate-free medium on embryo development and the viability of the produced embryos after transfer into the oviducts of recipient mares. Cumulus-oocyte complexes were collected via TUGA from pregnant mares and matured for 36 h. Before injection, sperm were immobilized by crushing the tail with the injection pipette. After ICSI, oocytes were cultured in either TCM199 (with glucose) or glucose-free, phosphate free medium for 48 h and then embryo development evaluated. The overall cleavage rate was 55% and no difference in embryos developing to the 2- to 3- or 4- to 8-cell stage was observed. After transfer 25% of the embryos established a pregnancy and produced normal foals. This study concluded that ICSI is the microinsemination technique of choice in the horse. Culture of early stage equine embryos in low glucose medium is beneficial as previously shown in the mouse (Chatot et al., 1989) and cattle (Ellington et al., 1990). Oocytes collected from pregnant mares are at the GV stage and allows reducing the variability in IVM periods to obtain good quality MII oocytes.

Overcoming infertility problems is the main application of ICSI in humans (Palermo et al., 1996a). In the horse, McKinnon et al. (2000) investigated the applicability of ICSI to produce pregnancies from infertile stallions. Cumulus-oocyte

complexes were recovered from mares via TUGA, and frozen-thawed sperm from a fertile and infertile stallion were used for injection. After injection, presumptive embryos were transferred within 6 h or cultured for 24 – 48 h before transfer into recipient mares. Embryo transfer within 6 h of injection resulted in 37.5% (3/8) pregnancies. Cleavage rate after 24 h of culture was 53% (8/15) and transfer of these embryos resulted in 12.5% (1/8) pregnancies. No difference in pregnancies produced between oocytes injected with sperm from fertile or infertile stallion was observed. Conclusions from this study were: the injected sperm stimulates oocyte activation and embryo development; transfer of embryos produced with sperm from infertile stallions can establish a pregnancy and produce a normal foal. Aspiration of pre-ovulatory follicles from valuable mares is a viable alternative for IVP embryos in horses.

Developmental competence of equine oocytes is influenced by the follicular size from which oocytes are collected, cumulus cells morphology of COCs and oocyte chromatin configuration (Hinrichs and Williams, 1997). Galli (2000), compared the embryo development of oocytes derived from follicles < 2 and > 2 cm in diameter, and with Cp or Ex cumulus cells. Following ICSI, oocytes were incubated in Io, cultured in SOF for 48 h and then transferred into sheep oviducts for 5 days. One hundred and 50% of COCs collected from follicles > 2 and < 2 cm had Cp respectively. Embryo development to the blastocyst stage was higher (30%) for Cp regardless of the follicle size they originated from, compared to Ex (11%). The authors concluded that COCs with Cp have higher developmental competence and the production of transferable embryos via ICSI in equine is comparable to IVF in bovine.

Use of Piezo-driven ICSI in the horse was first reported by Choi et al. (2002). The objectives of the study were to evaluate the oocyte activation rate after ICSI with fresh or frozen-thawed equine sperm and development *in vivo* (mare oviduct) or *in vitro*. In addition, bovine oocyte activation after injection with equine sperm (fresh or frozen-thawed) was investigated. Following injection (20 h) a group of oocytes were stained with Hoechst 33258 to assess activation and fertilization, and the rest were either transferred into a mare's oviduct or *in vitro* cultured for 96 h. Activation status was as follow, a) non-activated (MII oocyte with a condensed sperm head), b) activated but arrested (two polar bodies and de-condensing sperm head) and c) activated (two PN, syngamy or cleavage). Percentage of activated and fertilized oocytes after ICSI with fresh (91% and 71%, respectively) or frozen-thawed (93% and 60%, respectively) sperm were not different. Cleavage and development after 96 h of *in vitro* culture was not different between embryos derived from injection with fresh (72%) and frozen-thawed (55%) sperm. Similarly, cleavage and development after 96 h of *in vivo* culture was not different between fresh (75%) and frozen-thawed (64%) sperm. Although cleavage and development was not different between *in vitro* or *in vivo* culture, mean nuclei number was higher in *in vivo* (16) compared to *in vitro* (8) cultured embryos. Interspecies sperm injection of equine sperm into bovine oocyte resulted in 90% and 93% of activated and 81% and 80% of fertilized oocytes after injection with fresh or frozen-thawed sperm, respectively. The study demonstrated that exogenous activation is not necessary to obtain high rates of activation and fertilization in equine ICSI. The high rate of activation and fertilization may be due to increase damage to the sperm plasma membrane caused by the piezo pulses compared to scoring against the dish

bottom (Dozortsev et al., 1995; Palermo et al., 1996b). That incomplete activation was observed in up to 30% of injected equine oocytes may indicate reduced or shorter than the required stimulus to complete pronuclear formation.

The sperm factor (PLC ζ) induces Ca²⁺ oscillations responsible for oocyte activation (Fissore et al., 1998; Carroll, 2001); however was not known if this is similar in the horse. Whether stallion sperm carries and release PLC ζ , and if so, the pattern of the Ca²⁺ oscillations stimulated in horse oocytes was investigated by Bedford et al. (2003). In the first study, stallion sperm were processed to extract the sperm factor (Wu et al., 1997) and then mouse oocytes were injected with either stallion sperm factor or stallion sperm to investigate the presence and activity of PLC ζ . Then MII mouse and horse oocytes were injected with either stallion sperm factor or with stallion sperm to evaluate the induction and pattern of Ca²⁺ oscillations. Mouse oocytes displayed repetitive Ca²⁺ oscillation that lasted for up to 120 min after injection of stallion sperm extract or stallion sperm, and were similar to Ca²⁺ oscillations displayed in normal fertilization (Fissore et al., 1998). Parthenogenetic activation and development to blastocyst of mouse oocytes injected with stallion sperm factor was 94% and 73% respectively. Stallion sperm factor stimulated Ca²⁺ oscillations following injection into horse oocytes (88%) that lasted for up to 120 min and induced oocyte activation (77%). Conversely, a low percentage (38%) of horse oocytes injected with stallion sperm displayed Ca²⁺ oscillations and only 15% had Ca²⁺ oscillations for > 120 min. Nevertheless, 58% of the injected oocytes were activated after 20 – 24 h of culture. Results from this study demonstrated that the stallion sperm factor is released and consistently stimulates Ca²⁺ oscillations in mouse oocytes but not in the horse. To

elucidate the possible reasons why the stallion sperm factor was not released or partially released into the horse oocyte, the same researchers (Bedford et al., 2004) tested the effect of oocyte maturation (*in vitro* and *in vivo*) method and sperm treatment by either sonication or sperm membrane permeabilization to facilitate release of the sperm factor on the induction of Ca^{2+} oscillations. In addition, the release time of the sperm factor as well as the oocyte ooplasm involvement in this release was investigated. Stallion sperm injected into an *in vitro* or *in vivo* matured oocyte stimulated Ca^{2+} oscillations at the same rate (33% and 44%, respectively) and 50% of the *in vitro* and 85% of the *in vivo* matured oocytes were activated at 20 – 24 h. Ca^{2+} rises were observed in average at 38 min intervals and each rise lasted approximately 2.6 min. Sperm sonication or sperm membrane permeabilization did not increase the number of oocytes displaying Ca^{2+} oscillations (25% and 13%, respectively). The stallion sperm factor is rapidly released/degraded within the horse oocyte as stallion sperm removal within 30 min of injection and re-injection into a mouse oocyte stimulated short-duration Ca^{2+} oscillations in a limited number of oocytes (22%). Further investigation of the ooplasm involvement in the release and perhaps activation was evaluated after fusion of sperm-injected zona-free horse oocytes with mouse oocyte. Interestingly, Ca^{2+} oscillations were initially observed in the mouse oocyte and not in the sperm-injected horse oocyte; although, as fusion was completed (30 min), Ca^{2+} oscillations were observed in both oocytes. Conclusions from these studies were that a) stallion sperm carries the sperm factor able to induce Ca^{2+} oscillations when injected into mouse or horse oocytes, b) injection of a stallion sperm into a mare oocyte was unable to stimulate consistent and long-lasting Ca^{2+} oscillations, c) sperm decapitation by

sonication or sperm membrane permeabilization did not increase oscillations, d) the stallion sperm factor is rapidly released within the horse and mouse oocyte and d) a step downstream of the sperm factor release that activates it may be skipped during ICSI, causing the inconsistent stimulation of Ca^{2+} oscillations in horse oocytes or perhaps the required substrate (type and quantity) may not be available to the sperm factor to become fully functional.

Despite the ICSI related problems, this is the only reliable method to produce horse embryos *in vitro*. In the horse industry, ICSI clinical applications vary from increasing the number of embryos produced by a mare (Colleoni et al., 2007; Jacobson et al., 2010; Carnevale and Sessions, 2012) to salvage or preserve valuable mares (Hinrichs et al., 2012) and stallions (Choi et al., 2006) genetics after death. Production of embryos in live donors is performed by aspirating oocytes from the mares, ICSI and culture to the blastocyst stage (Galli et al., 2002; Colleoni et al., 2007; Jacobson et al., 2010). This procedure allows embryo production from mares that are either in competition or unable to carry a foal to term. Oocyte recovery per aspiration ranged from 54 – 58% (Colleoni et al., 2007; Jacobson et al., 2010), blastocyst development after ICSI and *in vitro* culture from 11.65% (Colleoni et al., 2007) to 63% (Jacobson et al., 2010), resulting in 0.4 – 0.85 blastocysts for trans-cervical transfer per oocyte aspiration session. Producing embryos from mares that unexpectedly die or have to be euthanized due to illness may offer the last chance to preserve this valuable genetic material. Recovered oocytes may be frozen and fertilized at a later stage (Tharasanit et al., 2006); however, the oocytes cytoskeleton and developmental competence is severely compromised. Thus, immediately after ovarian collection or transport, oocytes

are recovered and placed in IVM, followed by ICSI and IVC (Hinrichs et al., 2012). When the time from mare death to oocyte collection range from ≤ 2 h to 12 h, oocytes reaching MII have been 49%, with 19% of the injected oocytes reaching the blastocyst stage of development (1.3 blastocysts/mare) and 62% of the transferred blastocysts establishing pregnancies have been reported (Hinrichs et al., 2012).

In regards to the preservation of stallion's genetics, ICSI is an invaluable technique for stallions that have become infertile due to injury or illness or after death, and limited amounts of cryopreserved semen are available. It offers the ability to make an efficient use of semen as only one sperm is needed to produce a foal instead of hundreds of millions used in conventional artificial insemination. Efficient use of stallion cryopreserved semen for ICSI by cutting small pieces of a frozen straw for thawing (Altermatt et al., 2009) and re-freezing previously thawed semen (Choi et al., 2006) have been reported. Altermatt et al. (2009) cut and thawed straw sections of ~ 5 mm for ICSI instead of thawing a full straw. Choi and co-workers (Choi et al., 2006) compared the rate of blastocyst development of oocytes injected by sperm subjected to one or two freeze-thaw cycles. Also, oocyte development after ICSI with non-motile sperm was investigated. The freezing semen extender used in the second freezing cycle was different from the first cycle with the intention to replicate clinical situations where the original freezing extender used is unknown. Also, thawed semen was diluted 1:100 for the second freezing cycle. Thaw sperm motility decreased from 36% to 16% after two freezing cycles; however, blastocyst development with motile sperm after one freezing cycle (27%) was not different than after two freezing cycles (23%). Likewise, blastocyst development with non-motile sperm after two freezing cycles was not different (13%).

Furthermore, if semen from a sub-fertile stallion was used, sperm motility decreased from 22% to 3% after two freezing cycles; however, blastocyst development was similar (9%) when oocytes were injected with motile sperm after one or two freezing cycles. This study demonstrated that sperm frozen and thawed twice still maintain their fertilizing capacity. Dilution of 1:100 before re-freezing allows preservation of multiple straws for later embryo production via ICSI.

In livestock species, the main use of ICSI may not be to solve male infertility issues; instead this technique can help with biodiversity conservation, transgenic production or to solve IVF systems problems (Garcia-Rosello et al., 2009). ICSI has been commonly performed using fresh, fresh cooled or frozen-thawed sperm and freeze-dried sperm (Keskinetepe et al., 2002; Ward et al., 2003; Kwon et al., 2004; Abdalla et al., 2009; Choi et al., 2011). More recently in laboratory animals, sperm frozen without cryoprotectants have been shown to maintain the ability to produce offspring after ICSI (Lacham-Kaplan et al., 2003; Ward et al., 2003; Li et al., 2010). In livestock and endangered species, ICSI with sperm frozen without cryoprotectants has not been evaluated and may offer an alternative for biodiversity conservation and/or efficient use of cryopreserved semen.

Assessment of the Sperm Plasma Membrane and DNA Integrity

Plasma Membrane

During fertilization the sperm plasma membrane plays a fundamental role interacting with the oviductal epithelium (Ellington et al., 1993), CC and oolema (Rodríguez-Martínez, 2003). The term sperm viability has been commonly used when assessing the integrity of the plasma membrane, and sperm with intact plasma

membrane are called “live” whereas sperm with damaged plasma membrane are called “non-viable or dead”. The sperm plasma membrane integrity can be assessed using supravital stain and fluorophores. Supravital stain consist of dual staining the sperm with the plasma membrane permeable eosin and impermeable nigrosin; however, glycerol interferes with cell staining making this method not useful to assess cryopreserved sperm (Mixner J.P., 1954). Permeable fluorophores include CFDA which is an enzyme-based probe (Garner et al., 1986; Harrison and Vickers, 1990) and SYBR-14 which is a nucleic acid stain (Garner and Johnson, 1995). The population of sperm with damaged plasma membrane is obtained by adding impermeable nuclear stains to the sample. Commonly used stains are PI, ethidium homodimer-1, YoPro and Hoechst 33258 (Gillan et al., 2005). Using dual staining with CFDA and PI Garner et al (Garner et al., 1986) assessed mouse, bull, boar, dog, horse and human sperm plasma membrane integrity. With the dual stain, three sperm populations were observed, sperm with brightly red nuclei (dead), sperm with red nuclei but with some green within the acrosome and mitochondria (moribund) and brightly green fluorescence throughout the cytoplasm (live). In the study a correlation between CFDA fluorescence and progressive motility was found. However; enzyme-based fluorophores such as CFDA are non-fluorescent and become fluorescent after permeating through the plasma membrane and hydrolysis by intracellular esterases. Due to this, CFDA suffers from a time-dependency problem as it depends on enzyme substrate conversion and from a fluorescence emission latency after cell death, giving variability to the assessment (Garner and Johnson, 1995).

The most common fluorophores combination used to assess sperm plasma membrane integrity is the permeable SYBR-14 and the impermeable PI (Garner and Johnson, 1995; Merkies et al., 2000; Garner et al., 2001; Love et al., 2003; Len et al., 2010). Assessment is based on the two sperm populations observed. SYBR-14 permeates through an intact plasma membrane and display green fluorescence whereas PI can only access the nucleic acids in cells with damaged plasma membrane and display red fluorescence (Figure 2). In addition to assessment of the plasma membrane integrity, combination of more than two fluorophores may be used to assess multiple sperm structures using flow cytometry.

DNA

The main function of the sperm DNA is to transmit the male genetic information during fertilization. In the sperm, the chromatin is inactivated and highly compacted through the replacement of somatic histones by protamines to form linear, side-by-side arrays of chromatin (Ward and Coffey, 1991). This structural organization of the sperm chromatin is likely to be important for proper delivery of male genetic information (Ward and Coffey, 1991) and damage to the integrity of the sperm chromatin has been related to reduced fertility in bulls, boars, stallions and humans (Love and Kenney, 1998; Januskauskas et al., 2001; Evenson and Wixon, 2006; Bungum et al., 2007; Boe-Hansen et al., 2008).

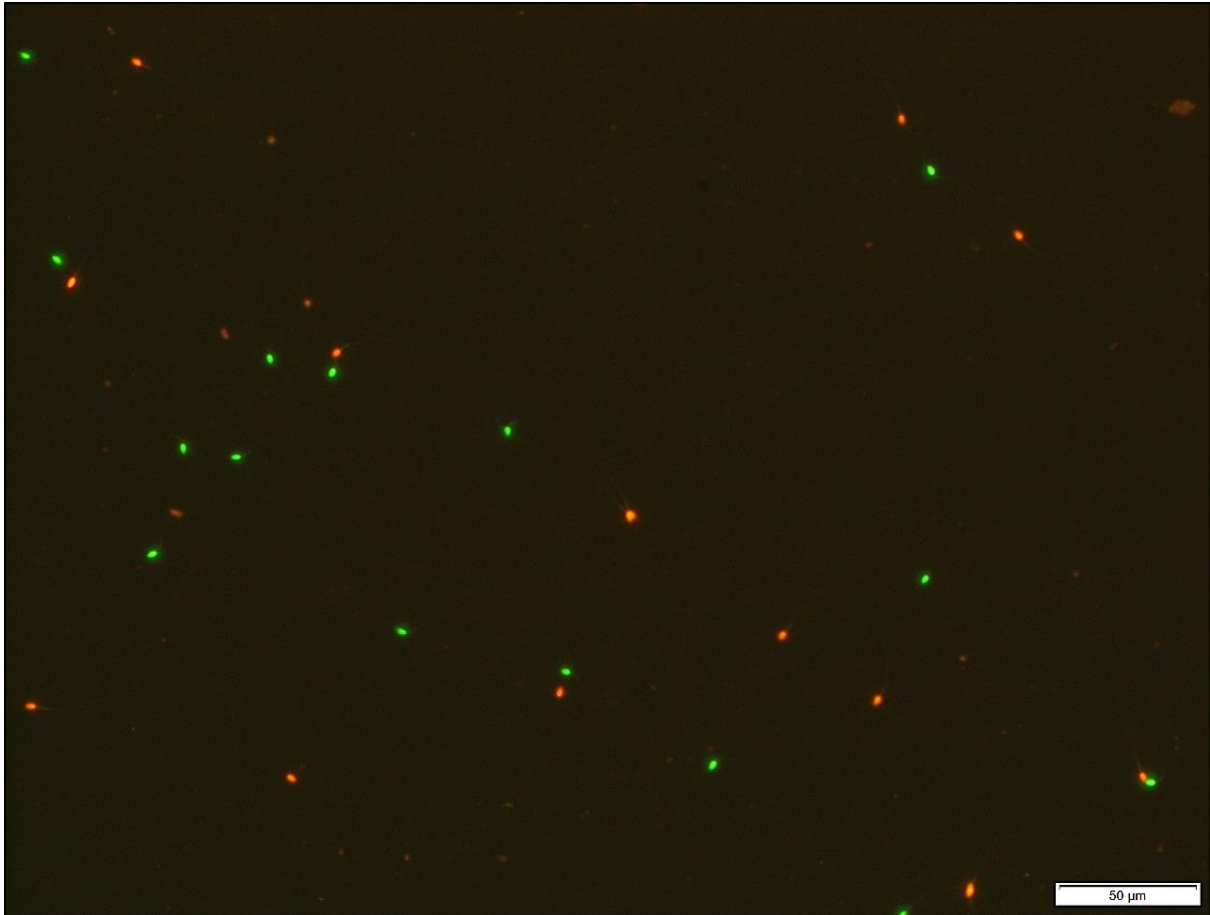


Figure 2: Stallion sperm stained with SYBR-14 and PI imaged under a fluorescent microscope. Sperm with intact plasma membrane display green fluorescence and sperm with damaged sperm display red fluorescence (Zeizz AX10 microscopy, Olympus cellSens™ imaging software, 200x).

The sperm DNA integrity may be assessed using fluorescent microscopy, flow cytometry and light microscopy. Methods include the AO test (Kapuscinski et al., 1983; Tejada et al., 1984), SCSA, Comet assay (Evenson and Wixon, 2006), Tunel (Sailer et al., 1995), and SCD test (Fernández et al., 2003). In the AO test, sperm are heated (100°C for 5 min) to denaturate DNA and then stained with AO. When AO binds to double stranded DNA it displays green fluorescent and display red fluorescent when bound to single stranded DNA. This method may be practical if fluorescence is evaluated with a flow cytometer; however, the test as postulated by Tejada et al (Tejada et al.,

1984) encountered some problems. The test requires a very exact concentrations of AO per DNA phosphate groups (Kapuscinski et al., 1983) and any increase or decrease of the ratio will cause all sperm to either fluoresce green or red causing the test to show extreme variations (Chohan et al., 2006). The SCSA also uses AO and is based on the principle that sperm with defective DNA is more susceptible to DNA denaturation. In SCSA, sperm are incubated in an acid detergent solution to cause DNA denaturation, followed by staining with AO. Then sperm populations with green (double stranded) or red (single stranded) are evaluated using flow cytometry.

In the Tunel assay, broken DNA is detected by labeling 3'OH terminals with biotinylated dUTP and transferred using deoxynucleotidyl transferase (Sailer et al., 1995), and sperm with braked DNA strands display fluorescence. In the Comet assay, sperm are mixed with melted agarose, lysed and subjected to horizontal electrophoresis. Braked DNA strands from a sperm head migrate out forming a comet like appearance. Although the Comet assay can determine the percentage of sperm with broken DNA, no thresholds have been established.

The SCD test is based on the chromatin halos formation after protein removal from the sperm nucleus by lysing solutions (Fernández et al., 2003). The halos represent relaxed DNA loops attached to the nuclear matrix and dispersion (increased size of the halo) indicates DNA damage (Figure 3). Samples may be stained with DAPI (Fernández et al., 2003), FITC conjugated 16-dUTP (Enciso et al., 2006) and PI (García-Macías et al., 2007) for fluorescent microscopy or Wright's staining solution for light microscopy (Fernández et al., 2005; Enciso et al., 2006; García-Macías et al., 2007) to assess halos size and discriminated between relaxed and fragmented DNA.

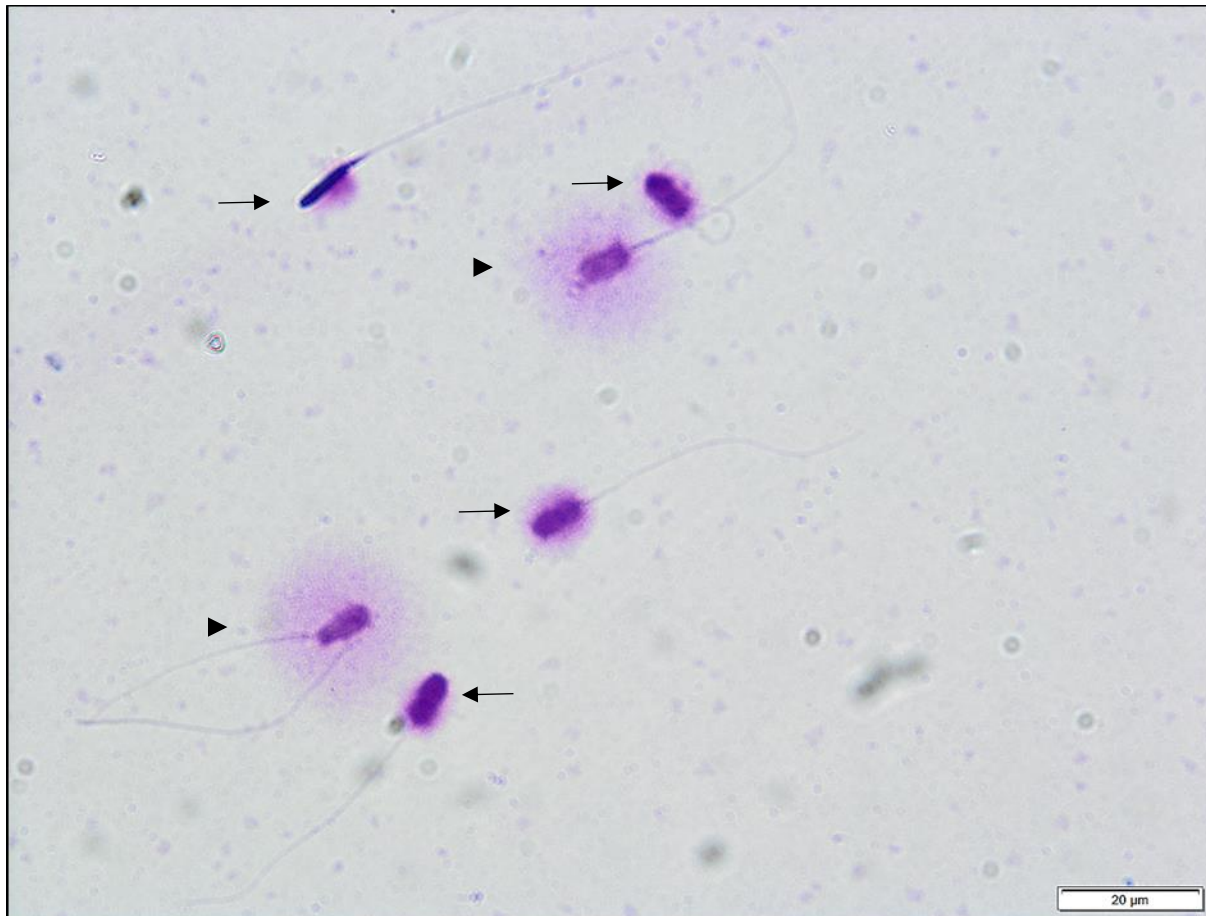


Figure 3: SCD of boar sperm stained with Wright's solution viewed under light microscopy. Note the sperm with fragmented DNA (big halos; arrowheads) and sperm with relaxed but not fragmented DNA (small halos; arrows) (Olympus BX31 microscope, Olympus cellSens™ imaging software, 1000x).

Human (Fernández et al., 2003; Fernández et al., 2005), stallion (López-Fernández et al., 2007), bull (Martínez-Pastor et al., 2009), boar (Enciso et al., 2006), ram (López-Fernández et al., 2010), buck, white tail deer, rabbit, donkey, rhinoceros and koala (Gosálvez et al., 2011) sperm have been analyzed with the SCD test under conventional laboratory conditions and test results have shown good correlation with fertility (Muriel et al., 2006). Although the power of the test may be inferior to the flow cytometric SCSA (Martínez-Pastor et al., 2009), determination of DNA fragmentation using the SCD test are comparable to the SCSA (Fernández et al., 2005; Chohan et al., 2006).

Influence of COCs Glucose Metabolism on Oocyte Maturation and Developmental Capacity

In mammals, oocytes remain arrested in prophase I of meiosis until the pre-ovulatory gonadotropin surge, or spontaneously resume meiosis once removed from the follicular environment (Pincus and Enzmann, 1935). Immature oocytes surrounded by CC forming the COC resume meiosis to complete final maturation characterized by the extrusion of the first polar body and cumulus expansion (Buccione et al., 1990; Eppig et al., 1996; Eppig, 2001). Following the pre-ovulatory gonadotropin surge, oocyte resumption of meiosis and final maturation are dependent on the cross talk communication between the surrounding cumulus cells and the oocyte through the transzonal cytoplasmic processes (TZP) which penetrate the ZP into the oolema ending in gap junctions (Albertini et al., 2001; Eppig, 2001; Sutton et al., 2003b; Gilchrist et al., 2004). The importance of this is reflected on the lower maturation and developmental competence of bovine (Sirard et al., 1988), mouse (Schroeder and Eppig, 1984), rat (Vanderhyden and Armstrong, 1989) and ovine (Staigmiller and Moor, 1984) oocytes matured in the absence of cumulus cells.

Most of our knowledge on COC metabolism is derived from IVM systems. Once removed from the follicular environment, a COC become dependent on the IVM medium to supply the necessary substrate, including electrolytes, amino acids, lipids and energy substrate for final maturation. In regards to energy substrate, glucose concentration in bovine (Leroy et al., 2004), swine (Chang et al., 1976), ovine (Nandi et al., 2008) and equine follicular fluid increase from small to large follicles (Table 1). Given the fact that glucose in IVM medium cannot be replenished by the surrounding follicular tissue, glucose concentrations in commonly used IVM media are at supra-physiological levels

Table 1: Glucose concentration in follicular fluid of small and large follicles in different mammals			
Species	Glucose (mmol/l)		Reference
	Small	Large	
Bubaline	2.04 ± 0.25	2.74 ± 0.21	Nandi et al. (2008)
Swine	4.11 ± 0.05	4.77 ± 0.33	Chang et al. (1976)
Dromedary	7.60 ± 0.22	4.28 ± 0.24	Ali et al. (2008)
Human		3.39 ± 0.91; 3.29 ± 0.09	Gull et al. (1999), Leese and Lenton (1990)
Murine		0.46 ± 0.08	Harris et al. (2005)
Caprine		1.4 ± 0.34	Herrick et al. (2006b)
Ovine	1.18 ± 0.13	1.64 ± 0.11	Nandi et al. (2008)
Bovine	2.01 ± 0.10	3.75 ± 0.18	Leroy et al. (2004), Sutton-McDowall et al. (2005)
	1.40 ± 0.20	2.30 ± 0.20	
Equine	4.72 ± 0.29	4.44 ± 0.15	Collins et al. (1997)

compared to follicular fluid. This is to avoid depletion during IVM and exposing maturing COCs to concentrations (< 2 mM) that adversely affects embryo development (Krisher and Bavister, 1998; Rose-Hellekant et al., 1998). As well as low glucose concentration, extremely high concentrations of glucose (> 10 mM) during IVM can adversely affects embryo development through the production of ROS and reduced concentrations of GSH (Hashimoto et al., 2000). The oocyte depends on CC to facilitate the glucose transport and metabolize the majority of the glucose (Sutton-McDowall et al., 2010) as well as to supply the oocyte with pyruvate (Downs and Hudson, 2000) because the oocyte has limited capacity to utilize glucose (Biggers et al., 1967; Downs and Utecht, 1999). During murine (Downs and Utecht, 1999) and bovine (Rieger and Loskutoff,

1994; Steeves and Gardner, 1999; Sutton et al., 2003a) COCs maturation, glucose consumption has a twofold increase. Increased glucose metabolism during maturation promotes meiotic resumption and improves subsequent developmental potential in cattle (Rose-Hellekant et al., 1998; Krisher and Bavister, 1999). In this regard, bovine COCs retrieved from matured cows utilize more glucose during IVM and have better developmental competence compared to pre-pubertal cows (Steeves and Gardner, 1999).

Cumulus-oocyte complexes that spontaneously resume meiosis are not dependent on the pre-ovulatory gonadotropin surge or gonadotropin presence in the IVM medium (Downs et al., 1996); however, during IVM, FSH but not LH stimulates CC glucose metabolism to promote oocyte maturation (Downs and Eppig, 1985; Downs et al., 1988; Downs et al., 1996; Van Tol et al., 1996). The FSH stimulation of COCs maturation is dependent on glucose because removal or prevention of glucose utilization blocks the stimulatory effect (Fagbohun and Downs, 1992; Downs et al., 1998). Furthermore, FSH-stimulated COCs produce paracrine/autocrine factors from the glucose metabolism involved in regulating and stimulating resumption of meiosis and oocyte maturation (Byskov et al., 1997; Downs and Hudson, 2000; Downs and Chen, 2008).

Glucose metabolism by COC promote oocyte maturation through signaling that follows four pathways: glycolysis (energy production), PPP, HBP and polyol pathway (Figure 4) (Sutton-McDowall et al., 2010). The rate at which glucose is metabolized during IVM follows a linear increase throughout maturation (Sutton et al., 2003a). Follicle stimulating hormone increase the HK activity within COCs to phosphorylate

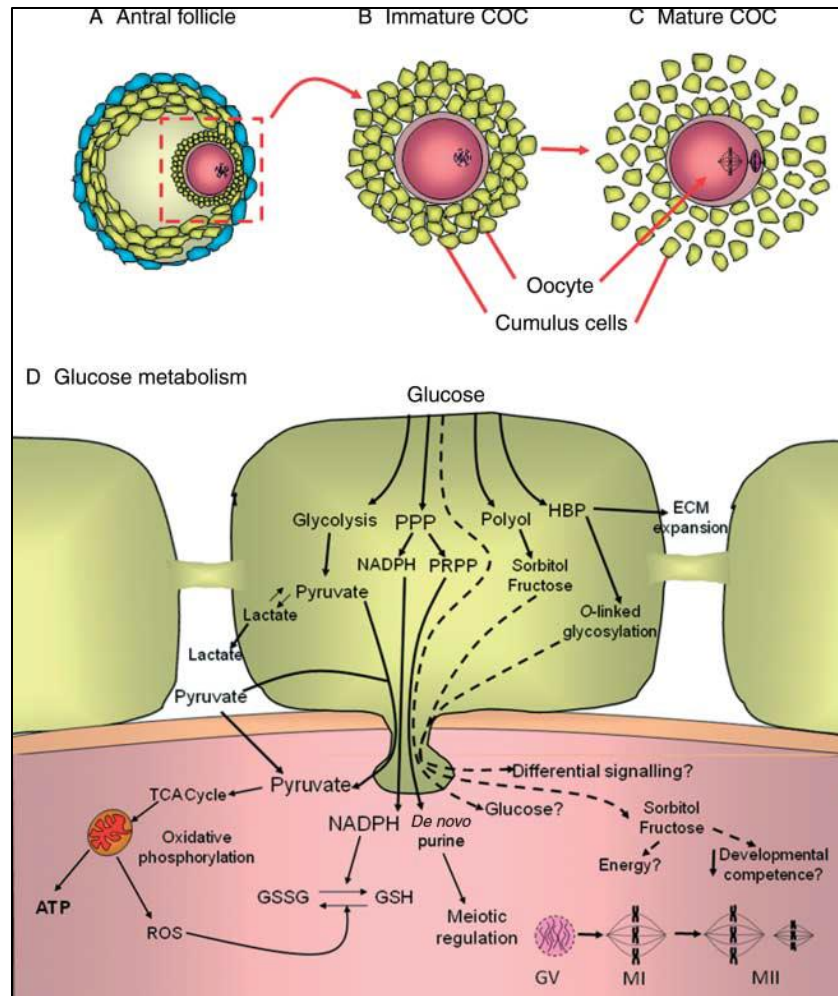


Figure 4: Glucose metabolism during COCs final maturation.

(A) Immature cumulus oocyte complexes (COCs; within red square) within antral follicles are characterised as having compact cumulus vestments and are arrested at prophase I (germinal vesicle stage, GV) of meiosis (B). Maturation occurs in response to gonadotrophin surges in vivo or release of the COC in vitro and is characterised by (C) expansion of the cumulus vestment and extrusion of the first polar body (metaphase II; MII). (D) Within the COC, glucose can be metabolised via four pathways. Glycolysis results in the production of pyruvate, which can be further metabolised via the tricarboxylic acid (TCA) cycle, followed by oxidative phosphorylation for energy production (ATP). The pentose phosphate pathway (PPP) produces NADPH for the reduction of the anti-oxidant glutathione (GSSG, oxidised glutathione; GSH, reduced glutathione). Phosphoribosylpyrophosphate (PRPP) is also produced by PPP and is a substrate for de novo purine synthesis, important for meiotic regulation within the oocyte. Products of the polyol pathway (polyol) include fructose and sorbitol. The hexosamine biosynthetic pathway (HBP) is important for producing substrates for extracellular matrices (ECM) of cumulus expansion and O-linked glycosylation (cell signaling). MI, metaphase I; ROS, reactive oxygen species. (From: The Pivotal role of glucose metabolism in determining oocyte development competence. Sutton-McDowall et al., 2010).

glucose into glucose-6-phosphate and either follows the glycolytic or the PPP pathway. The majority of glucose metabolized by the COC is through glycolysis (Downs and Utecht, 1999) for the production of pyruvate and lactate (Downs and Hudson, 2000; Harris et al., 2007). These metabolites are then transported into the oocyte through gap junctions. Pyruvate and lactate within the oocyte are further oxidized through the tricarboxylic acid cycle followed by oxidative phosphorylation in the mitochondria to produce more ATP (Steeves and Gardner, 1999). Oocyte consumption of pyruvate is associated with nuclear maturation (Downs et al., 2002).

In the PPP, glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, resulting in the production of nicotinamide adenine dinucleotide phosphate (NADPH), ribose-5-phosphate and fructose-6-phosphate (Downs et al., 1998; Sutton-McDowall et al., 2010). The NADPH protects the oocyte against ROS through the reduction of glutathione di-sulphide into the reactive GSH. Ribose-5-phosphate is further converted into phosphoribosylpyrophosphate (PRPP), substrate required for purine synthesis. De novo or salvage purine nucleotides synthesis is associated with the induction of germinal vesicle breakdown and meiotic resumption (Downs, 1997; Downs et al., 1998). Cumulus expansion is the result of glucose metabolism and synthesis of hyaluronic acid (Chen et al., 1990; Sutton-McDowall et al., 2004) to form the extra-cellular matrix. Fructose-6-phosphate enters the HBP and is converted into glucosamine-6-phosphate by the rate-limiting enzyme glucosamine-fructose-6-phosphate transaminase (Marshall et al., 1991) with end product of UDP-*N*-acetyl glucosamine which is further utilized for the synthesis of HA by hyaluronic acid synthase 2 or O-linked glycosylation. In somatic

cells, O-linked glycosylation regulates several biological processes such as nutrient sensing, stress response and chromatin remodeling (Vaidyanathan et al., 2014). During COC IVM, up-regulation of HBP by supplementing the IVM medium with glucosamine does not affect nuclear maturation or cleavage rates; however, it negatively affects pigs, cattle (Sutton-McDowall et al., 2006) and mouse (Schelbach et al., 2010) embryo development. Furthermore, supplementation with glucosamine alters cumulus expansion and may also interfere with the PPP (Schelbach et al., 2010).

Evidence regarding the role of glucose metabolism through the polyol pathway during COCs IVM is limited and derived from the studies of diabetes type I in mice (Colton et al., 2002; Colton et al., 2003; Colton and Downs, 2004). Glucose may enter this pathway when HK becomes saturated and oxidized into sorbitol by aldose reductase and sorbitol dehydrogenase. Increasing the flux of glucose into the polyol pathway affects the gonadotropin ovulation stimulation as the number of ovulations and MII ovulated COCs is decreased (Colton et al., 2002). Furthermore, IVM COCs resume meiosis earlier and faster, and are unresponsive to FSH stimulation. Additionally, CC-oocyte communication is deficient, have decrease PPP activity and limited synthesis of cAMP by CC (Colton et al., 2003; Colton and Downs, 2004).

CHAPTER 3 SPERM PLASMA MEMBRANE AND DNA INTEGRITY AFTER REFREEZING WITHOUT CRYOPROTECTANTS

Introduction

The PM plays a fundamental role in the sperm-oocyte interaction during normal fertilization (Lawrence et al., 1997; Talbot et al., 2003; Nixon et al., 2007) before the male DNA is delivered within the ooplasm. In normal fertilization, sperm PM interaction with the oocyte surface is required to stimulate the acrosome reaction and the generation of a signal transduction mechanism leading to oocyte activation (Schultz and Kopf, 1995; Lawrence et al., 1997). Following the sperm penetration and membrane fusion the male genetic information is released into the oocyte. Within the sperm, the chromatin is inactivated and tightly packed (Ward and Coffey, 1991) to prevent damage, important for proper delivery and subsequent embryo development.

The sperm PM is susceptible to damage during cryopreservation due to lipid bilayer separation (Holt and North, 1984; Quinn, 1985), osmotic stress (Noiles et al., 1993; Ball and Vo, 2001), membrane peroxidation (Aitken, 1995; Baumber et al., 2003) and intracellular ice formation (Mazur, 1977), and is associated with lower pregnancy rates after insemination with frozen semen. In addition, oxidative stress during cryopreservation can induce DNA fragmentation (Baumber et al., 2002); DNA damage or fragmentation has been associated with reduced fertility in bulls (Januskauskas et al., 2001), stallions (Love and Kenney, 1998), boars (Boe-Hansen et al., 2005) and humans (Evenson and Wixon, 2006). Although a sperm with an intact PM is necessary for normal fertilization, it is not for ICSI because the sperm-oocyte membrane interaction is surpassed. In fact, damage to the sperm plasma membrane during sperm

immobilization before ICSI is desired as it facilitates sperm nuclear de-condensation (Dozortsev et al., 1995) and fertilization (Palermo et al., 1996b). Moreover, removal of both the PM and acrosome from mouse sperm improves embryo development after ICSI (Morozumi et al., 2006).

The knowledge that sperm motility and PM integrity are not required characteristics for embryo production using ICSI (Kuretake et al., 1996; Wakayama et al., 1998; Ward et al., 2003) has led to the use of alternative sperm preservation methods including freeze-drying (Polge et al., 1949; Kusakabe et al., 2001; Keskinetepe et al., 2002; Abdalla et al., 2009; Choi et al., 2011) and cryopreservation without cryoprotectants (Wakayama et al., 1998; Lacham-Kaplan et al., 2003; Ward et al., 2003; Li et al., 2010). Mouse and rabbit sperm cryopreserved without cryoprotectants suffer plasma membrane damage (Wakayama et al., 1998) and is characterized by extensive round spots wounds and loss of the acrosome (Li et al., 2010). Conversely, the DNA and sperm proteins do not denaturise after cryopreservation without cryoprotectants (Jiang et al., 2005) and embryos produced show similar proportions of normal karyotype as do those with fresh sperm (Kusakabe et al., 2001; Ward et al., 2003).

The objectives of this experiment were to evaluate the sperm PM damage and DNA integrity of frozen-thawed bull and stallion sperm refrozen without further addition of cryoprotectants. We hypothesized that cryopreservation without cryoprotectants will damage the sperm plasma membrane but will not affect the DNA integrity.

Materials and Methods

Experimental Design

The experiment was designed to compare the sperm plasma membrane integrity and DNA integrity of bull and stallion frozen-thawed semen refrozen without further addition of cryoprotectants. One frozen semen straw from a fertile bull (n = 3) and stallion (n = 3) was thawed and sperm plasma membrane and DNA integrity assessed. The frozen-thawed semen (bull and stallion) was refrozen without cryoprotectants and then thawed to assess sperm plasma membrane and DNA integrity.

Sperm Preparation

A semen straw from a fertile bull frozen with cryoprotectants (7% GLY) was thawed in a 37°C water bath and 50 µL used to assess plasma membrane and DNA integrity (BFrozen). Then semen was prepared for IVF by layering over a discontinuous Percoll gradient (50%, 70% and 90%) in a 14-mL round bottom tube (Falcon®; Becton Dickson, VIC, AUS) containing H-SOF supplemented with 5 mg/mL BSA (Invitrogen; Auckland, NZ), 50 µg/mL caffeine (Sigma, MO, USA), 30 µg/mL glutathione (Sigma, MO, USA) and 20 µg/mL heparin (sperm medium) and centrifuged at 780 x g for 20 min. After centrifugation 120 µL of the selected sperm were placed into 200 µL of sperm medium and incubated at 38.6°C for 15 min. Following incubation, 1 µL was used for ICSI and the remainder was placed into a 1.5-mL cryogenic tube (Nalgene™, Thermo Fisher Scientific Inc., MA, USA). The cryogenic tube was immediately frozen by submerging into liquid nitrogen without cryoprotectant (BSnap).

A semen straw from a fertile stallion frozen with cryoprotectants (2.5% EY and 2.5% GLY) was thawed in a 37°C water bath and 50 µL used to assess plasma

membrane and DNA integrity (SFrozen). Then semen was subjected to swim up sperm selection. Two hundred μL of the thawed semen was layered under 600 μL of H-SOF supplemented with 4 mg/mL of BSA and amino acids (Walker et al., 1992) (swim up medium) in a 14-mL round bottom tube. The tube was then incubated at 38.6°C for 20 min. After incubation the top 400 μL were aspirated and placed in a 1.5-mL microcentrifuge tube (Corning, NY, USA). Then washed twice by centrifugation at 300 x g for 3 min and resuspended in swim up medium to a volume of 500 μL . One μL was immediately used for ICSI and the rest aspirated into a 0.5-mL straw and frozen by submerging into liquid nitrogen without addition of cryoprotectants (SSnap).

Plasma Membrane Integrity Assessment

Thawed semen was diluted to a concentration of $25 \times 10^6/\text{mL}$ with phosphate buffered saline (PBS). Twenty five μL of diluted semen was mixed with 2.5 μL of SYBR-14/PI fluorescent stain (Minitube, VIC, AUS) and incubated at 37°C for 10 min in the dark. After incubation, 10 μL of the mixture was placed on a slide and a glass coverslip. The slide was assessed under a fluorescent microscope (Zeiss AxioLab10; Zeiss, AUS) and images from a minimum of 3 different fields were obtained (cellSense™; Olympus, AUS). Sperm displaying green (intact plasma membrane) or red (damaged plasma membrane) fluorescence were counted from the images (Figure 5). A minimum of 400 sperm were analyzed per treatment group.

DNA damage assessment

The DNA integrity was evaluated using the sperm chromatin dispersion assay (SCD) (Sperm-Halomax® kit; ChromaCell SL, Madrid, Spain) as previously described (López-Fernández et al., 2007). Thawed semen was diluted to $10 \times 10^6/\text{mL}$ with PBS.

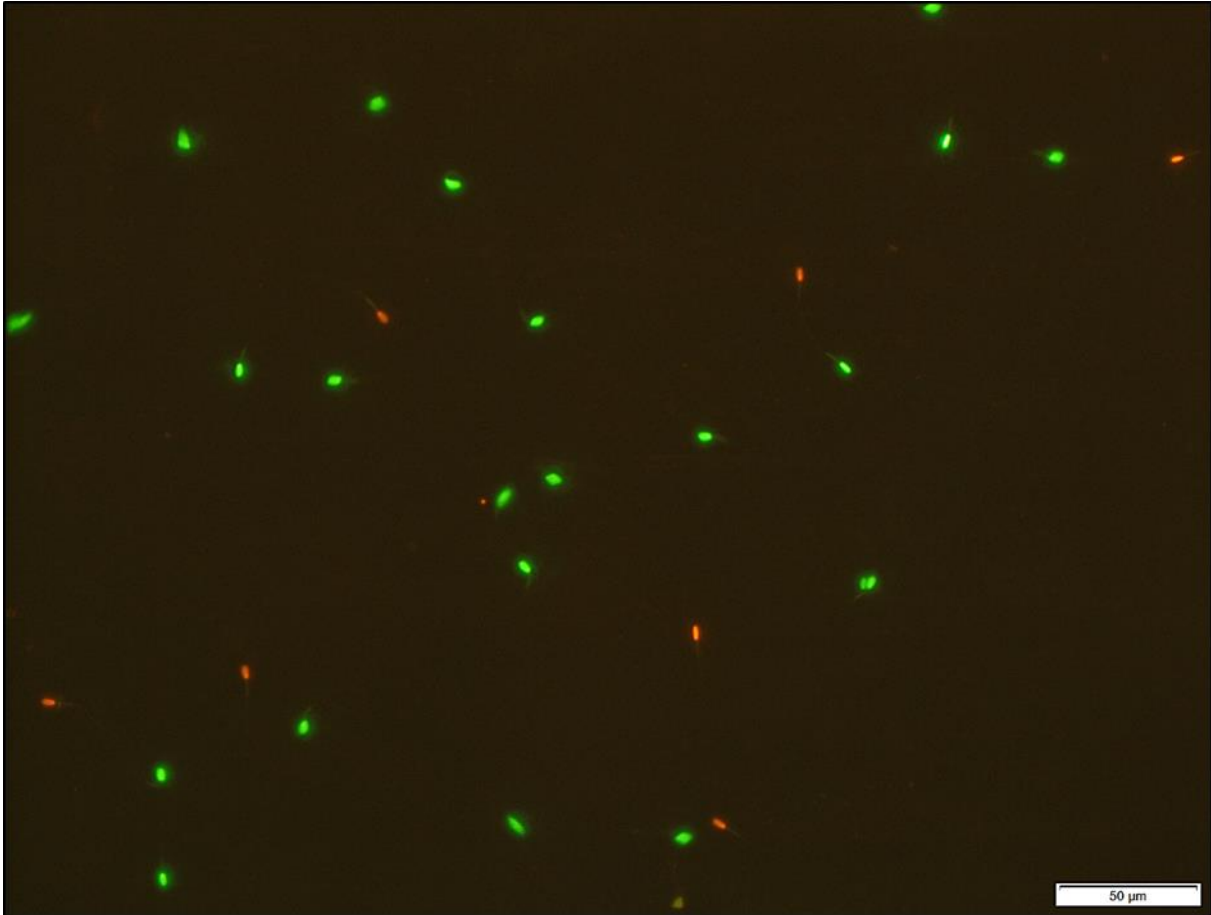


Figure 5: Frozen-thawed bull sperm (BFrozen-thawed) stained with SYBR-14/PI. Sperm with intact plasma membrane stained green and sperm with damaged plasma membrane stained red (Zeiss Axiolab1 microscope, Olympus cellSense™ imaging software, 200x).

Twenty five μL of diluted semen was mixed with 25 μL of low melting agarose. Ten μL of the agarose-semen mixture was placed on a pre-treated slides (provided by the Halomax® kit), covered with a coverslip and placed in the refrigerator (4 - 6°C) on a pre-cooled metallic plate for 5 min. After 5 min when the agarose-semen mixture has solidified, the coverslip was carefully removed and the slide placed in a tray with 10 mL of lysing solution for five min. Then the slide was thoroughly rinsed with dH_2O for five min and dehydrated in a series of increasing concentrations of ethanol (70%, 90% and 100%). Slides were air dried and stained with a mixture of PBS/Wright's solution

(50%/50%) and allowed to air dry. The degree of DNA fragmentation (sDFI, % of sperm with fragmented DNA) was assessed by placing the slides under a light microscope (BX41; Olympus, AUS) at 1000x magnification and counting the sperm with non-fragmented (small halos) or fragmented (large halos) DNA (Figure 6).

Statistical Analysis

The effect of refreezing semen without cryoprotectants on sperm PM and DNA integrity was evaluated using Chi-square analysis (SAS 9.4, SAS Institute Inc., NSW, AUS). A value of $p < 0.05$ between treatments was considered significant.

Results

An effect of treatment on plasma membrane integrity was observed. Cryopreservation without cryoprotectants damaged the sperm PM (Figures 7 and 8). Frozen-thawed bull and stallion sperm PM integrity ($62.8 \pm 0.02\%$ and $57.2 \pm 0.03\%$, respectively) was higher ($p < 0.001$) compared to snap frozen-thawed (0% and $1.4 \pm 0.01\%$, respectively) (Figures 9 and 10).

No increase in sperm DNA fragmentation was observed after bull and stallion frozen-thawed semen was refrozen by direct plunging into liquid nitrogen without the addition of cryoprotectants (Figures 11 and 12).

Discussion

Our results confirm the hypotheses that bull and stallion PM was damaged by refreezing without cryoprotectants while the DNA was not affected under this conditions. Our results are similar to those reported in mouse (Wakayama et al., 1998; Kusakabe et al., 2001; Ward et al., 2003) and rabbit (Li et al., 2010) sperm frozen without

cryoprotectants, and to bull sperm immobilized by snap freezing in liquid nitrogen before ICSI (Horiuchi et al., 2002).

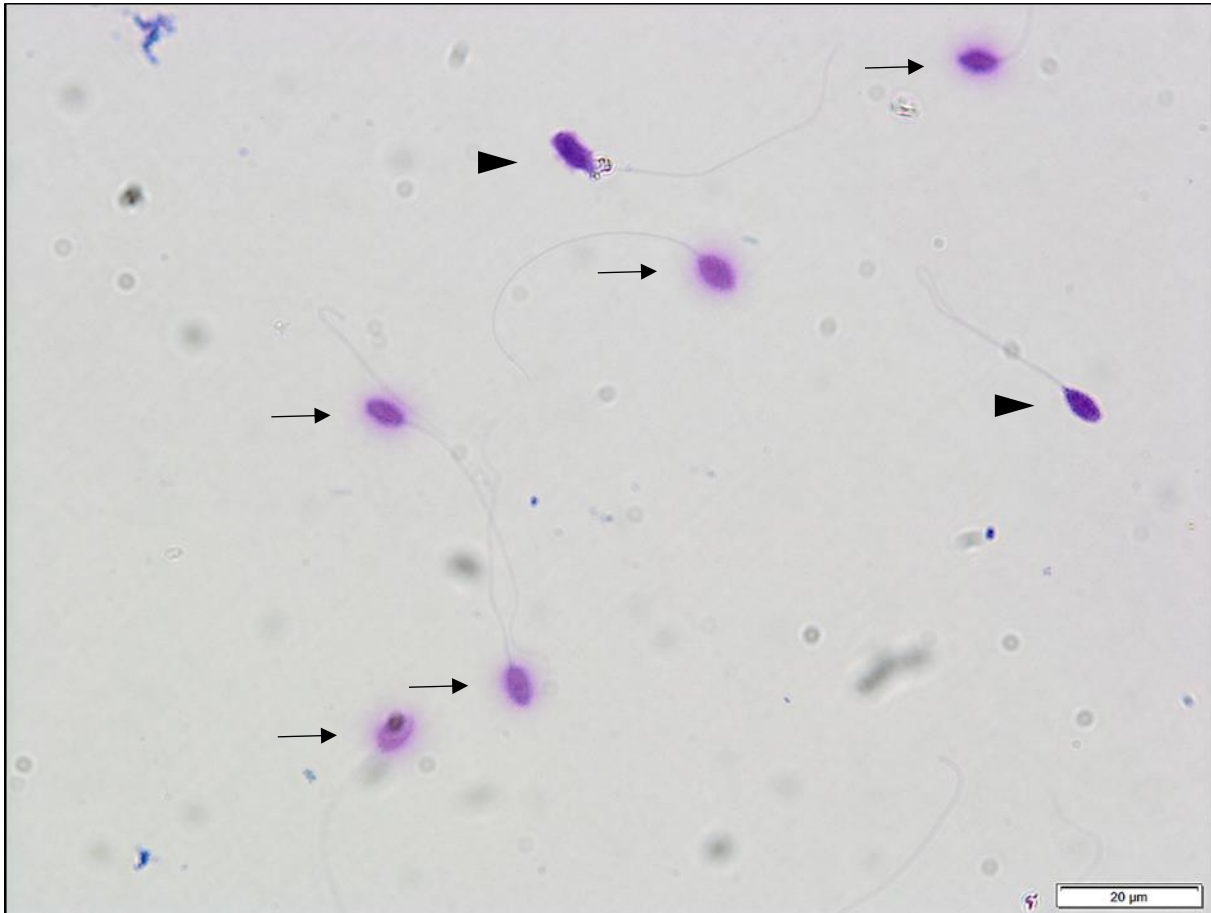


Figure 6: Stallion sperm chromatin dispersion (SCD) assay using the Halomax® kit and stained with Wright's solution under light microscopy. Two population of sperm are observed, sperm with small (arrow) and no (arrow head) halos. These two populations are considered without sperm DNA fragmentation. No sperm with big halo (defragmented DNA) was observed (Olympus BX41 microscope, Olympus cellSense™ imaging software, 1000x).

When sperm is slow-frozen at an optimal cooling rate of $\sim 60^{\circ}\text{C}/\text{min}$ to the final storage temperature of -196°C , several physical events take place. During cooling from 37°C to subzero temperatures, the plasma membrane undergoes rearrangement of the lipid bilayer (Quinn, 1985). At -5°C the cell and surrounding medium remain unfrozen and are super cooled due to the solutes present. Between -5°C to -15°C , extracellular

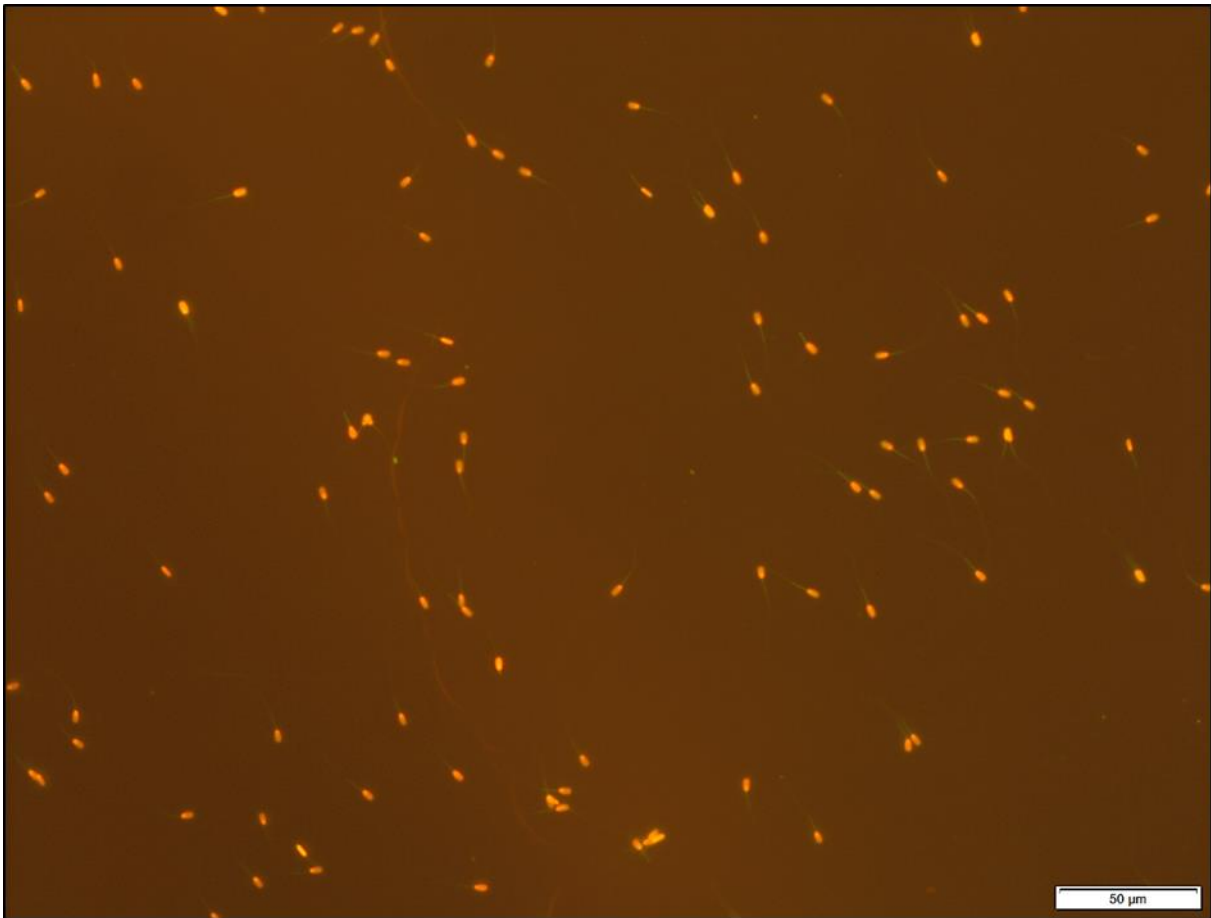


Figure 7: Bull sperm refrozen without further addition of cryoprotectants (BSnap-thawed) and stained with SYBR-14/PI. Notice all the sperm have a damaged plasma membrane and stained red (Zeiss Axiolab1 microscope, Olympus cellSense™ imaging software, 200x).

ice formation begins and to maintain the system in osmotic equilibrium, the sperm will dehydrate as a response to the increased extracellular osmolality (Mazur, 1977). During thawing, the physical events listed are reversed and the osmolality and cell volume will return to the pre-freezing state (Mazur and Cole, 1989). All these physical changes may cause sperm damage (mostly to the PM); thus the addition of cryoprotectants (non-permeating and permeating) is a common practice to decrease sperm damage during freezing; nevertheless sperm damage still occur and the sperm fertility decreased (Watson, 2000). Conversely, when sperm are ultra-rapidly frozen (vitrification), very high

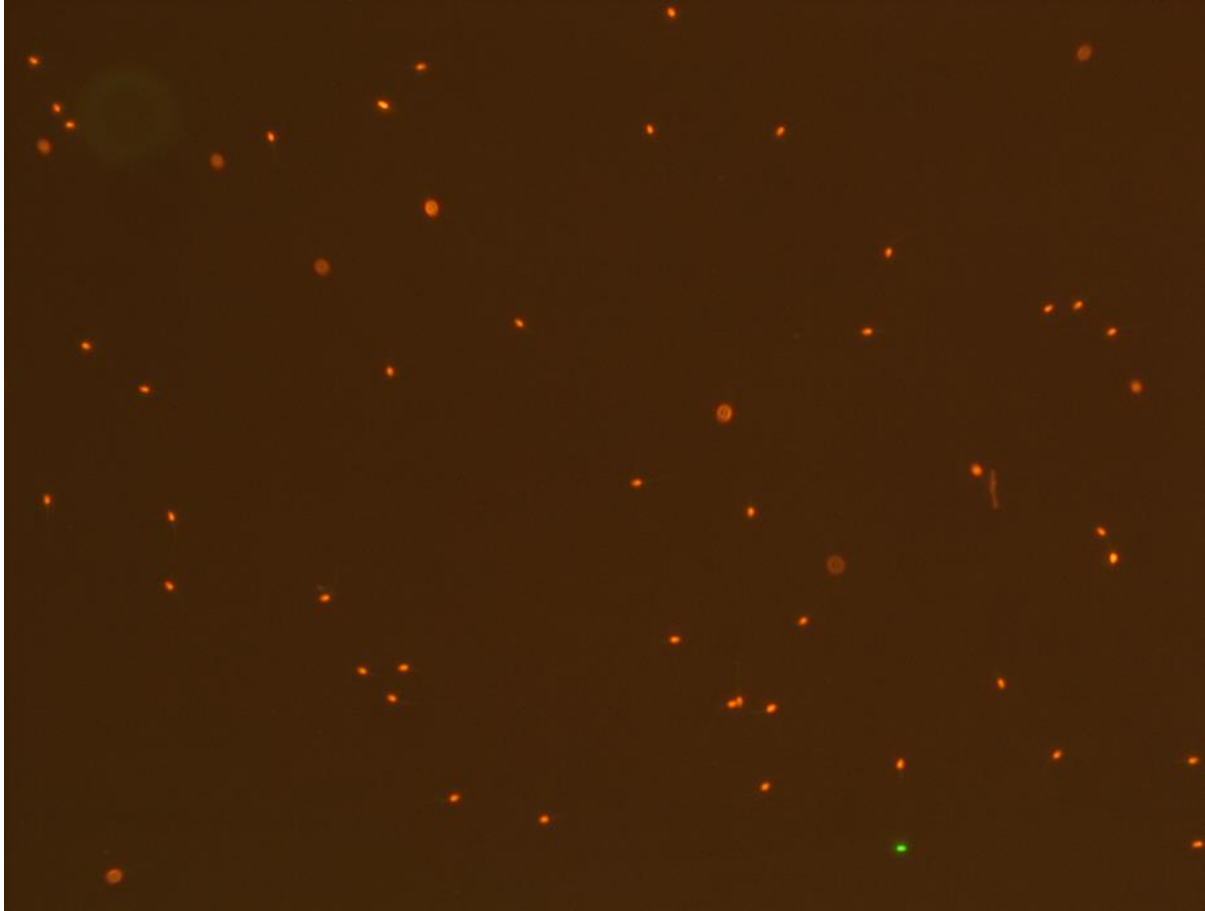


Figure 8: Stallion sperm refrozen without further addition of cryoprotectants (SSnap-thawed) and stained with SYBR-14/PI. Notice almost all the sperm have a damaged plasma membrane and stained red (Zeiss AxioLab1 microscope, Olympus cellSense™ imaging software, 200x).

cooling and warming rates ($> 40000^{\circ}\text{C}/\text{min}$) are used. Using these cooling and warming rates, the probability of cell damage by crystal formation is minimal (Isachenko et al., 2003). In the study herein, refreezing without cryoprotectants was performed in 0.5 mL straws compared to the recommended 20 μL sperm solution films (Isachenko et al., 2004) and damaged 100% of bull and 98.6% of stallion sperm PM as assessed by SYBR-14/PI dual staining. Damage to the sperm PM could have been caused by either refreezing at supraoptimal cooling rates and/or warming rates, causing lipid bilayer

separation (Quinn, 1985), intracellular ice formation (Mazur, 1977) or osmotic stress (Meryman et al., 1977; Ball and Vo, 2001), or by a combination of these processes.

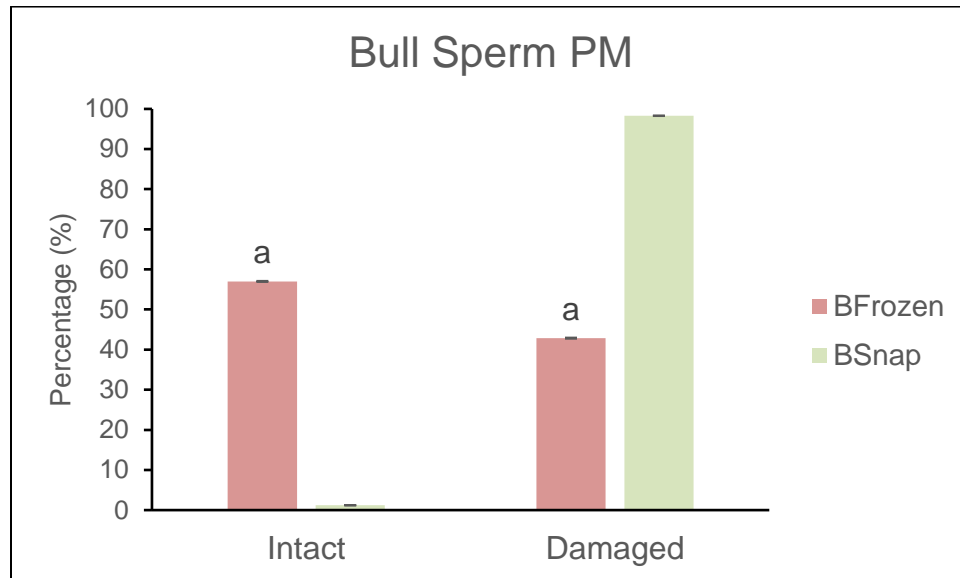


Figure 9: Plasma membrane (PM) integrity percentage (\pm SEM) of frozen-thawed (BFrozen-thawed) and refrozen (BSnap-thawed) bull sperm assessed by SYBR-14/PI. (a) Denotes difference ($p < 0.05$) between treatments.

During sperm maturation histones are replaced by protamines and the DNA is tightly packed in toroids (Ward and Coffey, 1991). As sperm travels through the epididymis, the chromatin is further compacted by cross-link disulphide bonds (Barratt et al., 2010). Bull and stallion bind to protamines-bound DNA is $\geq 85\%$ (Bench et al., 1996) and it is suggested that protamine-bound DNA is less susceptible to damage (Wakayama et al., 1998). Supporting this are the studies showing that mouse sperm with similar protamines-bound DNA as bull and stallion (Bench et al., 1996) is less susceptible to damage after sonication (Kuretake et al., 1996) compared to human sperm (Martin et al., 1988) with less protamines-bound DNA (73%) (Bench et al., 1996). In the study herein, DNA integrity was evaluated with the SCD assay and no increased in DNA damaged was observed. The SCD test has shown strong relationship with the

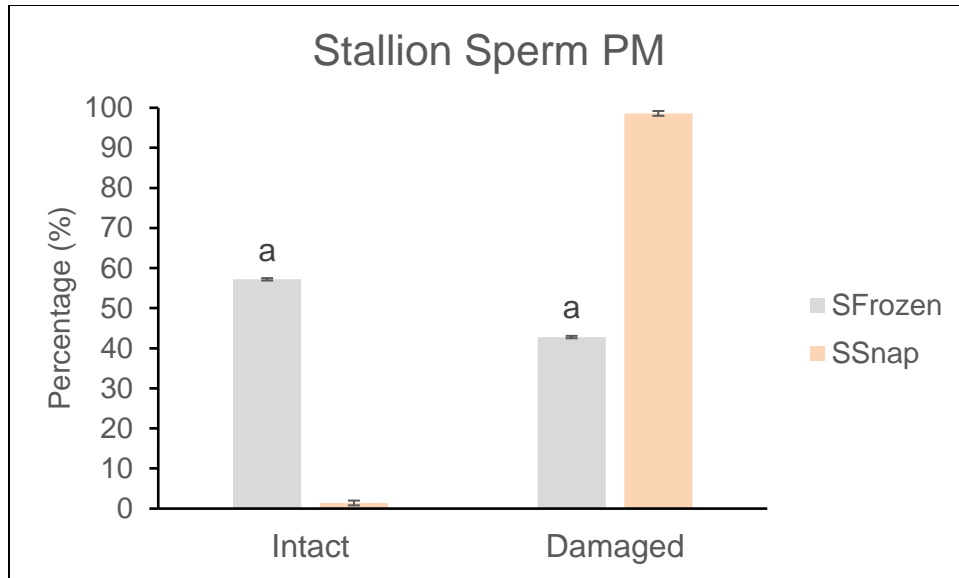


Figure 10: Plasma membrane (PM) integrity percentage (\pm SEM) of frozen-thawed (SFrozen-thawed) and refrozen (SSnap-thawed) stallion sperm assessed by SYBR-14/PI.

(a) Denotes difference ($p < 0.05$) between treatments.

SCSA (Chohan et al., 2006); however the number of sperm assessed and the inter-observer variability of the assay have raised concerns to its statistical strength (Evenson and Wixon, 2005). We assessed the DNA integrity in 600 sperm and did not observed sperm with large halo; yet is possible this was due to the low number of cells compared to the > 5000 sperm evaluated with a flow cytometer in SCSA. Nevertheless, results from our study also support that bull and stallion sperm with high protamine-bound DNA is less susceptible to damage by external stressors.

In conclusion, bull and stallion sperm refrozen without cryoprotectants and thawed will suffer PM damage; however the DNA integrity is unaffected. Freezing or refreezing sperm without cryoprotectants seems to be a good alternative when preparing sperm for ICSI as it causes sperm PM damage but the genetic material remains intact.

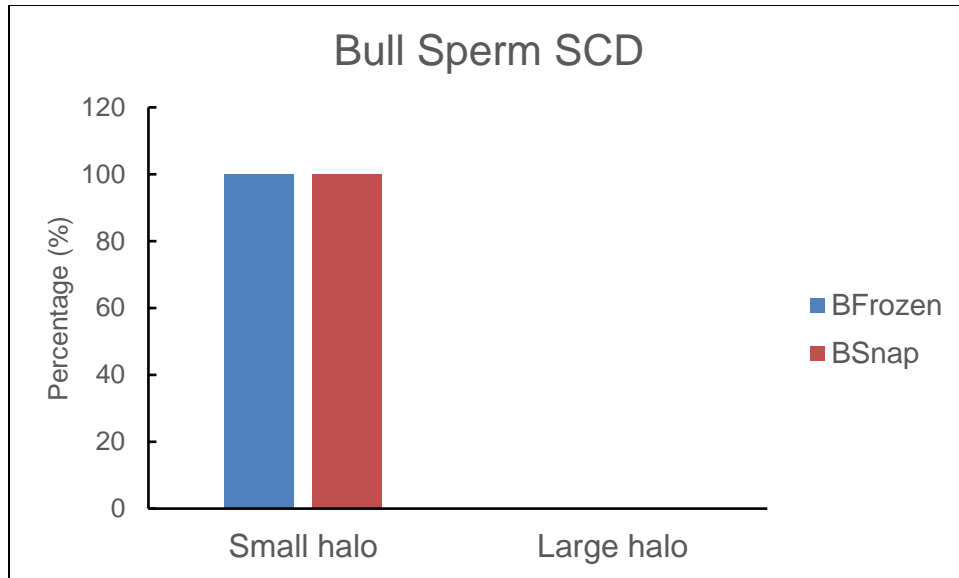


Figure 11: Percentage (\pm SEM) of BFrozen-thawed and BSnap-thawed sperm with non-fragmented (Small halo) or fragmented (Large halo) DNA assessed by the Halomax® test.

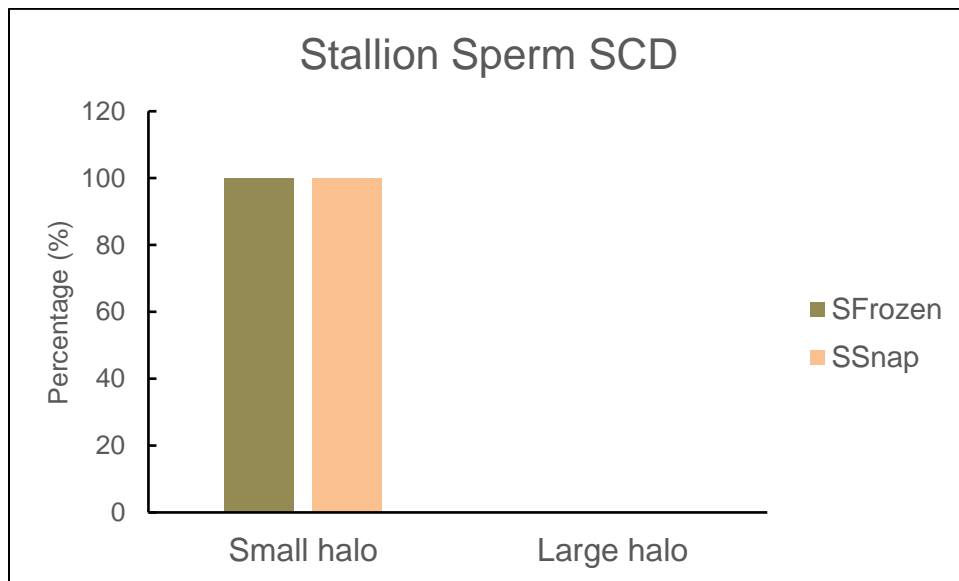


Figure 12: Percentage (\pm SEM) of SFrozen-thawed and SSnap-thawed sperm with non-fragmented (Small halo) or fragmented (Large halo) DNA assessed by the Halomax® test.

CHAPTER 4

ACTIVATION OF BOVINE OOCYTES AFTER INTRACYTOPLASMIC SPERM INJECTION (ICSI) WITH BULL AND STALLION FROZEN-THAWED SPERM REFROZEN WITHOUT CRYOPROTECTANTS

Introduction

Semen cryopreservation has enabled the large-scale propagation of genetic material from selected males and the preservation of endangered species genome (Wildt, 2000). In endangered species, cryopreserved semen is limited and in domestic species males may become infertile due to injury, disease, or death; thus limiting the source of semen for cryopreservation. Moreover, a proportion of sperm suffer damage during cryopreservation resulting in lower fertility (Watson, 2000). Tens to hundreds of million sperm are commonly packed in one straw of frozen semen. In assisted reproductive techniques such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) only a small portion of a thawed straw is used and the rest is generally discarded.

In an effort to optimize the use of cryopreserved semen and reduce wastage of valuable genetics, the unused semen portion has been subjected to a second freezing cycle (Polcz et al., 1998; McCue et al., 2004). Comparable conception rates have been achieved in cattle after artificial insemination with frozen-thawed (33%) and frozen-thawed/refrozen (28%) semen (Maxwell et al., 2007). Furthermore, percentage of embryos developing to the blastocyst stage in cattle (34%, IVF) (Underwood et al., 2010), ovine (62%, IVF) (Hollinshead et al., 2004) and horses (23%, ICSI) (Choi et al., 2006) with frozen-thawed/refrozen semen have been reported.

Intracytoplasmic sperm injection is the method of choice for the *in vitro* embryos production in the horse as marginal success is achieved with IVF (Zhang et al., 1990; Dell'Aquila et al., 1996; Dell'Aquila et al., 1997a; Hinrichs et al., 2002). In cattle, IVF systems have been commonly used to produce embryos *in vitro* (Farin and Farin, 1995; Blondin et al., 1997; Farin et al., 1997) as ICSI systems were considered inefficient (Suttner et al., 2000). Nevertheless, recent improvements in cattle ICSI systems have yielded 20 – 30% of embryos developing to blastocysts (Horiuchi et al., 2002; Wei and Fukui, 2002); developmental rates comparable to some (30 – 35%) (Farin et al., 1997; Holm et al., 1999; Alm et al., 2005; Underwood et al., 2010) but lower than other (>50%) (Hussein et al., 2006) IVF systems. Interestingly, sperm motility and PM integrity are not requirements for oocyte activation and embryo production by ICSI (Wakayama et al., 1998; Ward et al., 2003; Choi et al., 2006), and permits the investigation of sperm cryopreservation methods where sperm PM integrity and motility is not maintained. Sperm cryopreservation methods that have been explored are freeze-drying (Polge et al., 1949) and freezing without cryoprotectants (Wakayama et al., 1998). Oocyte activation and embryo production after ICSI with freeze-dried sperm have been reported in pigs (Kwon et al., 2004), cattle (Keskinetepe et al., 2002; Abdalla et al., 2009), mouse (Kusakabe et al., 2008) and horses (Choi et al., 2011). Fresh sperm frozen-thawed once without cryoprotectants has been used to activate oocytes, produce embryos and normal offspring after ICSI in mouse (Wakayama et al., 1998; Lacham-Kaplan et al., 2003) and rabbit (Li et al., 2010).

The birth of a calf with sperm immobilized (killed) by freezing to -20°C (Goto et al., 1990) and oocyte activation with sperm immobilized by plunging into liquid nitrogen

(Horiuchi et al., 2002) before ICSI have been reported. Currently, the ability of bull and stallion frozen-thawed sperm refrozen and stored without the addition of cryoprotectants to activate cattle oocytes after ICSI is unknown. The objective of this study was to evaluate the ability of bull and stallion frozen-thawed sperm stored refrozen without the addition of cryoprotectants to activate bovine oocytes after ICSI. We hypothesize that bull and stallion sperm stored refrozen without cryoprotectants will activate bovine oocytes after ICSI; however at a lower rate than that of sperm frozen with cryoprotectants.

Material and Methods

Experimental design

This experiment was designed to compare the ability of bull and stallion sperm frozen with cryoprotectants against bull and stallion sperm frozen-thawed and refrozen without the addition of cryoprotectants to activate *in vitro* matured bovine oocytes after ICSI. Cumulus-oocyte complexes (COCs) were aspirated from ovaries collected in a local abattoir and matured *in vitro*. After *in vitro* maturation (IVM), COCs were stripped of their cumulus cells and oocytes with a polar body were selected for ICSI. The Frozen-thawed (Bull: n = 7 replicates and n = 59 oocytes; Stallion: n = 6 replicates and n = 50 oocytes) and Snap-thawed (Bull: n = 6 replicates and n = 66 oocytes; Stallion: n = 6 replicates and n = 52 oocytes) treatment groups consisted of the injection of a sperm into the oocyte ooplasm. The sham treatment group (Bull: n = 4 replicates and n = 17 oocytes; Stallion: n = 5 replicates and n = 26 oocytes) consisted of injecting oocytes with a small volume (5 μ L) of a 5% PVP in H-SOF and served as parthenogenetic activation control for the study. Injected oocytes were cultured for 24 h and the

activation status assessed under a fluorescent microscope after culture in a (9:1) glycerol/PBS solution containing 2.5 µg of Hoechst 33258.

Cumulus-Oocytes Complexes (COCs) Collection and IVM

Ovaries were collected from a local abattoir and transported to the laboratory (1 h) in PBS at 33°C. Upon arrival to the laboratory, ovaries were rinsed with warm (37°C) PBS and all visible follicles were aspirated using a 10 mL syringe with an 18 G disposable needle. Cumulus-oocyte complexes were collected in H-TCM199 (Sigma, MO, USA) supplemented with 5% (v/v) fetal calf serum (FCS) and 100 µg/mL of streptomycin sulphate (CSL Ltd., Victoria, AUS) and 100 U/mL penicillin G (CSL Ltd., Victoria, AUS). Aspirated COCs (Figure 13) with at least 3 – 4 layers of CC's and a homogeneous cytoplasm were recovered from the aspiration fluid using a stereomicroscope, rinsed three times with sodium bicarbonate-buffered TCM199 supplemented with 20% (v/v) FCS, 5 µg/mL FSH (Folltropin; Bioniche Health Science Inc., Quebec, CA), 5 µg/mL hCG (Chorulon®; Intervet Australia Pty Ltd, Victoria, AUS) and 1 µg/mL estradiol 17β (Sigma, MO, USA) (maturation medium). Twenty five COCs were then transferred into each culture well (Nunc Inc., IL, USA) containing 600 µL of maturation medium covered with 300 µL of mineral oil (Sigma, MO, USA). Cumulus-oocyte complexes were matured at 38.6°C in a humidified atmosphere of 5% CO₂ in air. After 20 – 22 h of maturation COCs were denuded by vortexing for 3 min in a 15-mL conical centrifuge tube containing 1 mL of a 0.2% (w/v) hyaluronidase SOF. Denuded oocytes with an extruded polar body (MII, metaphase II) were selected (Figure 14) and washed three times with H-SOF supplemented with 4 mg/mL BSA (Invitrogen Corporation, Auckland, NZ) and amino acids (arginine 90 µM, aspartic acid 22 µM,

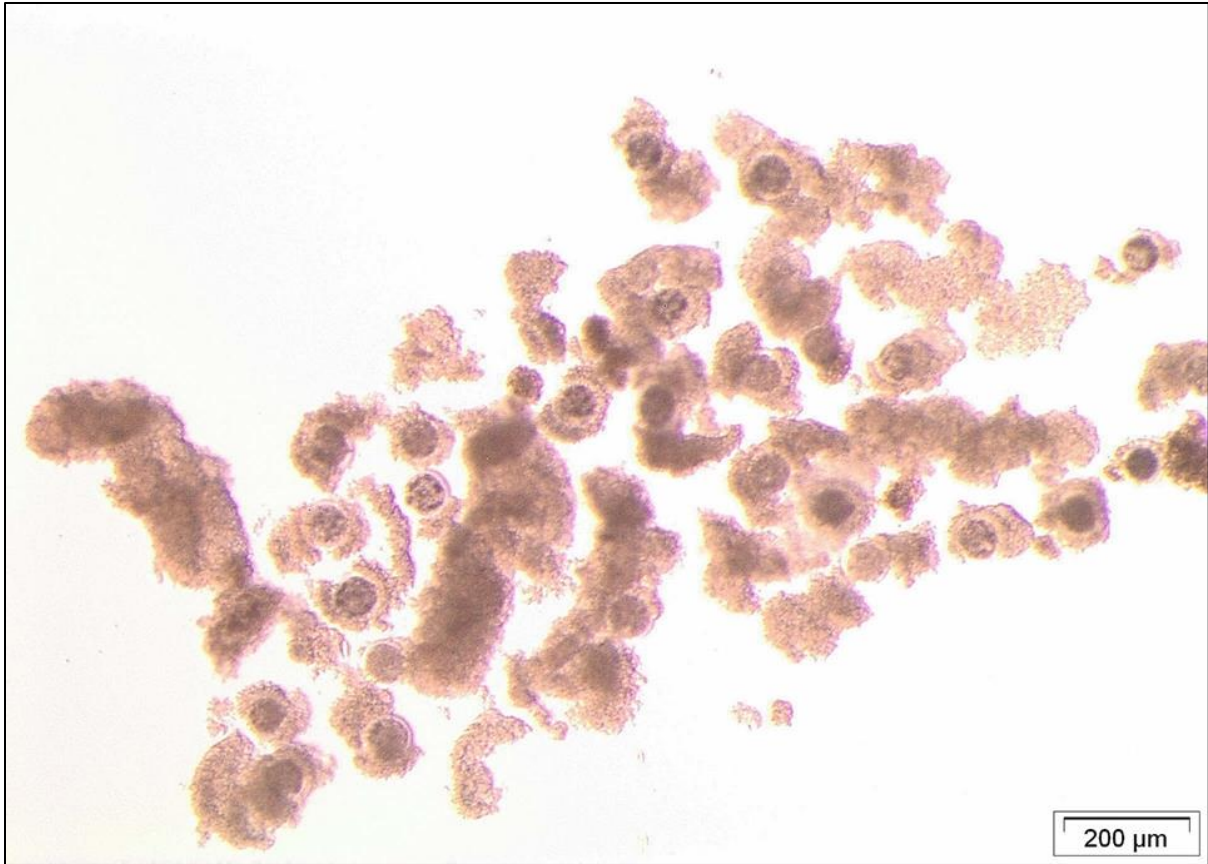


Figure 13: Bovine COCs recovered after follicular aspiration in aspiration medium at 37°C and viewed under an inverted microscope (Olympus SZX7, analySIS getIT imaging software, 40x magnification).

asparagine 24 μM, alanine 431 μM, cysteine 40 μM, glutamic acid 54 μM, glutamine 101 μM, glycine 1390 μM, histidine 42 μM, isoleucine 97 μM, leucine 194 μM, lysine 194 μM, methionine 55 μM, ornithine 19 μM, phenylalanine 95 μM, serine 9 μM, taurine 49 μM, threonine 9 μM, tyrosine 89 μM and valine 264 μM) at sheep oviductal fluid concentration (Walker et al., 1996) (holding medium) and left in the same medium until transferred for ICSI.

Sperm Preparation

A 0.5 mL semen straw frozen with cryoprotectants (7% GLY) (BFrozen-thawed) from a fertile bull was thawed in a 37°C water bath for 30 s and prepared for use in IVF

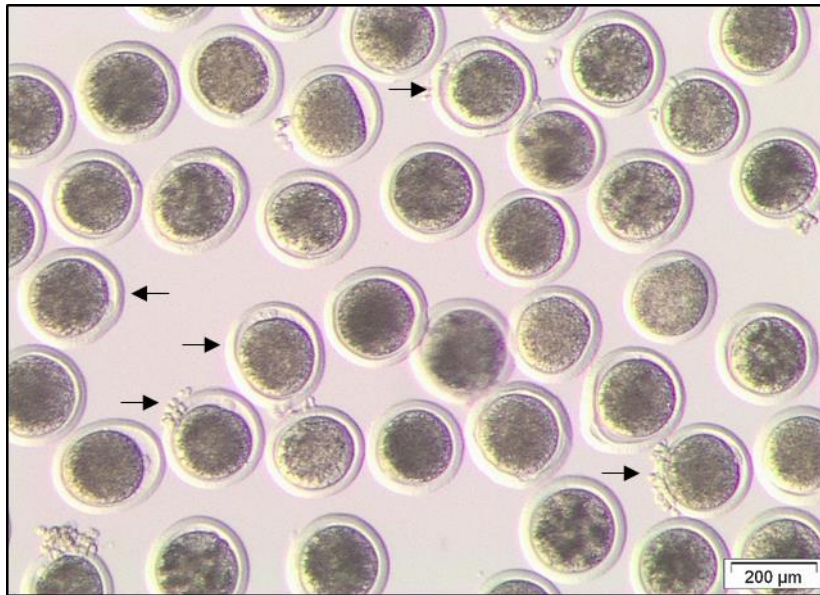


Figure 14: Denuded bovine oocytes in IVM medium at 37°C after 20 – 22 h of culture. Oocytes with a PB (arrow) were selected for ICSI (Olympus SZX7, analySIS getIT imaging software, 100x magnification).

by layering over a discontinuous Percoll gradient (50%, 70%, 90%) in a 14-mL round bottom tube (Falcon®; Becton Dickson, Victoria, Australia) containing H-SOF supplemented with 5 mg/mL BSA (Invitrogen Corporation, Auckland, NZ), 50 μg/mL caffeine (Sigma, MO, USA), 30 μg/mL glutathione (Sigma, MO, USA) and 20 μg/mL heparin (sperm medium) and centrifuged at 780 x g for 20 min. After centrifugation, 120 μL of the selected sperm were placed into 200 μL of sperm medium and incubated at 38.6°C for 15 min. Then 1 μL was immediately used for ICSI and the remainder of the sperm solution was placed into a 1.5-mL cryogenic tube (Nalgene™, Thermo Fisher Scientific Inc., MA, USA), which was immediately frozen by submerging into liquid nitrogen (BSnap-thawed) and stored for at least a week before use for ICSI.

A 0.5-mL semen straw frozen with cryoprotectants (2.5% EY and 2.5% GLY) (SFrozen-thawed) from a fertile stallion was thawed in a 37°C water bath for 30 s. The frozen-thawed stallion semen was then subjected to swim up sperm selection. Two

hundred μL of the thawed semen was layered under 600 μL of H-SOF supplemented with 4 mg/mL of BSA (swim up medium) concentration in a 14-mL round bottom tube. The tube was then incubated at 38.6°C for 20 min. After incubation the top 400 μL were aspirated and placed in a 1.5-mL microcentrifuge tube (Corning, NY, USA). Semen was washed twice by centrifugation at 300 x g for 3 min, the supernatant removed and sperm pellet re-suspended into 500 μL of swim up medium. One μL of the sperm suspension was immediately used for ICSI and the remainder aspirated into a 0.5-mL straw that was then frozen by submerging into liquid nitrogen (SSnap-thawed). Snap frozen samples were stored for at least one week before use for ICSI.

Intracytoplasmic Sperm Injection (ICSI)

The ICSI was performed as previously described by Kimura and Yanagimachi (Kimura and Yanagimachi, 1995a), using the Piezo injection system (PrimeTech PMAS-CT-150; Sutter Instrument®, CA, USA). The injection pipette (PIEZO-8-25; Humagen Pipets, VA, USA) had an 8- μm inner diameter (I.D.), a 25° angle bend, 6-mm taper from the bend to the tip and a blunt opening. A small volume of mercury (~ 1 μL) was placed in the proximal end of the pipette and connected to the oil-filled microinjection pipette holder leaving a small air column (~ 0.5 mm). The mercury was then pushed to the distal end applying forward pressure by filling the microinjection pipette with oil. The holding pipette (MPH-LG-35; Humagen Pipets, VA, USA) had a 30- μm I.D., a 35° angle bend, 1-mm taper from the bend to the tip and a blunt opening. The holding pipette was connected to an oil-filled Narishige microinjector.

Sperm injection was performed on a lid of a 100-mm petri dish (Falcon®; Becton Dickson, Victoria, Australia) at room temperature (23 – 24°C), using an inverted

microscope (Olympus IX70, Victoria, AUS) equipped with Hoffman optics at 200x magnification. Ten to fifteen MII oocytes were transferred into a 40- μ L drop of holding medium. One μ L of the sperm-thawed or snap-thawed sperm suspension was placed into a 5 μ L drop of H-SOF containing 5% (w/v) PVP (Sigma, St. Louis, MO, USA) under oil. Each MII oocyte was picked up with the holding pipette by applying gentle suction and the oocyte rotated to orient the PB to either the 6 or 12 o'clock position. Motile sperm from the frozen-thawed sample were immobilized by applying a few pulses with the Piezo drill to the sperm tail before injection. Immotile sperm from the snap-thawed treatment group were similarly treated before injection. The injection pipette was then placed in contact with the ZP at the 3 o'clock position and the ZP drilled (speed = 5 and intensity = 7) while applying light negative pressure. Following ZP perforation, the injection pipette was retrieved and the piece of ZP expelled into the medium. The injection pipette was then re-inserted through the hole in the ZP and the sperm advanced close to the tip of the pipette. The injection pipette was advanced until reaching the center of the oocyte and a single Piezo pulse (speed = 3 and intensity = 5) was applied to break the oolema. The sperm with minimum amount of medium was then expelled into the ooplasm and the injection pipette removed while applying light negative pressure. Sham injections were performed in the same manner without sperm injection. After injection oocytes were held at room temperature for 15 – 20 min to allow the broken membranes to heal before transfer into 600 μ L SOF medium supplemented with 6 mg/mL BSA, 50 μ g/mL myo-inositol (Sigma, St. Louis, MO, USA) and amino acids at sheep oviductal fluid concentration, covered with 300 μ L mineral oil, and incubated at 38.5°C under a 5% CO₂ in air atmosphere.

Oocyte Activation Assessment

To determine the activation status after 20 - 24 h of culture, oocytes were mounted on a slide with 10 μ L of a 9:1 glycerol:PBS solution containing 2.5 μ g Hoechst 33258 (Sigma, St. Louis, MO, USA), incubated in the dark for 10 min and examined under a fluorescent microscope. Oocytes in MII, with a visible condensed sperm head within the ooplasm were considered non-activated (Figure 15). Oocytes in MII with a decondensing sperm head or one PN were considered activated but arrested (Figure 16). Oocytes with two pronuclei or cleaved with the presence of nuclei in each blastomere were considered normally fertilized (Figure 17).

Statistical Analysis

The difference in the proportion of activated, arrested and non-activated oocytes between sperm treatment groups was assessed using Chi-Square analysis (SAS 9.4, SAS Institute Inc., New South Wales, AUS). If the value of any parameter was less than ten, Fisher's exact test was used. Significance difference was considered at a value of $p < 0.05$.

Results

Activation was assessed 20 - 24 h post-ICSI following Hoechst 33258 staining. The proportion of activated oocytes after injection with BFrozen-thawed, BSnap-thawed or BSham treatment groups was not significantly different ($p = 0.570$). The proportion of arrested and non-activated oocytes between the BFrozen-thawed and BSnap-thawed treatment groups was not significantly different; however the proportion of activated and arrested oocytes was higher than the Sham treatment group ($p < 0.001$) (Table 2).

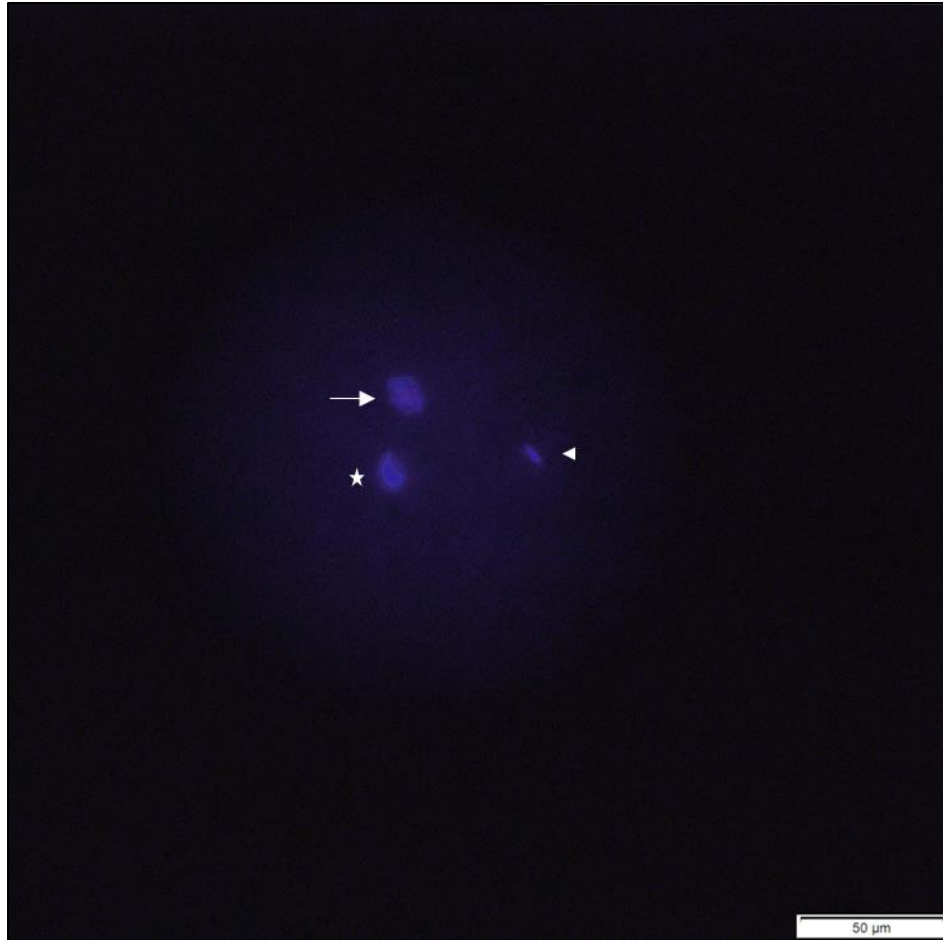


Figure 15: Bovine oocyte 24 h after ICSI with BFrozen-thawed sperm, stained with Hoechst 33258 and observed under a fluorescent microscope. Note the presence of a PB (star), MII spindle (arrow) and sperm head (arrow head). This oocyte was considered non-activated (Olympus IX70, Olympus cellSense™ imaging software, 200x).

There was an effect of sperm treatment on the proportion of activated oocytes after ICSI with stallion sperm ($p < 0.001$). The proportion of oocytes activated after injection with the SFrozen-thawed treatment group was higher than for the SSnap-thawed and Sham treatment groups. The proportion of arrested oocytes after injection with SSnap-thawed treatment group was higher ($p < 0.001$) than for the SFrozen-thawed and Sham treatment groups. The proportion of non-activated oocytes injected with the Sham treatment group was higher than the SFrozen-thawed and SSnap-thawed treatment groups ($p < 0.001$) (Table 3).

Table 2: Proportion (%) of activated, arrested and non-activated bovine oocytes after ICSI with bull frozen-thawed (BFrozen-thawed) and snap-thawed (BSnap-thawed) sperm

<u>Treatment</u>	<u>No. oocytes</u>	<u>Activated</u>	<u>Arrested</u>	<u>Non-activated</u>
BFrozen-thawed	59	9 (15.3%) ^a	21 (35.6%) ^a	29 (49.2%) ^a
BSnap-thawed	66	6 (9.1%) ^a	32 (48.5%) ^a	28 (42.4%) ^a
Sham	17	0 (0%) ^a	0 (0%) ^b	17 (100%) ^b

Different superscripts (a,b) within columns indicate statistical differences ($p < 0.05$).

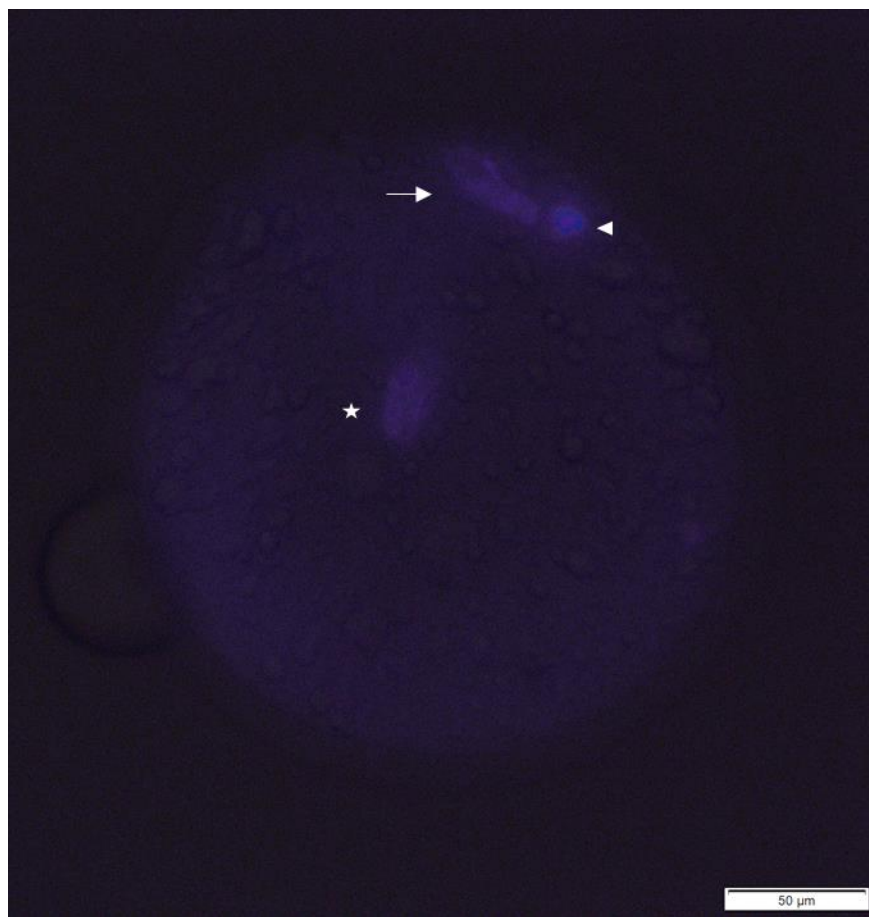


Figure 16: Bovine oocyte 24 h after ICSI with SFrozen-thawed sperm, stained with Hoechst 33258 and observed under a fluorescent microscope. Note the presence of decondensed spindle (arrow) a male PN (star), and 1st PB (arrowhead). This oocyte was considered activated but arrested (Olympus IX70, Olympus cellSense™ imaging software, 400x).

Table 3: Proportion (%) of activated, arrested and non-activated bovine oocytes after ICSI with stallion frozen-thawed (SFrozen-thawed) and snap-thawed (SSnap-thawed) sperm

<u>Treatment</u>	<u>No. oocytes</u>	<u>Activated</u>	<u>Arrested</u>	<u>Non-activated</u>
SFrozen-thawed	50	17 (34.0%) ^a	8 (16.0%) ^a	25 (50%) ^a
SSnap-thawed	52	4 (7.7%) ^b	21 (40.4%) ^b	27 (51.9%) ^a
Sham	26	0 (0%) ^b	0 (0%) ^c	26 (100%) ^b

Different superscripts (a,b,c) within columns indicate statistical differences ($p < 0.05$).

Discussion

Results from the study confirm our hypothesis and demonstrated the ability of bull and stallion sperm refrozen without the addition of cryoprotectants to activate bovine oocytes; although at a lower rate compared to frozen-thawed sperm. Oocyte activation with BSnap-thawed sperm in our study was higher (9.1%) than the 5.9% reported by Goto et al. (1990) but lower than the 62.2% reported by Horiuchi et al. (2002) with killed sperm and with or without oocyte chemical activation respectively. Reported bovine oocyte activation after Piezo-driven ICSI with bull frozen-thawed sperm without exogenous activation range from 70% to 86% (Katayose et al., 1999; Wei and Fukui, 2000; Horiuchi et al., 2002; Wei and Fukui, 2002). Oocyte activation in the present study were lower after ICSI with BFrozen-thawed (15.3%) compared to previous reports. This difference may be due in part (at least for the Bfrozen-thawed) by the oocyte activation criteria used in our experiment. In previous studies an oocyte was considered activated when one PN was present (Katayose et al., 1999; Wei and Fukui, 2002). Using this criteria, 50.9% of injected oocytes would have been considered activated in the study herein.

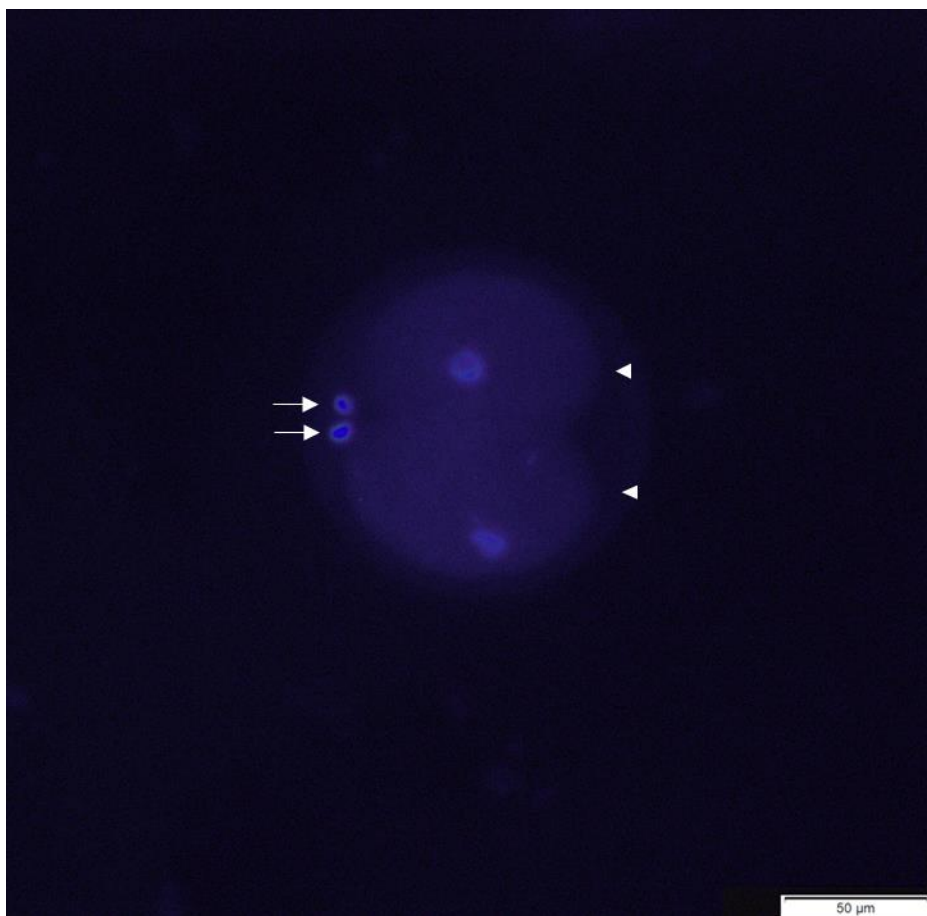


Figure 17: Bovine oocyte 24 h after ICSI with BFrozen-thawed sperm, stained with Hoechst 33258 and observed under a fluorescent microscope. Note the presence of two blastomeres with nuclei in each of them (arrowheads) and two polar bodies (arrows). This oocyte was considered activated (Olympus IX70, Olympus cellSense™ imaging software, 200x).

Reported bovine oocyte activation after Piezo-driven ICSI with bull frozen-thawed sperm without exogenous activation range from 70% to 86% (Katayose et al., 1999; Wei and Fukui, 2000; Horiuchi et al., 2002; Wei and Fukui, 2002). Oocyte activation in the present study were lower after ICSI with BFrozen-thawed (15.3%) compared to previous reports. This difference may be due in part (at least for the Bfrozen-thawed) by the oocyte activation criteria used in our experiment. In previous studies an oocyte was considered activated when one PN was present (Katayose et al., 1999; Wei and Fukui, 2002). In the study herein, 50.9% of oocytes would have been considered activated.

Bovine oocytes have proven to be an alternative to equine oocytes for the study of stallion sperm oocyte activation ability after ICSI (Choi et al., 2002). Bovine oocyte activation (presence of 2 PN or cleavage) rates of 90% and 93% after Piezo-driven ICSI with fresh or frozen-thaw stallion sperm, respectively, have been reported (Choi et al., 2002), which is higher compared to our results with either SFrozen-thawed (34%) or SSnap-thawed (7.7%) sperm. Parthenogenetic activation after ICSI of 4 – 10% has been reported in cattle (Keefer et al., 1990; Catt and Rhodes, 1995; Chen and Seidel Jr, 1997) and it may be argued that activation in this study was a result of parthenogenic activation; however this may seem unlikely considering none of the sham injected oocytes were activated.

Damage to the sperm plasma membrane facilitates sperm head de-condensation (Dozortsev et al., 1995), promotes perinuclear theca disassociation from the sperm nucleus (Katayama et al., 2005) and is a required step before ICSI (Dozortsev et al., 1995; Palermo et al., 1996b). Commonly, the sperm plasma membrane is damaged by scoring the sperm tail against the bottom of the dish (Catt and Rhodes, 1995; Dozortsev et al., 1995) or by applying Piezo pulses to the sperm tail (Kimura and Yanagimachi, 1995a; Katayose et al., 1999). We expected similar rates of activation (> 90%) as previously reported after ICSI using sperm refrozen without cryoprotectants (Ward and Coffey, 1991; Wakayama et al., 1998; Li et al., 2010); however, oocyte activation with BSnap-thawed (9.1%) was not different than BFrozen-thawed (15.2%) but SSnap-thawed (7.7%) was lower than SFrozen-thawed (34%). Freezing sperm without cryoprotectants damages the plasma membrane and causes loss of the acrosome (Li et al., 2010). Refreezing previously frozen-thawed sperm in the absence of

cryoprotectants caused the desired damage to the sperm plasma membrane (Chapter 2); however, an increased disruption of the perinuclear theca may have also been exerted (Martínez et al., 2006). The PLC ζ is associated with the PT (Fujimoto et al., 2004) and we can speculate that increased damage to the PT occurred following refreezing without cryoprotectants and may have negatively affected the release or activity of PLC ζ , causing the low rates of oocyte activation with the BSnap-thawed and SSnap-thawed sperm observed in the study.

The low rates of oocyte activation in the present study may also be attributed to the difference in activation stimulus observed between species after sperm injection (Catt and Rhodes, 1995). Patterns of Ca²⁺ oscillations are abnormal in cattle (Malcuit et al., 2006b) and horse (Bedford et al., 2004) oocytes following ICSI. Bull or stallion sperm injected into mouse oocytes induce Ca²⁺ oscillations required to stimulate oocyte activation; conversely, if mouse or bull sperm are injected into bovine oocytes and/or mouse or stallion sperm are injected into equine oocytes, they inconsistently or fail to stimulate Ca²⁺ oscillations required for oocyte activation (Bedford et al., 2003; Bedford et al., 2004; Malcuit et al., 2006b). In addition, the events of sperm-oocyte interaction during normal fertilization precede the PT dissolution and release of PLC ζ (Sutovsky et al., 1997). Intracytoplasmic sperm injection bypasses the sperm-oocyte membrane interaction and result in abnormal patterns of Ca²⁺ oscillations (Tesarik et al., 1994; Sutovsky et al., 1996; Nakano et al., 1997; Bedford et al., 2004; Malcuit et al., 2006b). Lack of sperm-oocyte membranes interaction during ICSI also affects the incorporation of the sperm head into the oocyte cytoplasm leading to incomplete or delayed dissolution of the PT (Sutovsky et al., 2003) causing an abnormal or delayed oocyte

activation (Tesarik et al., 1994; Kurokawa and Fissore, 2003) and development (Kurokawa and Fissore, 2003; Sutovsky et al., 2003). These factors may have contributed to the low activation rates reported herein.

The proportion of activated but arrested oocytes was similar between BFrozen-thawed (35.6%) and BSnap-thawed (48.5%) sperm and was higher in SSnap-thawed (40.4%) than SFrozen-thawed (16%). These results suggest that sperm re-frozen without cryoprotectants still have the capacity to stimulate oocyte activation but perhaps some of the components necessary to complete the activation process are lost, become dysfunctional or are inactivated during refreezing. Likewise, it is possible that thawed sperm with a damaged plasma membrane suffered cytoskeletal damage caused by contact with the surrounding medium as does the DNA (Tateno et al., 2000).

The 5% PVP concentration was chosen over the conventional 10% PVP since PVP may interfere with sperm nucleus de-condensation and delay the initiation of Ca^{2+} oscillations (Dozortsev et al., 1995). In addition, decreasing the PVP concentration has been shown to improve pronuclear formation (Wei and Fukui, 2000). In the study herein, an increase in pronuclear formation was not observed. We noted that with the lower PVP concentration lubrication of the injection pipette was insufficient and sperm often adhered to the pipette (more frequently with the snap-frozen sperm). As a result, control of the sperm within the injection pipette was problematic and often times caused the injection of increased amounts of medium during release of the sperm within the oolema.

Bovine and equine oocytes have very dark cytoplasm and compromise the ability to visualize sperm delivery within the oolema. Oocyte centrifugation to polarize the lipids

clarifies the oolema and facilitates visualization, improving delivery of the sperm with minimal amount of fluid within the oolema during injection (Chen and Seidel Jr, 1997; Rho et al., 1998; Wei and Fukui, 2000). Our results may have possibly improved if we had centrifuged the oocytes; however, ICSI is a laborious and time consuming technique and we did not do this step due to time constraints. Only one researcher was performing all the procedures during the injection sessions and adding another step would have increased the time per session outside acceptable parameters. This highlights the importance of developing a team before starting a research or clinical service involving ICSI.

In conclusion, bull and stallion sperm refrozen without cryoprotectants have the ability to activate oocytes after ICSI; however at lower rates when compared to frozen-thawed sperm. This differs from what is observed in mouse (Wakayama et al., 1998; Lacham-Kaplan et al., 2003; Ward et al., 2003) and rabbit (Li et al., 2010) where oocyte activation is similar between fresh and frozen-thawed sperm. This difference may be explained by the species difference in oocyte ability to become activated after ICSI (Catt and Rhodes, 1995). Technical improvements in our ICSI technique may improve the rate of oocyte activation and may reveal the real potential of using re-frozen sperm without cryoprotectants. Investigating the effect of refreezing bull and stallion sperm without cryoprotectants on the sperm cytoskeleton can further help to elucidate the factors responsible for the abnormal patterns of oocyte Ca^{2+} oscillations after ICSI in these species. Refreezing sperm without cryoprotectants is a simple process and an attractive alternative for the efficient use of sperm, either to preserve material from

valuable males or for the conservation of endangered species through embryo IVP using ICSI, and warrants further investigation.

CHAPTER 5

ACTIVATION OF EQUINE OOCYTES AFTER INTRACYTOPLASMIC SPERM INJECTION (ICSI) WITH FROZEN-THAWED STALLION SPERM REFROZEN WITHOUT CRYOPROTECTANTS

Introduction

Semen cryopreservation is a method to exploit and preserve the genetic potential of males. The ability of semen to withstand cryopreservation varies between stallions (Tischner, 1979; Amann and Pickett, 1987; Vidament et al., 1997) and stallions with a post-thaw motility of <30% are commonly excluded from freezing programs. In the horse industry, stallions are commonly selected as potential breeders based on their athletic performance and not for their fertility, semen quality or cryosurvival; therefore, stallions of outstanding performance with good semen cryosurvival ($\geq 30\%$ post-thaw motility) and acceptable pregnancy rates post-insemination ($\geq 50\%$) represent a valuable asset both financially and for genetics preservation. However, the reproductive lifespan of a stallion can be suddenly terminated if the stallion becomes infertile due to injury, disease or death.

Efficient use of cryopreserved semen in these cases, including endangered species, come to be extremely important. Assisted reproductive techniques such as IVF or ICSI use minimal number of sperm to produce an offspring and are reasonable alternatives to AI for the efficient use of cryopreserved semen. Intracytoplasmic sperm injection is the preferred method for embryo IVP in the horse as only two foals have been produced through IVF (Bezard et al., 1989; Palmer et al., 1991). Even though only a few sperm are needed per ICSI session, a considerable amount of frozen-thawed semen is commonly discarded. To avoid this, stallion thawed semen has been refrozen

(McCue et al., 2004; Choi et al., 2006) and re-used in following ICSI sessions (Choi et al., 2006), or only small sections of a frozen straw are cut and thawed for ICSI (Altermatt et al., 2009).

Mare oocytes injected with refrozen motile and refrozen non-motile sperm developed to the blastocyst stage (23% and 13%, respectively) (Choi et al., 2006), suggesting that stallion sperm motility preservation after cryopreservation is not required for ICSI and that refrozen sperm maintain the ability to fertilize (Aba et al., 1997; Wakayama et al., 1998). Supporting this are the reports of a calf (Goto et al., 1990) and bovine embryos (Horiuchi et al., 2002) produced by bull sperm killed (flash frozen-thawed) immediately before ICSI. Likewise, embryos have been produced after injection of lyophilized (immotile) sperm in pigs (Kwon et al., 2004), cattle (Keskinetepe et al., 2002), mice (Kusakabe et al., 2001) and horses (Choi et al., 2011). Moreover, sperm frozen without cryoprotectants have successfully produced offspring in the mouse (Wakayama et al., 1998; Lacham-Kaplan et al., 2003; Ward et al., 2003) and rabbit (Li et al., 2010).

We previously showed that stallion sperm refrozen without cryoprotectants have the ability to activate cross-species oocytes (cow); although at a lower rate when compared to sperm frozen once. Hence, the objective of this study was to assess the ability of frozen-thawed stallion sperm refrozen without the addition of cryoprotectants and stored to activate equine oocytes. We hypothesized that frozen-thawed and refrozen without cryoprotectants stallion sperm will activate mare oocytes; however sperm refrozen without cryoprotectants will activate oocytes at a lower rate than sperm frozen subjected to only one freezing cycle.

Material and Methods

Experimental Design

This experiment was designed to compare the ability of stallion sperm frozen with cryoprotectants (Frozen-thawed) against stallion frozen-thawed sperm refrozen without cryoprotectants (Snap-thawed) to activate *in vitro* matured equine oocytes after ICSI. Cumulus-oocyte complexes were aspirated and/or scrapped from ovaries collected at a local abattoir. After IVM, COCs were stripped from their CC's and oocytes with a polar body were selected for ICSI. The Frozen-thawed (n = 5 replicates and n = 36 total oocytes) and Snap-thawed (n = 5 replicates and n = 36 total oocytes) treatment groups consisted of the injection of a sperm into the oocyte ooplasm. The Sham treatment group (n = 3 replicates and n = 13 total oocytes) consisted of injecting oocytes with a small volume (~5 µL) of a 5% PVP H-SOF and serving as a parthenogenetic activation control for the study. Injected oocytes were cultured for 24 h and the activation status assessed.

Cumulus-Oocytes Complexes (COCs) Collection and IVM

Ovaries were collected from a local abattoir and transported (~ 2.5 h) to the laboratory in a Styrofoam™ box at room temperature (~20 - 25°C). Upon arrival to the laboratory, ovaries were rinsed with PBS at room temperature (22 – 25°C). Ovaries were dried with gauze and the tunica albuginea trimmed. A COC recovery was attempted from each visible follicles by a combination of scraping and aspiration using a 10-mL syringe with an 18-G disposable needle containing 0.5 mL of H-TCM199 with Earle's salts (Sigma, MO, USA) supplemented with 5% (v/v) FCS and 100 µg/mL of streptomycin sulphate (CSL Ltd., Victoria, AUS) and 100 U/mL penicillin G (CSL Ltd.,

Victoria, AUS) (aspiration medium). The ovary was then carefully sliced with a scalpel blade. Follicles observed after slicing the ovary were opened with a scalpel blade and scraped using a 0.5 cm bone curette, the curetted contents were then rinsed into a petri dish with aspiration medium. Cumulus-oocyte complexes were searched and selected using a stereomicroscope (Figure 18) , rinsed three time with sodium bicarbonate-buffered TCM199 supplemented with 10% (v/v) FCS, 1 μ L/mL insulin-transferrin-selenium (ITS; Gibco™; Life Technologies, VIC, AUS), 1 mM sodium pyruvate, 100 mM cysteamine, 0.1 mg/mL FSH (Folltropin; Bioniche Health Science Inc., Quebec, CA) and 2% antibiotic-antimycotic (Anti-Anti; Gibco™, Life Technologies, Victoria, AUS) (Gambini et al., 2012) (maturation medium). Cumulus-oocyte complexes with signs of degeneration were discarded. Twenty five COCs were then transferred into a culture well (Nunc Inc., IL, USA) containing 600 μ L of maturation medium covered with 300 μ L of mineral oil (Sigma, MO, USA). Cumulus-oocyte complexes were matured at 38.6°C in a humidified atmosphere of 5% CO₂ in air. After 24 – 30 h of maturation COCs were denuded by gently pipetting in a 0.2% (w/v) hyaluronidase SOF. Denuded oocytes with an extruded polar body (MII, metaphase II) were selected (Figure 19) and washed three times with H-SOF supplemented with 4 mg/mL BSA (Invitrogen Corporation, Auckland, NZ) and amino acids (arginine 90 μ M, aspartic acid 22 μ M, asparagine 24 μ M, alanine 431 μ M, cysteine 40 μ M, glutamic acid 54 μ M, glutamine 101 μ M, glycine 1390 μ M , histidine 42 μ M, isoleucine 97 μ M, leucine 194 μ M, lysine 194 μ M, methionine 55 μ M, ornithine 19 μ M, phenylalanine 95 μ M, serine 9 μ M, taurine 49 μ M, threonine 9 μ M, tyrosine 89 μ M and valine 264 μ M) at sheep oviductal fluid concentration (Walker et al., 1996) (holding medium) and left in the same medium until transferred for ICSI.

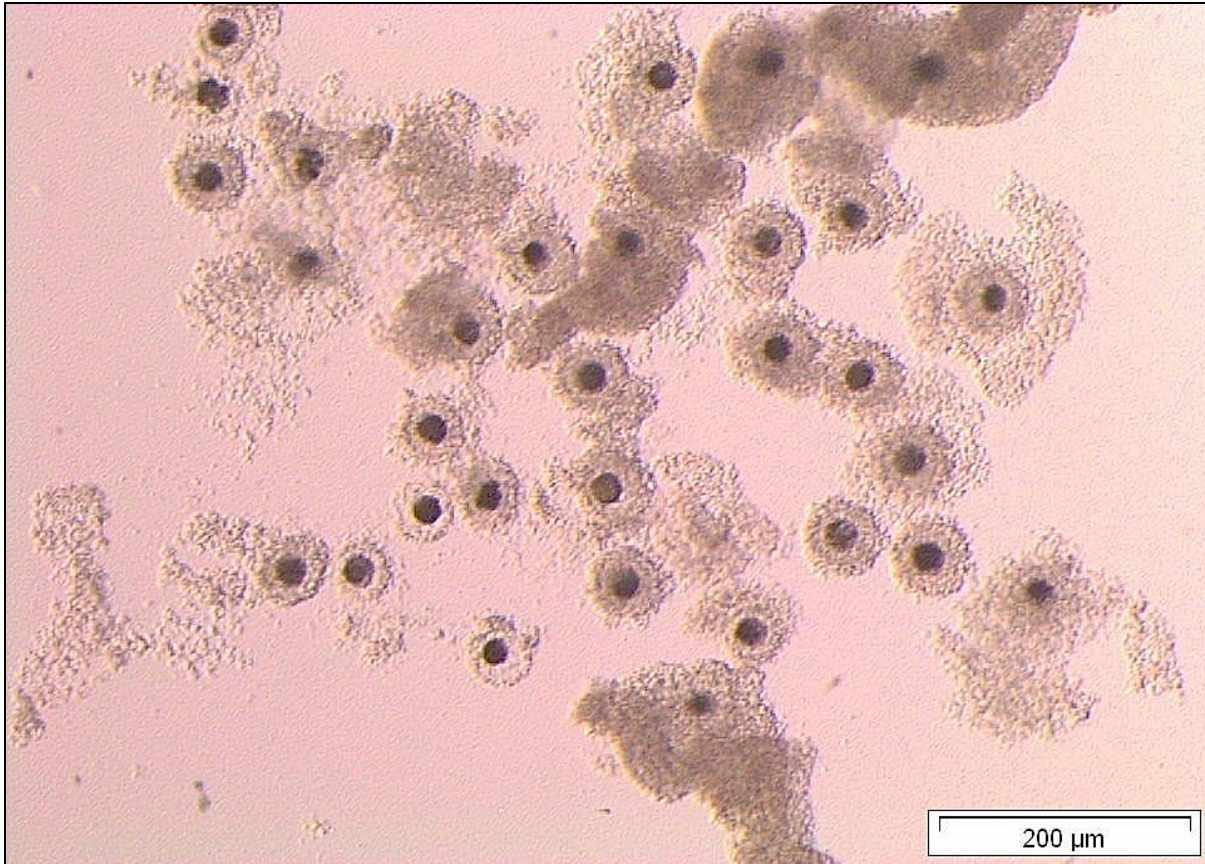


Figure 18: Equine COCs recovered after follicular aspiration/scraping in aspiration medium at room temperature and viewed under an inverted microscope (Olympus SZX7; analySIS getIT imaging software, 40x magnification).

Sperm Preparation

A semen straw (0.5 mL) from a fertile stallion frozen with cryoprotectants (2.5 v/v EY and 2.5% v/v GLY) (Frozen-thawed) was thawed in a 37°C water bath for 30 s. The semen was then subjected to swim up for sperm selection. Two hundred μL of the thawed semen was layered under 600 μL of H-SOF supplemented with 4 mg/mL of BSA and amino acids at sheep oviductal fluid concentration (swim up medium) in a 14-mL round bottom tube. The tube was then incubated at 38.6°C for 20 min. After incubation the top 400 μL were aspirated and placed in a 1.5-mL microcentrifuge tube (Corning, NY, USA). The semen was then washed twice by centrifugation at 300 x g for 3 min,



Figure 19: Denuded equine oocytes in IVM medium after 24 - 30 h of culture. Oocytes with a PB (arrows) were selected for ICSI (Olympus SZX7; analySIS getIT imaging software, 100x magnification).

supernatant removal and the sperm pellet re-suspended in swim up medium up to 500 μ L. A 1- μ L aliquot of the sperm suspension was immediately use for ICSI and the rest aspirated into a 0.5 mL straw and frozen by submerging into liquid nitrogen (Snap-thawed) until used for ICSI.

Intracytoplasmic Sperm Injection (ICSI)

The ICSI was performed as previously described by Kimura and Yanagimachi (1995a) using the Piezo injection system (PrimeTech PMM-150FU; Sutter Instrument, CA, USA). The injection pipette (LRC-OD8TA15; The Pipette Company, Thebarton, AUS) had a 6- μ m inner diameter (I.D.), a 15° angle bend, 1-mm taper from the bent to

the tip and a blunt opening. A small volume of mercury (~ 1 μ L) was placed in the proximal end of the pipette and connected to the oil-filled microinjection pipette holder leaving a small air column (~ 0.5 mm). The mercury was then pushed to the distal end applying forward pressure by filling the microinjection pipette with oil. The holding pipette (LHC-TA20; The Pipette Company, Thebarton, AUS) had a 30- μ m I.D., a 20° angle bend, 1-mm taper from the bent to the tip and a blunt opening. The holding pipette was connected to an oil-filled Narishige microinjector.

Sperm injection was performed on a lid of a 100 mm petri dish (Falcon®; Becton Dickson, VIC, Australia) at room temperature using an inverted microscope (Olympus IX70, VIC, AUS) equipped with Hoffman optics at 200x magnification. Five to ten MII oocytes were transferred into a 40- μ L drop of holding medium. A 1- μ L aliquot of the Sperm-thawed or Snap-thawed sperm suspension was placed into a 5- μ L drop of 5% (w/v) PVP (Sigma, St. Louis, MO, USA) H-SOF under oil. Each MII oocyte was picked up with the holding pipette by applying gentle suction and the oocyte rotated to orient the PB to either the 6 or 12 o'clock position. Motile sperm from the Frozen-thawed treatment group were immobilized by applying a few pulses with the Piezo drill to the sperm tail before loading into the injection pipette. Immotile sperm from the snap-thawed treatment group were similarly treated before injection. Then the injection pipette was placed in contact with the ZP at the 3 o'clock position and the ZP drilled (speed = 5 and intensity = 7) while applying light negative pressure. Following ZP perforation, the injection pipette was retrieved and the piece of ZP expelled into the medium. The injection pipette was then re-inserted through the hole in the ZP and the sperm advanced close to the tip of the pipette. The injection pipette was advanced until

reaching the center of the oocyte and a single Piezo pulse (speed = 3 and intensity = 5) was applied to break the oolema. The sperm with minimum amount of medium was then expelled into the ooplasm and the injection pipette removed while applying light negative pressure. Sham injections were performed in the same manner without sperm injection. After injection oocytes were held at room temperature for 15 – 20 min to allow the broken membranes to heal before transfer for culture.

Injected oocytes were cultured in DMEM/F-12 (Gibco™; Life Technologies, VIC, AUS) supplemented with 10% (v/v) FCS, 1 µL/mL ITS and 1% Anti-Anti (culture medium), at a 10 µL medium per oocyte ratio and covered with mineral oil. Oocytes were cultured at 38.5°C under a 5% CO₂, 5% O₂ and 90% N₂ atmosphere for 20 – 24 h.

Oocyte Activation Assessment

To determine the activation status after 20 - 24 h of culture, oocytes were mounted on a slide with 10 µL of a 9:1 glycerol:PBS solution containing 2.5 µg Hoechst 33258 (Sigma, St. Louis, MO, USA), incubated in the dark for 10 min and examined under a fluorescent microscope. Oocytes in MII, with a visible condensed sperm head within the ooplasm, were considered non-activated. Oocytes in MII with a de-condensing sperm head or one PN were considered activated but arrested. Oocytes with two pronuclei or cleaved with the presence of nuclei in each blastomere were considered activated.

Statistical Analysis

The difference in the proportion of activated, arrested and non-activated oocytes between sperm treatment groups was assessed using Chi-Square analysis (SAS 9.4, SAS Institute Inc., NSW, AUS). Where the value of any parameter was less than ten,

Fisher's exact test was used. Significant difference was considered at a value of $p < 0.05$.

Results

There was an effect of sperm treatment on the activation of oocytes after ICSI ($p < 0.001$). The proportion of activated oocytes in the Frozen-thawed group was higher than the Snap-thawed and Sham treatment groups. The proportion of arrested oocytes in the Snap-thawed treatment group was higher ($p < 0.001$) than the Frozen-thawed group and the Sham treatment group was the lowest. The proportion of non-activated oocytes was higher ($p < 0.001$) in the Sham treatment group compared to the Frozen-thawed and Snap-thawed treatment groups (Table 4).

Table 4: Activation (%) of Equine Oocytes 24 h after ICSI with Stallion Frozen-thawed and Snap-thawed Sperm

<u>Treatment</u>	<u>No. oocytes</u>	<u>Activated</u>	<u>Arrested</u>	<u>Non-activated</u>
Frozen-thawed	36	15 (41.7%) ^a	15 (41.7%) ^a	6 (16.7%) ^a
Snap-thawed	36	0 (0%) ^b	24 (64.9%) ^b	12 (35.1%) ^a
Sham	13	0 (0%) ^b	0 (0%) ^c	13 (100%) ^b

Different superscripts (a,b,c) within columns indicate statistical differences ($p < 0.05$).

Discussion

Results from the experiment did not support the hypothesis that stallion sperm refrozen without the addition of cryoprotectants have the ability to activate equine oocytes following ICSI. This is opposite from what has been observed in the mouse where high oocyte/activation rates (87 – 95%) with normal offspring are produced (Wakayama et al., 1998; Lacham-Kaplan et al., 2003; Ward et al., 2003) or in cattle

were 62.2% of the oocytes became activated after injection of killed sperm (Horiuchi et al., 2002). Oocytes from large domestic animals oocytes (e.g. cattle and horses) are less permissive to activation after ICSI compared to other mammals (e.g. humans and rodents) (Catt and Rhodes, 1995) and this species difference may in part explain the difference between our results and those previously reported for laboratory animals; however it does not explain the difference observed with bull sperm killed immediately before ICSI (Horiuchi et al., 2002).

In our study, sperm were subjected to two freezing cycles (the second without the addition of cryoprotectants), compared to one cycle in the mouse studies. It is possible that refreezing previously frozen-thawed sperm impaired the function or integrity of sperm compartments or the cytoskeleton involved in the oocyte activation signaling pathway. The idea of snap freezing sperm without cryoprotectants had two purposes. First, this process immobilizes the sperm. Second, refreezing damages the PM before ICSI which is a requisite to facilitate sperm nucleus de-condensation (Dozortsev et al., 1995; Palermo et al., 1996b). In addition, removal of the sperm PM and acrosome before ICSI accelerates oocyte activation and improve embryo development in the mouse (Morozumi et al., 2006). In our study, refreezing previously thawed sperm caused damage to the sperm PM (Chapter 3). Additionally, stallion sperm refrozen without cryoprotectants may have lost the acrosomes as is observed with rabbit sperm (Li et al., 2010). However, freezing and thawing bull sperm alters the PT (Martínez et al., 2006), the structure that carries PLC ζ (Fujimoto et al., 2004). It is plausible to think that refreezing previously frozen-thawed sperm may have increased alterations or caused complete loss of the PT and was perhaps responsible for the high

rates of activated but arrested oocytes in the SSnap-thawed group (64.9%). Other possibilities are 1) leakage of PLC ζ following damage of the sperm PM (Bedford et al., 2004), 2) increase PM permeability allowed media-induced damage of the cytoskeleton or other oocyte activation pathways (Tateno et al., 2000).

Interestingly, in a previous experiment (Chapter 4) stallion sperm refrozen without cryoprotectants were able to induce oocyte activation when injected into bovine oocytes. This suggests that even if refreezing increased alterations to the PT and perhaps reduced the amount of PLC ζ , there was still sufficient stallion PLC ζ to activate bovine oocytes. Supporting this idea are previous studies showing that the activity (Cox et al., 2002) and amount of PLC ζ (Ito et al., 2008) required to induce inter-species oocytes activation is different compared to intra-species. While the difference in the amount of PLC ζ required may explain the difference in oocyte activation between experiments, the failure of refrozen stallion sperm to completely activate homologous oocyte is difficult to explain.

The sperm contains all the components required to activate oocytes (Palermo et al., 1992; Kimura and Yanagimachi, 1995a; Arienti et al., 1998; Galli et al., 2003; Choi et al., 2004b) after ICSI, provided the sperm nucleus remains intact (Polge et al., 1949; Kusakabe et al., 2001; Ward et al., 2003). During normal fertilization PLC ζ is released within the oolema after sperm-oocyte membrane interactions, stimulating oscillatory waves of Ca²⁺ (Fissore et al., 1992; Saunders et al., 2002; Ducibella and Fissore, 2008); however sperm-oocyte membrane interactions are skipped during ICSI and abnormal Ca²⁺ oscillations are observed after ICSI in the horse (Bedford et al., 2004) and cattle (Malcuit et al., 2006b). Incomplete or delayed released of the PLC ζ (Sutovsky

et al., 2003) or lack of some downstream signals (Bedford et al., 2004; Malcuit et al., 2006b) may be responsible for abnormal (Tesarik et al., 1994; Kurokawa and Fissore, 2003) or in our case low or complete failure to stimulate oocyte activation.

Oocyte activation in the Frozen-thawed treatment group was 41.7%, which is lower than activation rates after Piezo-driven ICSI in previous studies (50 – 60%) (Choi et al., 2002; Bedford et al., 2004; Choi et al., 2004a; Colleoni et al., 2007). In these studies, *in vivo* matured oocytes or oocytes originating from Expanded (Ex) COCs were used for ICSI and *in vivo* matured COCs have higher activation rates after ICSI compared to *in vitro* matured COCs (Dell'Aquila et al., 1997a; Choi et al., 2004a). We did not select for COC with Ex cumulus morphology, and matured all non-degenerated COCs together (Ex and Cp); therefore MII oocyte originating from either the Ex or Cp COCs were injected and possibly explains the lower oocyte activation rate in the study herein.

Ovaries for the study were collected during the Southern hemisphere summer and early fall (February – April) and time from ovarian collection to COCs placement into IVM was 6 – 8 h. Season does not appear to affect the meiotic competence of recovered COCs (Hinrichs and Schmidt, 2000); however the elapsed post mortem time to COC placement into IVM and time in culture affects the oocyte meiotic and developmental competence (Hinrichs et al., 2005). Compacted COCs storage within the ovary for 5 – 9 hours before culture increased oocyte degeneration; yet the proportion of viable oocytes progressing to the MII stage after 24 h of culture was similar compared to COCs placed in culture immediately. In COCs (Cp and Ex) stored in the ovary before placement into IVM, the culture time (24 to 36 h) did not affect the proportion of oocytes

reaching the MII stage; however when Cp COCs were cultured for only 24 h, oocytes had lower developmental competence compared to oocytes cultured for 36 h (Hinrichs et al., 2005). As mentioned before, we placed all COCs (Cp and Ex) collected into IVM and after 24 h of culture we started denuding COCs. Every oocyte at the MII stage was selected for ICSI; therefore it is possible that oocytes from Cp COCs cultured for only 24 h and with lower developmental competence were injected. This may have caused or have been part of the reason why in the study herein, Frozen-thawed and Snap-frozen sperm, had low ability or fail to activate equine oocytes.

The Snap-thawed sperm tended to stick to the bottom of the dish as well as to the inside of the injection pipette. A 10% PVP solution is commonly used to slow down sperm and lubricate the inside of injection pipette (Kimura and Yanagimachi, 1995b; Choi et al., 2002; Horiuchi et al., 2002; Jacobson et al., 2010). We used a 5% PVP concentration as recommended by Wei and Fukui (2000); however the increased adhesive characteristic of the Snap-thawed sperm and the lower PVP concentration reduced injection pipette lubrication, causing the researcher performing ICSI to have difficulties controlling the movement of the sperm within the injection pipette. Due to this technical difficulty, increased amounts of fluid may have been injected during sperm delivery in some instances, perhaps causing delayed sperm nucleus de-condensation (Dozortsev et al., 1995; Wei and Fukui, 2000) and decreasing oocyte activation rates.

In conclusion, frozen-thawed stallion sperm refrozen without cryoprotectants was unable to completely stimulate oocyte activation after ICSI into mare oocytes. The exact reason for this failure is unknown but increased damage to sperm compartments or the cytoskeleton involved in the cascade of events of oocyte activation may have been

responsible. Freezing or refreezing stallion sperm in the absence of cryoprotectants is an easy procedure that has potential for the efficient use of cryopreserved sperm and/or for cryopreservation of endangered species where animals are at a remote distance and facilities with specialized semen freezing equipment are not readily available. Moreover, as maintenance of sperm integrity (e.g. PM and motility) is not necessary for ICSI, it opens the opportunity of preserving sperm and producing offspring from stallions of genetic value but excluded from freezing programs due to unacceptable post-thaw semen quality.

In addition, investigating the effects of refreezing previously frozen-thawed stallion semen on the sperm ultrastructure to elucidate the possible reason(s) responsible for the failure in oocyte activation observed is warranted. Also, evaluating the capability of fresh stallion sperm receiving 1 freezing cycle without cryoprotectants in stimulating oocyte activation and embryo development may unveil the value of this simple cryopreservation method for stallion semen preservation. Although factors within the sperm may have been responsible for the low oocyte activation rate in this study, other factors such as IVM may have affected the oocyte developmental capacity (Sutton et al., 2003b) and deserve further investigation.

CHAPTER 6

GLUCOSE UTILIZATION BY EQUINE CUMULUS-OOCYTE COMPLEXES (COCs) DURING IN VITRO MATURATION (IVM)

Introduction

Advances on *in vitro* embryo production (IVP) in the horse are hindered compared to other species. Reasons include low number of ovary available for COCs collection, low number of antral follicles per ovaries (Hinrichs et al., 1993; Dell'Aquila et al., 2001), difficulty to recover COCs from antral follicles (Hawley et al., 1995) and difference in meiotic and developmental competence of recovered COCs (Hinrichs and Schmidt, 2000; Dell'Aquila et al., 2003; Choi et al., 2004a).

Oocyte final maturation is a critical stage with profound implications on embryonic developmental competence (Sirard, 2001). The low number of equine COCs available amplifies the importance of providing appropriate IVM conditions to stimulate a high proportion of oocytes progressing to MII with high developmental competence. Energy metabolism of COCs during IVM plays a pivotal role on meiotic resumption, progression to MII and developmental competence (Biggers et al., 1967; Rose-Hellekant et al., 1998; Downs and Hudson, 2000; Sutton et al., 2003b; Herrick et al., 2006a; Thompson, 2006; Krisher et al., 2007). Glucose is the preferred substrate utilized by COCs during IVM and glucose metabolism is stimulated by FSH (Fagbohun and Downs, 1992; Downs et al., 1996; Van Tol et al., 1996); however, the oocyte has limited capacity to metabolize glucose and the majority of the glucose is metabolized by the CCs to produce ATP, pyruvate and lactate which are transported to the oocyte via gap-junctions and then metabolized by the oocyte (Biggers et al., 1967; Downs and Utecht, 1999; Downs and Hudson, 2000; Harris et al., 2007; Thompson et al., 2007). In

addition to glycolysis, glucose metabolism by the PPP pathway plays a fundamental role in the resumption of meiosis (GVBD) through the synthesis of purines (Downs, 1997; Downs et al., 1998). The importance of glucose metabolism through glycolysis and the PPP to produce metabolites for oocyte consumption and regulation of meiosis is supported by the fact that COCs matured in medium that enhance the metabolism of glucose have higher meiotic and developmental competence compared to COCs showing low rates of glucose consumption (Downs et al., 1998; Rose-Hellekant et al., 1998; Krisher and Bavister, 1999; Downs and Hudson, 2000; Sutton-McDowall et al., 2004). Pyruvate and lactate are further metabolized within the oocyte through the TCA cycle (Steeves and Gardner, 1999) to produce more ATP.

Oocyte ATP concentration is correlated with viability and developmental potential of human (Van Blerkom et al., 1995), mouse (Leese et al., 1984) and bovine (Stojkovic et al., 2001; Sutton-McDowall et al., 2012) oocytes. Viable oocytes with high developmental capacity have ATP concentrations of ≥ 2 pmol (Van Blerkom et al., 1995; Stojkovic et al., 2001; Sutton-McDowall et al., 2012). In the horse, only one study has reported that oocyte ATP concentration between younger (< 12 years) and older (> 12 years) mares before IVM (40.5 pmol and 57.6 pmol, respectively) which were not different and did not change after IVM (Hendriks et al., 2015). The concentrations reported were much higher than those reported in humans, mouse and bovine oocytes. An ATP molecule is generated from ADP by oxidative phosphorylation and the ADP:ATP ratio reflects the metabolic activity of a cell. Cells with low ADP:ATP ratio are engaged in oxidative phosphorylation and associated with cell proliferation whereas

cells with high ADP:ATP ratio have low metabolic activity and is associated with apoptosis (Crouch et al., 1993; Bradbury et al., 2000).

In the horse a plethora of research has investigated the effect of supplementing the IVM medium with follicular fluid (Dell'Aquila et al., 1997b; Bogh et al., 2002; Caillaud et al., 2008), growth factors (Carneiro et al., 2001; Lorenzo et al., 2002; Pereira et al., 2013), antioxidants (Deleuze et al., 2010) and hormones (Carneiro et al., 2001; Tremoleda et al., 2003) or co-culture with follicular cells (Tremoleda et al., 2003) during maturation. Also the effect of cumulus morphology, chromatin configuration and follicle size (Hinrichs and Schmidt, 2000) on meiotic competence and development of equine oocytes has been extensively evaluated; however, the effect of all the aforementioned supplements or CCs morphology on the metabolic pathway utilized by equine COCs during IVM and the implications on embryo development has not been investigated. Understanding the effect of different supplements to the IVM medium on COC metabolism has led to the improvement of maturation and developmental competence of bovine oocytes (Sutton-McDowall et al., 2005).

The objectives of this study were to investigate the glucose uptake and metabolic pathway utilized during equine COCs IVM. Also, estimate the viability and developmental competence of equine oocytes by measuring the ATP production and ADP/ATP ratio after IVM. We hypothesized that equine COCs metabolize glucose via glycolysis and that oocytes matured under the IVM conditions of this study will be viable with high developmental capacity.

Material and Methods

Experimental Design

The objective of the study was to assess the metabolic pathway of equine COCs by measuring the glucose uptake and lactate production after 30 h of IVM. In addition, the oocyte ATP concentration and the ADP:ATP ratio was also evaluated. Ovaries were collected from a local abattoir and transported (2.5 h) to the laboratory. COCs were collected by aspiration/scraping and divided into compacted (Cp) or expanded (Ex) COCs. The Cp and Ex COCs were further divided into COCs with only the corona radiata or corona radiata with additional layers of CCs. After IVM COCs were removed from the IVM medium and denuded. The spent medium from each treatment group was recovered and the glucose and lactate content analyzed with a chemical analyzer. The denuded oocytes ADP and ATP concentrations analyzed with a bioluminescence assay.

Cumulus-Oocyte Complexes (COCs) Collection and IVM

Ovaries were collected from a local abattoir and transported to the laboratory in a Styrofoam™ box. Upon arrival to the laboratory, ovaries were rinsed with sterile saline at room temperature (22 – 25°C), dried with gauze and the tunica albuginea trimmed. The COCs collection from each visible follicles was attempted by a combination of scraping and aspiration using a 10 mL syringe with an 18 G disposable needle containing 0.5 mL of Vitro Wash (IVF Vet Solutions, Adelaide, AUS) supplemented with 4% mg/mL of fatty acid-free BSA (FAF BSA; ICPbio Ltd., Auckland, NZ) (wash medium). The ovary was then carefully sliced with a scalpel blade and follicles observed after slicing were opened with the scalpel blade and scraped using a 0.5 cm bone curette. The curette content was rinsed into a petri dish with wash medium. Recovered

COCs were assigned into each treatment group using a stereomicroscope, rinsed three times with sodium bicarbonate-buffered TCM199 supplemented with 10% (v/v) FCS, 1 $\mu\text{L}/\text{mL}$ insulin-transferrin-selenium (ITS; Gibco™; Life Technologies, VIC, AUS), 1 mM sodium pyruvate, 100 mM cysteamine, 0.1 mg/mL FSH (Puregon; Organon, Netherland) and 2% antibiotic-antimycotic (Anti-Anti; Gibco™, Life Technologies, VIC, AUS) (Gambini et al., 2012) (maturation medium). Treatment groups were: COCs with compacted CCs (Cp-CCs), COCs with compacted corona radiata only (Cp-corona), COCs with expanded CCs (Ex-CCs) and COCs with compacted corona radiata only (Ex-corona). Groups of 5 COCs of similar type were matured in 50 μL of maturation medium covered with mineral oil (Sigma, MO, USA) at 38.6°C in a humidified atmosphere of 5% CO_2 in air. After 30 h of maturation COCs were removed from maturation medium and denuded by gently pipetting in a 0.2% (w/v) hyaluronidase of wash medium. Then the denuded oocytes were placed into 1.5 mL microcentrifuge tubes (Eppendorf®, Sigma-Aldrich, Castle Hill, NSW, AUS), plunged into liquid nitrogen and stored at -80°C until analysis. An aliquot (50 μL) of maturation medium before (fresh) and spent maturation medium from each group were placed into 1.5 mL microcentrifuge tubes plunged into liquid nitrogen and stored at -80°C until analysis.

Glucose, Lactate and ADP:ATP ratio

Fresh and spent maturation medium were thawed, then glucose and lactate concentration (mmol/COC) was determined using a chemical analyzer (COBAS Integra 400 plus, Roche Diagnostics Ltd., Switzerland). The difference in glucose and lactate concentration between the fresh and spent medium was considered the glucose uptake and lactate production, respectively. Stored oocytes were thawed and the intra-oocyte

ATP and ADP concentrations as well as the ADP:ATP ratio were determined using a bioluminescent assay (ADP/ATP ratio assay kit, Sigma-Aldrich, MO, USA), based on the measurement of light generated (RLU's: relative light units) by the luciferin-luciferase reaction (Bradbury et al., 2000) (Figure 20). The RLU's detected are proportional to the ATP. The ADP/ATP ratio is calculated after three RLU's measurements. The first represent the ATP concentration (RLU_A), the reaction is allowed to decay for 10 min and a second measurement is taken (RLU_B: residual light), then the ADP enzyme is added to convert ADP into ATP and the third measurement (RLU_C) represents the ADP concentration. A standard curve with ATP concentrations ranging from 0 to 90 pmol/μL was generated for the analysis. The ADP/ATP ratio was calculated by the following formula:

$$\text{ADP/ATP ratio} = (\text{RLU}_C - \text{RLU}_B) / \text{RLU}_A$$

Groups of 5 oocytes from each treatment group were simultaneously analyzed on a single 96-well plate and the RLU's from each well was measured with a luminometer (Glo-Max®, Promega Corp., WI, USA).

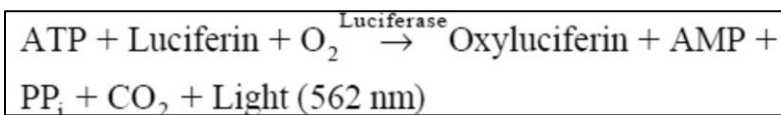


Figure 20: Luciferin-luciferase reaction.

Statistical analysis

The effect of the presence and absence of CCs and COCs type (Cp and Ex) on glucose uptake and lactate production was analyzed by a 2x2 factorial analysis of variance. The effect of the presence and absence of CCs and COCs type (Cp and Ex) on the ADP and ATP concentration, and ADP:ATP ratio of denuded oocytes was also

analyzed by a 2x2 factorial analysis of variance (SAS 9.4, SAS Institute Inc., NSW, AUS). A value of $p < 0.05$ between groups was considered statistically significant.

Results

No interaction between the presence and absence of CCs on COCs type (Cp and Ex) glucose uptake ($p = 0.05$) or lactate production ($p = 0.13$) was observed (Figures 21 and 22). An interaction within COCs type (Cp and Ex) was observed but only in the Cp groups. The Cp-CCs (0.78 ± 0.09 mmol/COC) glucose uptake was higher ($p = 0.02$) than Cp-corona (0.42 ± 0.09 mmol/COC). No interaction within COCs type on lactate production was observed.

No interaction ($p = 0.06$) was observed between the presence and absence of CCs on oocyte ATP concentration (Figure 23). An interaction between the presence and absence of CCs on COCs type (Cp and Ex) was observed. The Cp-corona (0.38 ± 0.08 pmol/COC) ATP concentration was higher ($p = 0.02$) than Cp-CCs (0.08 ± 0.04 pmol/COC) but not different than Ex-corona (0.17 ± 0.05 pmol/COC) and Ex-CCs (0.24 ± 0.04 pmol/COC). Ex-CCs ATP concentration was also higher ($p = 0.04$) than Cp-CCs. No interaction was observed between the presence of CCs and COCs type (Cp and Ex) on ADP concentration ($p = 0.42$) (Figure 24) or ADP:ATP ratio ($p = 0.52$) (Figure 25).

Discussion

In glycolysis, two molecules of lactate are produced per molecule of glucose. The molecules of lactate produced per molecule of glucose consumed by equine COCs in this study was consistent with glycolysis and confirms our hypothesis. To our knowledge, this is the first study reporting the glucose uptake of equine COCs during IVM. Glucose consumption after 30 h of IVM was in the range of 0.28 – 0.93

mmol/COC. Reported glucose consumption in the mouse was 2.3 nmol/COC after 18 h of culture (Downs et al., 1996). Sutton-McDowall et al. (2004), calculated the glucose

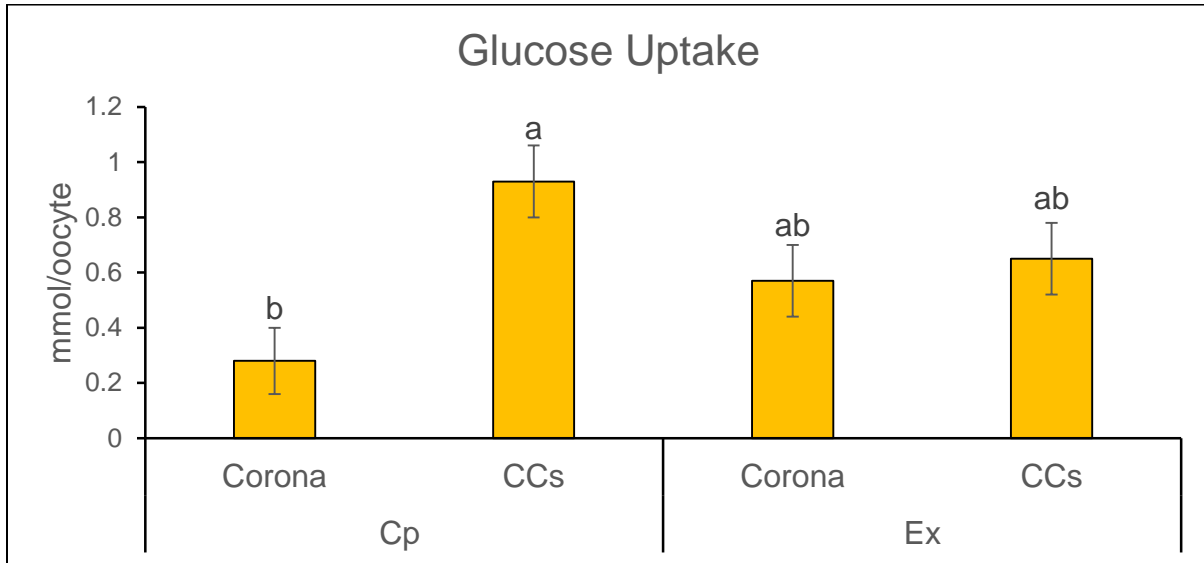


Figure 21: Effect of the presence and absence of CCs (Corona and CCs) and COCs type (Cp and Ex) on Glucose uptake (LSMean ± SEM) by equine COCs after IVM for 30 h.

Different letters (a, b) between bars represent significant difference ($p < 0.05$).

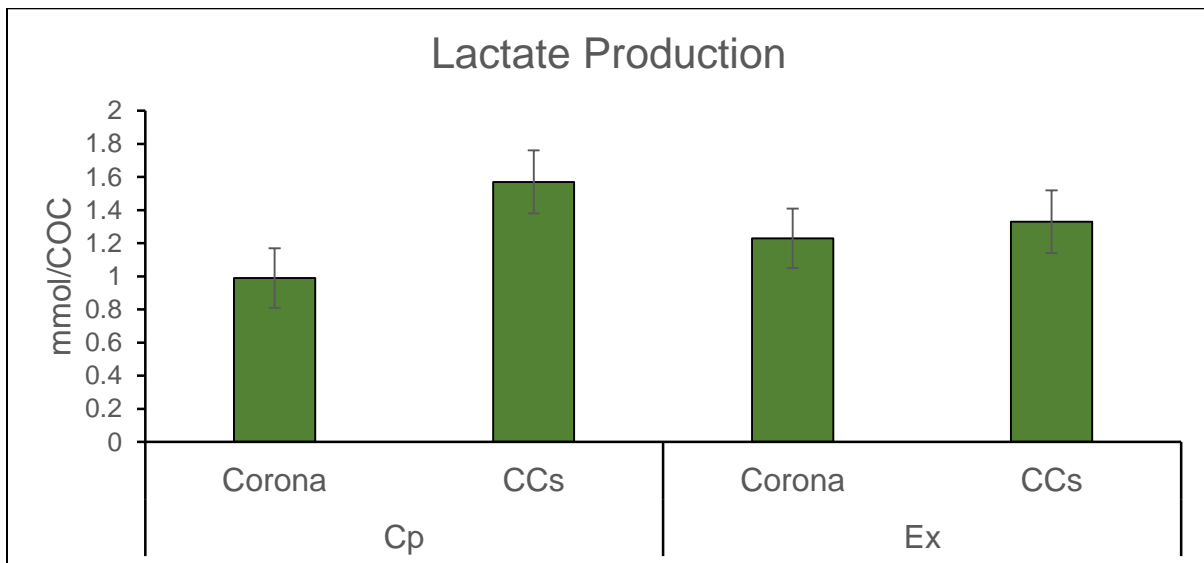


Figure 22: Effect of the presence and absence of CCs (Corona and CCs) and COCs type (Cp and Ex) on Lactate production (LSMean ± SEM) by equine COCs after IVM for 30 h.

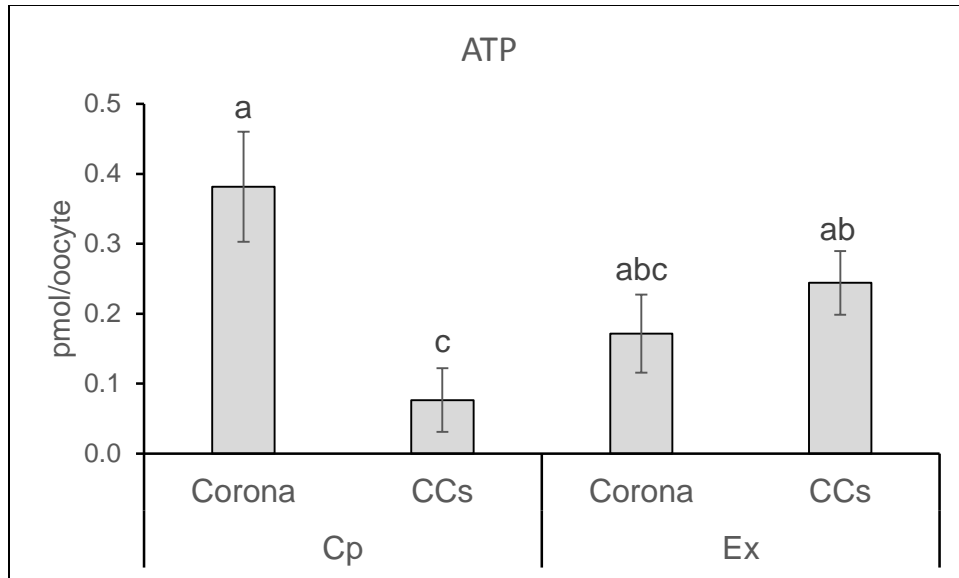


Figure 23: Effect of the presence and absence of CCs (Corona and CCs) and COCs type (Cp and Ex) on the ATP concentration (LSMean \pm SEM) of equine oocytes after IVM for 30 h.

Different letters (a, b, c) between bars represents significant difference ($p < 0.05$).

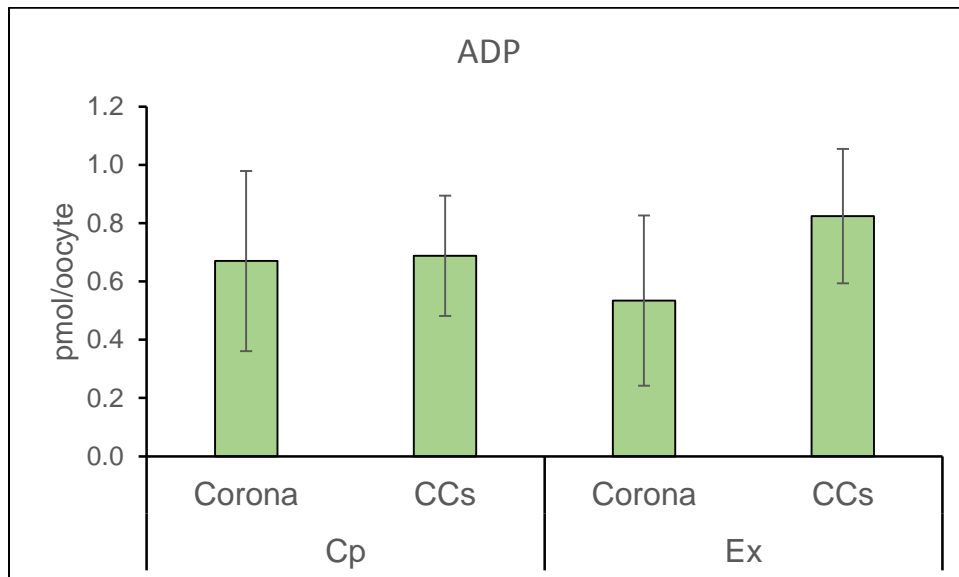


Figure 24: Effect of the presence and absence of CCs (Corona and CCs) and COCs type (Cp and Ex) on the ADP concentration (LSMean \pm SEM) of equine oocytes after IVM for 30 h.

consumption of bovine oocytes to be 0.38 mmol/COC after 24 h of IVM. Our results suggest that equine COCs glucose consumption during IVM is comparable to that of bovine COCs. Glucose consumption between treatment groups was not different

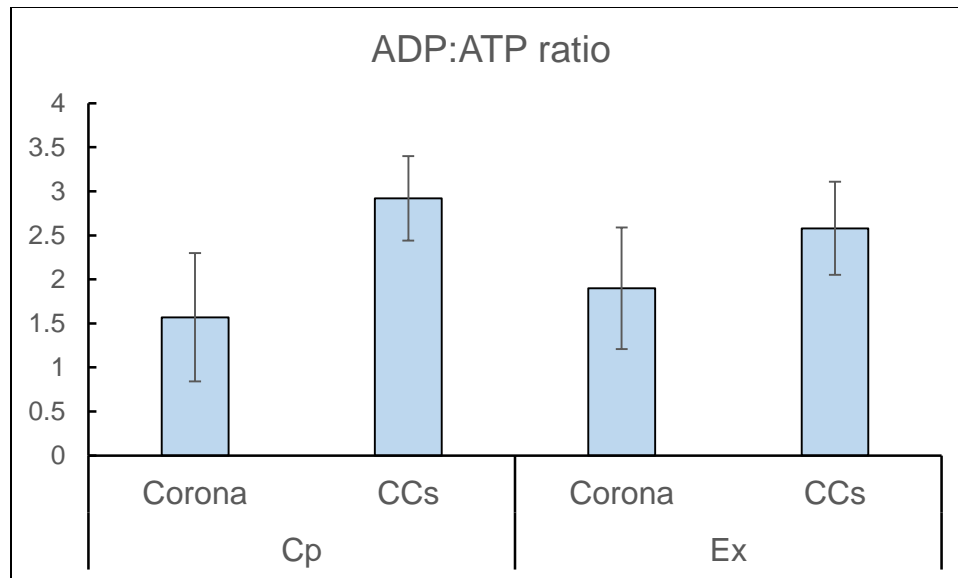


Figure 25: Effect of the presence and absence of CCs (Corona and CCs) and COCs type (Cp and Ex) on the ADP:ATP ratio (LSMean ± SEM) of equine oocytes after IVM for 30 h.

regardless of the presence of additional CCs; however within the Cp treatment groups, Cp-CCs consumed more glucose than Cp-corona. It is possible that having extra layers of CCs allowed for more glucose consumption or because Cp COCs are derived from viable follicles at a juvenile stage (Hinrichs and Williams, 1997), they have increased metabolism thereby increasing glucose consumption. Conversely, as Ex COCs are derived from atretic follicles (Hinrichs and Williams, 1997), they may have less energetic demands; resulting in similar glucose consumption by Ex COCs regardless of the presence or absence of additional CCs.

There were missing values in our data and LSMeans was used to reflect the estimated means from the model. Results from our study reject the hypothesis that oocytes matured under our IVM conditions will be viable and possess high developmental competence. The highest ATP concentration was observed in the Cp-corona (0.38 ± 0.08 pmol/oocyte), followed by Ex-CCs (0.24 ± 0.05 pmol/oocyte), Ex-

corona (0.17 ± 0.06 pmol/oocyte) and the lowest in the Cp-CCs (0.08 ± 0.05 pmol/oocyte). This ATP concentration is lower than the 40.5 – 57.6 pmol/oocyte previously reported in equine (Hendriks et al., 2015); however our findings are more in accordance to what has been reported in humans, murine and cattle (Leese et al., 1984; Van Blerkom et al., 1995; Sutton-McDowall et al., 2012). An ATP of > 2 pmol/oocyte after IVM is associated with viability and developmental competence. Hence, ATP concentrations from our study (0.08 – 0.38 pmol/oocyte) suggest that the IVM conditions were not appropriate and may have compromised oocyte viability and developmental competence.

Results from the ADP:ATP ratio may not completely support the conclusion that oocytes matured under the IVM conditions of the study herein are compromised. A relationship between the metabolic activity and developmental competence exists in bovine oocytes, in which higher proportions of oocytes with an ADP:ATP ratio of ≤ 2 reach the blastocyst stage (Sutton-McDowall et al., 2012). The ADP:ATP ratios in our study were in the range of 1.5 – 2.9 and not different between treatment groups, and oocytes derived from COCs with only the corona radiata had ADP:ATP ratios of < 2 (Cp-corona = 1.57 and Ex-corona = 1.91).

Follicle stimulating hormone is a common IVM medium supplement, as it stimulates glucose metabolism (Downs and Eppig, 1985; Van Tol et al., 1996) and the production of paracrine/autocrine factors regulating and stimulating the resumption of meiosis (Byskov et al., 1997; Downs and Chen, 2008). Follicle stimulates glucose consumption by two-fold during IVM of mouse (Downs and Utecht, 1999) and cattle (Rieger and Loskutoff, 1994; Sutton et al., 2003a) COCs. The IVM media for the horse

is commonly supplemented with FSH; however the effect on COC metabolism is unknown (Dell'Aquila et al., 1996; Choi et al., 2004a; Hinrichs, 2010). A higher proportion of COCs progress to MII (57%) when IVM medium was supplemented with FSH compared to COCs cultured without FSH (41%) (Tremoleda et al., 2003). From our results we may hypothesize that the increase in COCs progressing to MII observed by Tremoleda et al. (2003) was due to increased glucose uptake due to the presence of FSH as previously shown in the mouse (Downs et al., 1988) and cow (Sutton-McDowall et al., 2004).

In conclusion, equine COCs utilize glucose to supply their energetic during IVM. An increased number of CCs may have a beneficial effect on glucose uptake by Cp COCs. According to the oocyte ATP concentrations (< 2 pmol) additional CCs enhanced glucose metabolism but do not stimulate ATP production by the TCA cycle (Sutton-McDowall et al., 2012). The oocyte ATP concentrations after IVM suggest that the conditions used in this study may compromise oocyte viability and developmental competence. Although oocyte ATP concentrations were low, the ADP:ATP ratios of COCs with only corona (< 2) and CCs (> 2 but < 3) suggest these oocytes have high or slightly compromised developmental competence. COCs with only the corona radiata do not have an increased uptake of glucose but at the same time the glycolytic flow was not altered as they still produced two molecules of lactate. It is interesting that their ATP production was higher (Cp-corona) to similar (Ex-corona) and the ADP:ATP ratio was < 2, two values associated with cell viability and increase developmental competence. It is tempting to suggest we should select COCs with only the corona radiata as they are viable and with high developmental competence following IVM; however none of the

matured oocytes were fertilized and cultured to assess development to support this notion. This is the first study evaluating equine COCs metabolism during IVM and more information is needed to evaluate if our current IVM systems are appropriate or require further improvements.

CONCLUSIONS

The main objective of these experiments was to demonstrate the ability of bull and stallion frozen-thawed sperm refrozen without the addition of cryoprotectants to activate intra- and interspecies oocytes following intracytoplasmic sperm injection (ICSI). Demonstrating the ability of sperm refrozen without the addition of cryoprotectants to activate oocytes will allow the efficient use and propagation of genetics from valuable males during their life and beyond. It will also be the platform for the development of fast and simple semen cryopreservation methods to preserve sperm from valuable males.

We demonstrated that bull and stallion frozen-thawed sperm refrozen without the addition of cryoprotectants have the ability to activate bovine oocytes following intracytoplasmic sperm injection (ICSI); although at a lower rate when compared to frozen-thawed sperm. Conversely, stallion frozen-thawed sperm refrozen without the addition of cryoprotectants was unable to activate equine oocytes following ICSI.

Refreezing sperm without the addition of cryoprotectants damaged the sperm plasma membrane (PM) which is desirable before ICSI. Damage to the sperm PM can be attributed to lipid bilayer separation, intracellular ice formation, osmotic stress, or by the combination of these processes as a result of supraoptimal cooling and/or warming rates. Conversely, no DNA damage was detected, which is also desirable for normal fertilization and embryo development. However, the validity of this result may be questioned as it has been suggested that the sperm chromatin dispersion test lacks statistical strength. Nevertheless; the fact that no DNA damage was observed after refreezing without the addition of cryoprotectants can be attributed to the high

protamines-bound DNA ($\geq 85\%$) of bull and stallion sperm which makes the chromatin less susceptible to damage.

Although refreezing caused some desirable effects (e.g. sperm plasma membrane damage), it may also have damaged the sperm cytoskeleton, disrupting the release and/or amount of PLC ζ to stimulate oocyte activation, or by inactivating sperm signaling pathways involved in the oocyte activation process. The increased damage to the sperm caused by refreezing may explain the difference in oocyte activation between frozen-thawed and refrozen sperm. Moreover, the use of 5% over 10% PVP for sperm suspension during ICSI caused technical difficulties, and less than ideal lubrication of the injection pipette for optimal control of sperm movement was attained. This problem sometimes caused the sperm within the injection pipette to become attached, and was often experienced with the refrozen sperm. The lack of appropriate lubrication of the injection pipette caused inconsistent delivery of minimal amount of fluid during sperm injection which disrupts oocyte activation and contributed to the lower oocyte activation rates.

The reason for the inability of refrozen stallion sperm to activate equine oocytes is difficult to explain. We previously discussed the reasons explaining the difference in oocyte activation rates between frozen-thawed and refrozen sperm, reasons that can also be responsible for the inability of refrozen stallion sperm to activate equine oocytes. In addition, the difference in stimulus required by oocytes from different species to become activated may add to the problem, and may explain why refrozen stallion sperm was able to activate bovine oocytes but unable to activate equine oocytes.

Cumulus-oocyte complex glucose metabolism during final maturation have a profound impact in oocyte developmental capacity; therefore we investigated if equine COCs metabolism under the conditions of our study would explain the activation failure of equine oocytes injected with refrozen stallion sperm. Equine COCs utilized and metabolized glucose through glycolysis during *in vitro* maturation. Studies have shown that equine COCs with multiple layers of cumulus cells have higher meiotic and developmental competence than COCs with only the corona radiata. In our study, the presence of extra layers of cumulus cells (CCs) appears to be beneficial to the compacted (Cp) equine COCs glucose consumption; however this increased consumption did not stimulate more ATP production by the oocyte, in fact the highest ATP production was observed in the oocytes from Cp equine COCs with only the corona radiata. This is the first study of equine COCs metabolism during *in vitro* maturation and no clear conclusions regarding its impact on equine oocyte activation/development can be made. Clearly, a considerable amount of information in equine COCs metabolism is needed before conclusions of its impact on oocyte activation/development, or sound recommendations to improve equine COCs *in vitro* maturation systems may be postulated.

The results from these studies are the platform for the development of protocols intended for the efficient use of semen from males of valuable genetics and endangered species. The process of freezing or refreezing sperm without the addition of cryoprotectants is very fast and no specialized freezing equipment is required. It also eliminates international restrictions imposed on semen frozen with animal-derived cryoprotectants. Freezing fresh bull and stallion sperm without cryoprotectants was not

investigated in these studies; however further investigation is warranted as the results may reveal the real potential of this freezing protocol for the production of embryos and offspring in these species. Developing an *in vitro* embryo production protocol for cattle and horses with sperm frozen without cryoprotectants and ICSI will allow the preservation of sperm from males that are not commonly considered for semen freezing programs due to their poor tolerance to conventional freezing protocols. Moreover, preservation of sperm from every male of valuable genetics will enrich the genetic variety within species.

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VITA

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