Sperm Decondensation and Male Pronuclear Formation in Bovine Intracytoplasmic Sperm Injection

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SPERM DECONDENSATION AND MALE PRONUCLEAR FORMATION IN BOVINE INTRACYTOPLASMIC SPERM INJECTION

A Thesis

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

The School of Animal Sciences

by

Lauren Nicole Gatenby
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LIST OF ABBREVIATIONS

1PN  1 pronucleus
2PN  2 pronuclei
BSA  Bovine serum albumin
DAG  1,2-diacylglycerol
DMAP 6- dimethylaminopurine
DTT  Dithiothreitol
ER  Endoplasmic reticulum
FITC-PNA  Fluorescein isothiocyanate-conjugated peanut agglutinin
HEP  Heparin
ICSI  Intracytoplasmic sperm injection
IP3  Inositol-triphosphate
IVF  In vitro fertilization
LPC  Lysophosphatidylcholine
MPF  Maturation promoting facto
P4  Progesterone
PBS  Phosphate Buffered Saline
PIP2  Phosphatidyl 4,5-bisphosphate
PLCz  Phospholipase C zeta
PVP  Polyvinylpyrrolidone
SOF  Synthetic Oviductal Fluid
ABSTRACT

This study assessed the effects both DTT and progesterone have on bovine spermatozoa to induce decondensation or the acrosome reaction in order to facilitate male pronuclear formation after ICSI. Sperm prepared by swim-up in a 5 mM concentration of DTT displayed time dependent morphological changes resulting in decondensation over a 6-hour period. An estimated 90% of treated sperm displayed early changes in morphology after the second hour of incubation. Sperm displaying partial decondensation of the nucleus was estimated as 42% by the 3rd hour and increased to 62% by the 5th hour of incubation. Fully decondensed sperm or sperm with completely dispersed DNA increased from 20% at hour 5 to 50% at hour 7. No such changes were seen in sperm not treated with DTT (p < 0.01). Progesterone was used to induce an acrosome reaction in sperm which were first capacitated with heparin (10 µg/mL) prior to treatment with progesterone (10 µM). The acrosome reacting population was measured using a conjugated FITC-PNA stain and observations using fluorescent microscopy and cytometric flow analysis. Sperm undergoing an acrosome reaction at hour 1 was 80.2% and increased to 89.3% after 2 hours. Sperm that completed the acrosome reaction increased from 49.8% at hour 1 and 62.5% at hour 2. Only 14.2% completed acrosome reactions in sperm not treated with progesterone (p < 0.05). Using these observations with DTT and progesterone treated sperm, ICSI was performed with both treatments to assess if rates of male pronuclei formation and successful fertilization could be improved. Injection of sperm treated with DTT resulted in 28.3% of embryos with 2 pronuclei (2PN) after activation and 16 hours of culture, compared to 6% in control injections (p < 0.001). ICSI with progesterone treated sperm also resulted
in a higher number of 2PN embryos, with 38.1% compared to 10.1% in control injections (p < 0.001). These results show that both DTT and progesterone have effects on sperm which allow for higher rates of male pronuclear formation after ICSI by utilizing either the physical approach of DTT or the sperm’s physiological response to progesterone.
Intracytoplasmic sperm injection (ICSI) has been a valuable tool in many species for its ability to not only overcome male factor infertility problems, but also to eliminate the risk of polyspermy commonly seen with in vitro fertilization (IVF), and can assist in implementing other technologies (Catt et al., 1995). For species with limited gamete availability ICSI has the potential of bypassing the need to optimize IVF conditions. However, bovine ICSI lacks the success rates that has been observed in other mammals (Águila et al., 2017). For example, in humans (Tesarik, 1998) and mice (Kimura et al., 1995) the injection of a spermatozoon directly into the cytoplasm of the oocyte is sufficient for activation and to initiate decondensation and formation of the sperm’s nucleus into the male pronucleus to join with the female genome to complete a diploid embryo. This is not the case for bovine ICSI, where injection of a spermatozoon is not sufficient for activation or sperm decondensation (Ock et al., 2003). While the exact reason has yet to be elucidated, it is suspected to be a combination of both oocyte and sperm factors. It has been shown that in vitro matured bovine oocytes may be deficient in glutathione, an essential antioxidant which plays a major role in sperm decondensation, giving the oocytes a reduced capacity to form a male pronucleus (Curnow et al., 2010). This is coupled with bovine sperm's resistance to decondensation, as there is a greater stability of the sperm nucleus due to a high density of protamine disulfide bonds and tightly packed nuclear structure compared to other species (Hutchison et al., 2017). Despite these challenges, ICSI has the potential to be a valuable tool in cattle reproduction. ICSI provides some advantages over IVF in
using genetic material more conservatively, as well as being able to improve male factor infertility. However, a more valuable application is perhaps in implementing genetic editing, CRISPR/Cas9, more efficiently and studying aspects of fertilization. Improving on this technology in cattle may also provide insight into improving ICSI for other species, including humans, and shed knowledge on fundamental events during fertilization.
CHAPTER II.
LITERATURE REVIEW

2.1. Intracytoplasmic Sperm Injection

The process of successfully combining mammalian oocytes and sperm \textit{in vitro} to create a viable embryo first began with rabbits in the 1950’s, which initiated a decades long cascade in reproductive studies (Chang et al. 1959). As the techniques of in vitro fertilization (IVF) became more efficient, this allowed for further application in a number of species, including bovine, to increase propagation of valuable genetics, assist with infertility, and implement other genetic technologies. (Ferre et al., 2019). However, IVF is not ideal for species prone to polyspermic fertilization, such as porcine, or for individuals with low sperm count or motility (Nakai et al., 2014; Simopoulou et al., 2016). In such cases, intracytoplasmic sperm injection (ICSI) has been a valuable tool for its ability to not only overcome male factor infertility, but also to eliminate the risk of polyspermy, or multiple sperm fertilizing a single oocyte (Catt et al., 1995; Salamone et al., 2017). ICSI could also be useful for species where optimization of IVF may not be practical because of scarcity of gametes.

ICSI is the process by which a single spermatozoon is isolated and immobilized, by a kinking of the tail, before injecting it directly into a mature oocyte with the polar body centered along the longitudinal axis to avoid disrupting crucial structures, such as the spindles and metaphase plate (Simopoulou et al., 2016; Rader et al., 2016). The kinking of the tail at the midpiece of the sperm before injection serves not only to immobilize the sperm and prevent movement after injection, but it also serves to initially
break the spermatozoa’s outer membranes (Simopoulou et al., 2016). The breaking of these membranes before injection has been shown to allow for greater frequency of fertilization after ICSI by allowing maternal access to the spermatozoa and the release of sperm factors for oocyte activation (Salamone et al., 2017). However, in bovine ICSI the kinking or disrupting of the midpiece or tail alone is not sufficient as it is in other species for fertilization or oocyte activation (Horiuchi and Numabe 1999). Compared to traditional IVF, where millions of sperm are needed for fertilization, ICSI is a more conservative approach in utilizing genetics, which is ideal if the semen is costly or is a limited resource, as is the case in male factor infertility as well as with sexed or genetically altered sperm, or in dead or endangered animals (Salamone et al., 2017). Since only one spermatozoon per injected oocyte is needed, each unit of frozen semen can be sectioned into multiple ‘ICSI-cuts’. Up to ten ‘ICSI-cuts’ can be obtained from a single straw of frozen semen that can be thawed separately for its use in separate ICSI procedures (Rader et al., 2016). Additionally, some authors maximize the use of valuable semen not only by using this strategy, but also by diluting and refreezing the semen into smaller quantities, for example, when a frozen semen store is limited or when expensive sex-sorted sperm straws are employed (Hamano et al., 1999; Rader et al., 2016; Canel et al., 2017). Semen from deceased animals can also be collected in emergency situations, by collecting immature sperm from the testicles or epididymis post-mortem (Salamone et al., 2017). As these sperm are not yet mature and have reduced motility, ICSI is a crucial methodology for utilizing and fertilizing with these sperm. This technique is also applicable in the case of azoospermia, or the condition in which no sperm are present in an ejaculate (Zheng et al., 2015). Sperm can be
harvested from the testicles to treat severe male infertility and used to fertilize an oocyte via ICSI (Zheng et al., 2015). In many species, including the rabbit (Hosoi and Iritani 1993), mouse (Kimura & Yanagimachi 1995), sheep (Catt et al., 1996), horse (Cochran et al., 1998), domestic cats (Pope et al., 1998), pigs (Kolbe and Holtz 2000), goats (Wang et al., 2003) and humans, ICSI is successful and has resulted in births of live offspring (Horiuchi and Numabe 1999; Salamone et al., 2017). However, bovine ICSI lacks the success rates that has been observed in other mammals (Goto et al., 1990; Águila et al., 2017). Interestingly, conventional bovine IVF, can achieve high success rates, leading to cleavage rates over 80%, blastocyst rates of over 40%, and pregnancy rates of transferred *in vitro* produced embryos of approximately 50%, depending on methodology (Ferre et al., 2019; Sanches et al., 2017). Bovine ICSI utilizing the same general procedures as with other species, generally results in blastocyst rates of less than 10% (Goto et al., 1990; Chung et al., 2000; Devito et al., 2010; Arias et al., 2014).

The great improvements in bovine IVF, and the inferior results observed with bovine ICSI emphasize the importance of re-examining the possible causes of these differences and highlights the need to study early fertilization events (Salomone et al., 2017). For reasons not completely known, the injection of a bovine spermatozoa into a fully matured bovine oocyte does not commonly result in fertilization or activation of the oocyte as it does in some other species. The lack of oocyte activation after sperm injection confirms incomplete fertilization after bovine ICSI, in contrast to humans (Tesarik, 1998) and mice (Kimura et al., 1995) where the injection of a spermatozoon directly into the cytoplasm of the oocyte is sufficient for activation and to initiate decondensation and formation of the male pronucleus which joins with the female.
genome to form a diploid embryo. Thus, after bovine ICSI, activation protocols must also be used for pronuclei formation and cleavage to occur to complete fertilization, despite this process readily occurring after injection in other species. The addition of activation protocols in bovine ICSI further complicates this technology with many studies claiming vast differences between protocols and reagents with results varying between labs and studies (Ock et al., 2003; Salamone et al., 2017; Suttner et al., 2000). Despite great efforts from numerous researchers, it is not known why bovine ICSI remains insufficient while IVF is successful (Salamone, 2017; Ock et al., 2003). As most processes occurring during fertilization and early embryogenesis are largely ubiquitous among most domestic animals, it is suspected that a key process lacking in bovine ICSI may shed light in improving other IVF/ICSI protocols. While IVF, in bovine, currently has the most commercial potential with high success rates, lower cost, and limited training requirements, increasing the efficiency of bovine ICSI could improve the implementation of genomic editing technologies (Ma et al., 2017). Currently, the most frequently used method to induce genomic editing in bovine oocytes, the CRISPER/cas9 system, is to first preform IVF and then subject the zygotes to microinjection at the pronuclear stage (Ma et al., 2017). However, this can result in mosaicism, or incomplete integration of these constructs into the embryos, and increases the overall handling and stress on the embryo (Ma et al., 2017). It has been shown, in humans, that earlier introduction of these gene editing constructs, at fertilization with ICSI, reduces the frequency of mosaicism and allows for better integration and higher efficiency, as well as reduces the total manipulations and handling of the embryos (Ma et al., 2017). While ICSI may not have an immediate commercial application for cattle, as it does in humans and horses,
the technology has potential value in exploring the mechanisms of fertilization, as well as for certain individuals where the sperm is a limited resource, and for implementation of genomic editing technologies.

2.2. Bovine ICSI Pitfalls and Limitations

Technical Issues

In addition to physiological constraints with bovine ICSI, there are also physical obstacles which make ICSI more difficult in bovine than in other species. Due to the high lipid content and presence of vacuoles, bovine oocytes appear much darker compared to that of mice or human. As a result, the actual injection process in bovine ICSI is blind, or obscured from view, making confirmation of sperm injection extremely difficult (Horiuchi and Numabe, 1999). While centrifugation can be used to localize these lipids and vacuoles to the periphery of the oocyte to allow for a clear injection field, centrifugation of oocytes has been shown to prematurely activate oocytes and cause extrusion of the second polar body prior to sperm injection in humans (Tatham et al., 1996). Another pitfall to this technique is that bovine spermatozoa are generally larger than that of other mammals and requires a larger injection pipette of 8-10 microns versus 4-5 microns typically used in human or mouse ICSI (Salisbury et al., 1978). The increase in pore size brought about by a larger injection pipette causes more damage that the oocyte must recover from (Horiuchi and Numabe 1999). Additionally, bovine oocytes have a highly elastic plasma membrane which requires forceful and vigorous aspiration of the cytoplasm to fully puncture (Horiuchi and Numabe 1999). With the difficulty in puncturing the oocyte, combined with the obstacle of visualizing the injection
within the oocyte itself, and the lack of success after ICSI, training of personnel for ICSI in bovine oocytes is troublesome, despite bovine oocytes being readily available.

Physiological Constraints

While the exact reasons for the lack of success in bovine ICSI has yet to be elucidated, it is suspected to be a combination of both oocyte and sperm factors. It has been shown that in vitro matured bovine oocytes may be deficient in glutathione, an essential antioxidant which plays a major role in sperm decondensation. This gives the in vitro matured oocytes a reduced capacity to form a male pronucleus (Curnow et al., 2010). For most species where ICSI is highly successful, the oocytes are first matured in vivo and then collected, fertilized by ICSI, and cultured in vitro. However, due to the difficulty of obtaining in vivo matured bovine oocytes, virtually all oocytes used for bovine ICSI and IVF are in vitro matured. Incomplete cytoplasmic maturation for in vitro matured oocytes compared to the in vivo matured oocyte could result in reduced developmental potential of the oocyte before fertilization occurs (Gomez et al., 2000). The overall glutathione deficiency in in vitro matured bovine oocytes reflect incomplete cytoplasmic maturation and is one difference between ICSI with bovine oocytes versus that of human or mouse. This may partially explain the bovine oocytes decreased ability to decondense the sperm head, as it is unable to compensate with the increased antioxidant demand with ICSI. During IVF, the sperm’s membranes are disrupted and weakened in the presence of cumulus cells and contact with the zona pellucida before integration into the oocyte. However, this does not occur during ICSI, and thus places a greater burden on the oocyte to reduce and decondense the sperm membranes. It has been observed that the injection of a bovine spermatozoa into an equine oocyte results
in the decondensation of the sperm head and formation of the male pronucleus (Li et al., 2003). These details implicate an oocyte factor in the limited success rates of bovine ICSI. However, this is also coupled with bovine sperm’s extreme resistance to decondensation. In bovine spermatozoa, there is a greater stability of the plasma membranes due to a high density of protamine disulfide bonds present, which are also accompanied by a more tightly compacted nuclear structure compared to other species (Hutchison et al., 2017). This prevents male pronuclear formation and activation of the oocyte post-ICSI. For male pronuclear formation to occur, the sperm must undergo a necessary change which includes the removal of sperm-specific protamines from the sperm’s nucleus. These protamines, held together by disulfide bonds, are responsible for maintaining the condensed inert state of the paternal genome. Upon removal, this process is followed by decondensation of the paternal chromatin and leads to an essential replacement of the paternal protamines for maternal histones, which then remodel and reassemble the chromatin into a male pronucleus (McLay and Clark, 2003). The protamine-disulfide bonds which hold the protamines intact are normally broken, or reduced, by glutathione within the oocyte during fertilization, following permeabilization from the vesiculation of the acrosome reaction. This would normally allow for nuclear decondensation and subsequent development. However, the persistence of the acrosome or its’ substructures over the anterior part of the injected sperm inhibits the exchange with the maternal nuclear proteins during sperm decondensation and causes insufficient sperm head decondensation (Sutovsky et al., 1997; Galli et al., 2003). As ICSI bypasses these critical processes with injection of the sperm directly into the cytoplasm of the oocyte, combined with a deficiency in
glutathione in *in vitro* matured bovine oocytes, additional mechanisms are necessary for successful fertilization, activation, and pronuclei formation to occur with ICSI in cattle.

While calves have been born using this technology, it still remains a highly inefficient and inconsistent technology (Solermo et al., 2017). The most common measure taken to assist bovine ICSI is pre-treating the sperm, prior to ICSI, to assist in decondensation. However, the exact physiological sequential processes the sperm requires before and during fertilization are not entirely known. If these processes could be replicated or simulated *in vitro*, then bovine fertilization and blastocyst rates by ICSI could potentially mirror those seen with traditional IVF. However, currently even with a pretreatment of the sperm, which could allow for decondensation, additional measures are still required to activate bovine oocytes for fertilization, the resumption of meiosis, and further development to occur (Salamone et al., 2017). With IVF or other methods where fertilization occurs, as the sperm fuses with the oocyte, sperm factor phospholipase C zeta (PLCz) is released and begins the process of oocyte activation. PLCz catalyzes the hydrolysis of phosphatidyl 4,5-bisphosphate (PIP$_2$), producing inositol-triphosphate (IP$_3$) and 1,2-diacylglycerol (DAG). The increase in IP$_3$ concentration is responsible for inducing calcium release from the endoplasmic reticulum (ER), the calcium store of the cell, and cause a cyclical rise in calcium which signals cyclin B and the proteasomes to decrease protein synthesis of maturation promoting factor (MPF). The decline of MPF in response to calcium oscillations results in a continuation of the cell cycle and allows for further development. In bovine oocytes, the sperm decondensing in response to treatment after injection, or the ICSI procedure alone, is not sufficient to activate the oocytes as it is in other species. In humans and
mice for example, a sham injection alone or simple insertion of an injection pipette into the oocyte can result in rise in calcium leading to activation and subsequent cleavage even without the presence of sperm (Dozortsev et al., 1995). In porcine ICSI, a sham injection is not as successful for oocyte activation as in humans, but the injection of a sperm is sufficient for both fertilization and activation, as it is in humans, mice, and horses as well (Catt et al., 1995). It is not clear why this is not the case in bovine oocytes, but various chemical methods for activation have been found to be successful after ICSI to result in a viable embryo. These methods using various concentrations, combinations, and exposure times to chemicals such as calcium ionophores like ionomycin, protein inhibitors such as cycloheximide or 6-dimethylaminopurine (DMAP), as well as ethanol, mimic the initial activation of the oocyte through calcium signaling and a time sensitive down regulation of protein synthesis. These 2 signals in succession promote progression of the oocyte though the cell cycle from metaphase II to anaphase and the first cell cleavage (Ock et al., 2003). Currently, fertilization of bovine oocytes via ICSI is dependent on such activation protocols. Most fertilization that occurs with bovine oocytes though natural conception or conventional IVF immediately results in oocyte activation and further development without additional treatment, as does ICSI in most other species. Yet in cattle, a crucial event of fertilization is missing during ICSI to result in complete fertilization. Furthermore, subjecting ICSI oocytes to these activation protocols also increases the occurrence of parthenogenic activation, or activation of only the maternal genome without incorporation of the male genome, as well as DNA fragmentation (Szczygiel and Ward 2002). These parthenogenetically activated oocytes can develop beyond the 2-cell stage and potentially up to the blastocyst stage, yet these
embryos lack the diploid nature of a viable embryo and only contain one set of chromosomes, making these embryos haploid and nonviable. Despite having a sperm injected, if the sperm does not decondense these embryos are not fertilized, yet morphologically resemble embryos that are successfully fertilized. The presence of parthenotes among true fertilized embryos may misconstrue true fertilization, cleavage, and blastocyst rates in studies and needs to be accounted for when examining embryo development after ICSI with activation to ensure the diploid nature of a viable successfully fertilized embryo. Moreover, the use of these activation protocols has been shown to produce high numbers of ICSI and parthenote embryos with chromosomal abnormalities (De La Fuente and King, 1998; Rho et al., 1998; Ross et al., 2008; Canel et al., 2010), explaining in part the low blastocyst rates and frequent pregnancy loss of transferred embryos after ICSI. Some argue that with proper treatment of the sperm prior to ICSI, oocyte activation should not be needed due to a more complete fertilization event occurring, similar to that in IVF (Salamone et al., 2017). However, without knowledge of the missing factor in bovine ICSI, treatments will continue to be explored to attempt in realizing the potential of this technology.

There has been a multitude of studies attempting to mimic the effects of capacitation, the acrosome reaction, and fertilization in order to improve bovine ICSI yet success is still limited, and results remain controversial (Salamone et al., 2017). Studies have been done using various sperm pretreatments, such as heparin with lysophosphatidylcholine to induce capacitation and the acrosome reaction (Gómez-Martínez et al., 2019), glutathione to mimic the oocytes natural reducing capacity (Lee 2015, Canel et al., 2017), various calcium ionophores to signal fertilization events within
the sperm (Jamil et al., 1981), and a multitude of other treatments in order to increase fertilization rates of bovine ICSI with limited success. One of the most successful methodologies initially discovered was the pretreatment of sperm with dithiothreitol (DTT), which was reported to lead to an increase in diploid blastocyst rates (Rho et al., 1998; Galli et al., 2003; Oikawa et al., 2016). However, DTT and other oxidizing and reducing compounds may increase the instances of DNA damage or fragmentation leading to poly- or mixoploidy embryos, or embryos with chromosomal abnormalities (Rho et al., 1998; Suttirojpattana et al., 2016). This, and the lack of any reported live births of DTT treated ICSI, highlights an importance to pursue treatments options which resemble physiological events to follow natural endogenous pathways for both the sperm and the oocyte to increase embryo viability and resemble in vivo conditions more closely. As mentioned previously, treatment options such as glutathione have been used to treat the sperm before injection. This treatment has been reported to increase blastocyst rates compared to DTT treated sperm (Lee et al., 2015). However, the diploid nature of these blastocysts was not evaluated and only marginal improvement was observed. Another study evaluating glutathione treatment of sperm in bovine ICSI also showed increases in blastocyst rates up to 20% however only approximately 62% of those had evidence of sperm decondensation (Canel et al., 2017). There remains a need for a treatment, or multiple treatments, which simulate the physiological processes of fertilization for the sperm in order to achieve consistent decondensation after injection to efficiently produce viable bovine embryos via ICSI.
2.3. Sperm Treatments for Bovine ICSI

Membrane Disruptors

Dithiothreitol (DTT) has been used as a reducing agent and as a pretreatment for sperm to reduce the protamine disulfide bonds located in the outer membrane (Galli et al., 2003). The complete chemical reduction of these bonds ultimately leads to the physical relaxation of the sperm chromatin structure and degradation of the outer membranes containing the nucleus, allowing for the maternal histones to access the male genome (Rho et al., 1998). This physical change in the chromatin structure brought about by DTT, have been shown to increase efficiency of bovine ICSI and rates of male pronuclei formation (Suttner et al. 2000, Rho et al., 1998; Galli et al., 2003). While results with DTT pretreatment also vary depending on method of activation, as well as maturation and culture conditions, some studies have shown an increase in diploid blastocyst rates to approximately 20% (Oikawa et al., 2016; Rho et al., 1998). However, using DTT as a pretreatment of sperm prior to fertilization may not be ideal and result in damage to the sperm’s DNA (Sekhavati et al., 2012). DTT in the oxidized form has a stable ring structure with an internal disulfide bond. The redox reaction that occurs with DTT contains two sequential thiol-disulfide exchange reactions, which closes the ring structure, leaving behind a reduced disulfide bond (Ates et al., 2009). This reaction is what dissociates the protamine, allowing for decondensation of bovine sperm over at least a 4-7-hour period, or the potential replacement of protamine for maternal histones after injection. As DTT continues this redox reaction, the morphology of the head and midpiece is altered, causing bending or folding of the head at the equatorial segment, swollen or bent midpieces, followed by partial to complete
decondensation during incubation in sperm-TALP media (Rho et al., 1998). These morphological changes indicate a weakening and breaking down of the membranes, which then allow for decondensation to take place. However, as DTT has a high propensity to complete its’ ring structure, it is therefore liable to undesired side reactions. Residues on the sperm can interact with DNA and proteins to prevent or reduce other essential disulfide bond formations. Contrastingely, DTT is also an oxidizing agent which may damage paternal chromosomes after exposure, as well as potentially interfere with the maternal genome and possibly add an additional stressor to the already reduced capacity of the in vitro matured bovine oocyte to handle oxidative stress after ICSI (Szczygiel and Ward, 2002; Ashibe et al., 2019). It was observed in these studies using DTT treated sperm for bovine ICSI, that the developmental capacity of the resulting embryos remains limited. This may be due to DTT toxicity and the effect of side reactions on essential cellular structures, DNA fragmentation, and damage to the paternal chromosomes (Szczygiel and Ward, 2002; Yamauchi et al., 2011). Results in these studies with DTT pretreatment also vary depending on method of activation, as well as maturation and culture conditions, but overall does increase the percentage of oocytes fertilized compared to nontreated sperm (Oikawa et al., 2016; Rho et al., 1998). However, these results are still generally not considered an efficient enough option for the practical application of bovine ICSI. The use of DTT to forcibly reduce bonds and allow for relaxation of chromatin and the nucleus of the sperm head imitates the decondensation of the sperm head brought during actual fertilization, yet there are many physiological differences in the way these two processes occur. These differences may contribute to the limited increase in blastocyst rates seen with DTT.
treatment and the lack of live births following this sperm treatment. In recent years, other membrane-disrupting agents have been tested, such as Triton X-100 (Lee and Yang, 2004), sodium hydroxide (Arias et al., 2014), dithiobutylamine (Suttirojpattana et al., 2016), lysolecithin (Morozumi et al., 2006; Zambrano et al., 2017) and methyl-β-cyclodextrin (Arias et al., 2017). Some of these treatments raised blastocyst rates, but male pronucleus formation was not improved in all cases and some showed detrimental effects to embryo development (Zambrano et al., 2016). While further optimization and study of the DTT and other membrane disrupting treatments for bovine ICSI, including the timing of injection and observing morphological differences after treatment may help improve this technique, another approach would be more beneficial to improving bovine ICSI efficiency.

**Physiological Pathway Manipulation**

Another process which can perhaps be more beneficial, yet is not fully explored, is utilizing the sperm’s physiological pathways to elicit a similar result as occurs with fertilization. Treatments using heparin in addition to lysophosphatidylcholine (LPC) or calcium ionophores, such as A23187, have been used to simulate capacitation and the acrosome reaction (Parrish et al., 1988). Both LPC and A23187 have been confirmed separately to induce the acrosome reaction and resulting vesiculation and fusion of the acrosomal membranes and release of acrosomal contents in bovine spermatozoa (Parrish et al., 1988). LPC is known as a fusogenic lipid which has been shown to cause fusion of the acrosomal membranes similar to the vesiculation process of the acrosome reaction (Parrish et al., 1988). The calcium ionophore A23187 has been shown to greatly increase calcium influx, signaling downstream pathways within the sperm to
initiate the acrosome reaction (Jamil et al., 1981). However, these treatments in conjunction with ICSI have not improved blastocyst rates; with blastocyst rates after ICSI of sperm treated with LPC or A23187 less than 10% in some studies (Gómez-Martínez et al., 2019). There are other treatments which activate the sperm’s physiological pathways in fertilization that have yet to be evaluated for bovine ICSI. For example, Miller et al. (2016), published the signaling pathways governing the membrane localized progesterone receptor on the head of the sperm. It has been noted previously, that progesterone is present in not only follicular fluid, but in the cumulus cells themselves (Romero-Aguirregomezcorta et al., 2019). It was not known that this source of progesterone could have an effect on condensed transcriptionally inactive spermatozoa. Most steroid hormone receptors, like those for progesterone, are located intracellularly, which would not be accessible due to the compact and condensed nature of a sperm cell (Lishko et al., 2011, Miller et al., 2016). Sperm have been observed to have one of the few known nongenomic external membrane bound progesterone receptors (Miller et al., 2016). It has been shown that use of progesterone treatment triggers sequential events in the sperm through the binding of progesterone to this surface receptor (Calogero et al., 2000). This binding causes significant influx of extracellular calcium through the activation of the sperm-specific calcium channel, CatSper, which begins an essential cascade needed for both hypermotility and the acrosome reaction to occur (Sun et al., 2017). Progesterone, found to be secreted by the cumulus cells in bovine oocyte complexes and also present in the follicular fluid released after ovulation, plays a role in vivo to remove capacitated sperm from the oviductal epithelium and initiate hypermotility (Romero-Aguirregomezcorta et al., 2019).
Once capacitated, spermatozoa’s cell surface receptors are able to interact with progesterone to immediately initiate a cascade of biochemical events, which result in the activation of intracellular pathways to cause an influx of extracellular calcium through the sperm-specific Catsper ion channel. The catsper ion channel has also been shown to play a crucial role in hyperactivation and motility (Tamburrino et al., 2014). Furthermore, the influx in calcium brought about by Catsper depolarizes the outer membranes and activates endogenous pathways which result in the dissociation of the acrosomal membranes and increase in cellular activity (Sun et al., 2017). This primes the sperm for acrosomal exocytosis, or the acrosome reaction, which removes the acrosomal and outer membranes of the sperm. This response can be replicated in vitro, by exposing sperm to progesterone to induce the acrosome reaction in bovine sperm (Calogero et al., 2000; Contreras et al., 1999). This methodology has been confirmed using staining to visualize the exposed acrosomal membranes, using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). FITC-PNA staining can be observed and measured once the plasma membrane is removed through capacitation and the acrosomal membranes are exposed. Once exposed, FITC-PNA then selectively binds to β-galactose terminals located exclusively within the outer acrosomal membrane, indicating an ongoing acrosome reaction (Cheng et al., 1996; Nagy et al., 2003). This can be used to measure affected sperm, as well as track progression of the acrosome reaction after exposure to progesterone. Using progesterone to induce the acrosome reaction has been shown to be successful as a pretreatment before ICSI to facilitate access to the sperm’s chromatin and allow for increased male pronuclear formation (Katayama et al., 2002). As progesterone is known to play a crucial role in
**vivo**, in the bovine female reproductive tract to initiate hyperactivity and the acrosome reaction, both crucial for fertilization to occur in vivo, it is suspected that progesterone may also assist in vitro (Romero-Aguirregomezcorta et al., 2019). While these steps may not be required in vitro with species, such as humans or mice, demonstrated by high rates of successful embryo development after ICSI. In cattle and other species with low rates of fertilization after ICSI due to failure of the sperm to decondense, induction of the acrosome reaction and the subsequent increase in permeability of the outer membranes was shown to increase fertilization after ICSI (Tesarik, 1998; Kimura. et al., 1995). Progesterone as a treatment prior to bovine ICSI may provide a missing key difference between fertilization with IVF versus ICSI, as progesterone is present in the cumulus cells and influences downstream pathways for fertilization. While this has been shown with similar acrosome reaction inducing agents, such as LPC and calcium ionophore A23187, these do not utilize a natural endogenous pathway within the sperm, but only partially manipulate other signaling pathways to lead to an effect (Gómez-Martínez. 2019). In human, porcine, and mouse spermatozoa, progesterone has been shown to induce an acrosome reaction (Contreras 1999; Katayama et al., 2002; Parinaud et al., 1992). It has also been shown that progesterone treatment of mouse sperm can increase fertilization rates after sub-zonal sperm injection compared to non-treated controls, with lower cytotoxic effects than A23187 (Parinaud et al., 1992). Similarly, with porcine spermatozoa, progesterone treatment has been shown not only to induce vesiculation of the acrosomal membranes consistent with the acrosome reaction, but was also shown to increase male pronuclei formation after ICSI to 64% compared to 31% for nontreated sperm injection (Katayama et al., 2002). These studies
show such treatment should be evaluated in bovine ICSI to see if any improvements in efficiency can be made. Eventually, the exact necessary sequential series of events the sperm undergoes during fertilization will be discovered. Using bovine ICSI with sperm treatments has the potential to help understand which signals are critical for the sperm to gain the ability to fertilize and activate the oocyte and should continue to be evaluated.
CHAPTER III.
SPERM DECONDENSATION AND MALE PRONUCLEAR FORMATION FOLLOWING BOVINE INTRACYTOPLASMIC SPERM INJECTION

3.1. Introduction

Intracytoplasmic sperm injection (ICSI) has been a valuable tool in many species for its ability to not only overcome male factor infertility problems, but also to eliminate the risk of polyspermy commonly seen with in vitro fertilization (IVF), and can assist in implementing other technologies (Catt et al., 1995). For species with limited gamete availability, ICSI has the potential of bypassing the need to optimize IVF conditions. It has also been shown, in humans, to improve upon current genome editing technologies (Ma et al., 2017). However, bovine ICSI lacks the success rates that has been observed in other mammals (Águila et al., 2017). There have been both sperm and oocyte factors identified to play a part in the lack of success with bovine ICSI, including hyper-condensed sperm as well as a glutathione deficiency within in vitro matured oocytes. However, the overall lack of sperm decondensation is the limiting factor in ICSI success in cattle. Without sperm decondensation, successful fertilization post-ICSI is impaired, as the oocyte histones responsible for forming the nuclear structure of the male pronucleus cannot bind to transcriptionally inactive chromatin present before the sperm undergoes an essential morphological change. This required change includes the removal of sperm-specific protamines from the sperm’s nucleus, responsible for holding the condensed inert state of the paternal genome. With the removal of the protamines, decondensation of the paternal chromatin occurs (McLay and Clarke, 2003) This then leads to a replacement of the paternal protamines with maternal histones, which remodel and reassemble the chromatin into a transcriptionally active
male pronucleus. (McLay and Clark, 2003). The disulfide bonds which hold the protamines intact are normally broken, or reduced, by glutathione during these processes during fertilization following the acrosome reaction and fusion with the oocyte plasma membrane, allowing for nuclear decondensation and subsequent development. However, because ICSI bypasses these critical processes with injection of the sperm directly into the cytoplasm of the oocyte combined with a deficiency in glutathione in in vitro matured bovine oocytes, additional measures must be taken for successful fertilization and pronuclei formation to occur. The most common approach is pre-treating the sperm, prior to ICSI, to assist in decondensation. Dithiothreitol (DTT) has been used as a reducing agent and as a pretreatment for sperm to reduce the protamine disulfide bonds located in the outer membrane (Galli et al., 2003). The complete chemical reduction of these bonds ultimately leads to the physical relaxation of the chromatin structure and degradation of the outer membranes containing the nucleus, eventually resulting in a decondensation of the sperm DNA (Rho et al., 1998). This physical change in the chromatin structure brought about by DTT, has been shown to increase efficiency of bovine ICSI and rates of male pronuclei formation (Suttner et al., 2000; Rho et al., 1998). However, it was observed in these studies that the developmental capacity of the resulting embryos remains limited. There is also a concern with DTT toxicity and the effect of residues on formation of essential structures, which can result in undesirable side reactions, DNA fragmentation and damage to the paternal chromosomes (Szczygiel and Ward, 2002; Yamauchi et al., 2011). Results with DTT pretreatment also vary depending on method of activation, as well as maturation and culture conditions, but some studies have resulted in approximately a 10-15%
diploid blastocyst rate (Oikawa et al., 2016; Rho et al., 1998). Another process, not wholly explored, is utilizing the sperm’s physiological pathways to illicit a similar result. Using progesterone, part of the natural endogenous pathway of sperm capacitation, triggers sequential events in the sperm through the binding of progesterone to surface receptors (Calogero et al., 2000). This binding causes an influx of extracellular calcium through the activation of the sperm-specific calcium channel, CatSper, which begins an essential cascade needed for both hypermotility and the acrosome reaction to occur (Sun et al., 2017). Progesterone, secreted by the cumulus cells in bovine oocyte complexes and also present in the follicular fluid released after ovulation, plays a role in vivo to remove capacitated sperm from the oviductal epithelium and initiate hypermotility (Romero-Aguirregomezcorta et al., 2019). This primes the sperm for acrosomal exocytosis, or the acrosome reaction, which removes the acrosomal and outer membranes of the sperm. This response can be replicated in vitro to induce the acrosome reaction in bovine sperm with exposure to progesterone (Calogero et al., 2000; Contreras et al., 1999). With staining, using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), this process can be observed and measured once the plasma membrane is removed following capacitation. Once removed, FITC-PNA then selectively binds to the exposed β-galactose terminals located within the outer acrosomal membrane, indicating an ongoing acrosome reaction (Cheng et al., 1996; Nagy et al., 2003). This can be used to measure the population of affected sperm, as well as track progression of the acrosome reaction after exposure to progesterone. Using progesterone to induce the acrosome reaction has been shown, in other species, to be successful as a pretreatment before ICSI to facilitate access to the sperm’s
chromatin and allow for increased pronuclear formation (Katayama et al., 2002). This study aims to measure the effects of both DTT and progesterone on bovine spermatozoa in experiments 1 and 2, respectively, after treatment through tracking and observation of morphological changes, staining with Hoechst for DTT treated sperm and FITC-PNA for progesterone treated sperm, as well as cytometric flow analysis of progesterone treated sperm. In experiments 3 and 4, ICSI was performed to evaluate DTT and progesterone, respectively, as pretreatment for sperm prior to ICSI in bovine oocytes by assessing rates of pronuclear formation 16 hours after fertilization. While DTT pretreatment of sperm has been investigated with bovine ICSI, to our knowledge, no such studies have been done investigating the effects of progesterone sperm pretreatment for bovine ICSI. These experiments will begin to examine these effects to further elucidate on critical processes of fertilization and if the efficacy of bovine ICSI can be improved in this manner.

3.2. Materials and Methods

Chemicals and Reagents

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte and Semen Allocation

Oocytes used were abattoir derived and commercially sourced through DeSoto Genetics (TN, USA). Semen used was commercially processed Holstein semen cryopreserved in standard egg-yolk tris buffer.
**DTT Treatment and Evaluation**

Frozen semen was thawed by immersion in a 37°C water bath for 30 seconds before being washed in 2 mL of non-capacitating media (BO-SemenPrep; IVF scientific) by centrifugation for 5 minutes at 400 x g, to remove extenders. A swim-up was then prepared and the pellet was layered under 2 mL sperm-TALP (Cassion, Smithfield, TN) containing a 5 mM concentration of DTT. Control sperm swim-up was not supplemented with DTT. After 1-hour incubation, the top 0.5 mL was removed and washed twice in the same non-capacitating medium. Sperm were then cultured in sperm-TALP at 5% CO\textsuperscript{2} at 37°C and either diluted for injection or observed hourly for 7 hours under phase contrast at 400x to assess the progression of decondensation after exposure to DTT. Changes in morphology of DTT treated and non-treated sperm were observed through a combination of phase-contrast and Hoffman Modulation Contrast microscopy. To confirm DNA dispersal, DTT treated sperm were stained with Hoechst 33342 (1 µg/mL) and viewed under fluorescence microscopy. The proportion of sperm affected by DTT was estimated by a single observer and categorized into different stages of decondensation based on progressive time-dependent changes in morphology. Stage 1 includes sperm which have a folded head at the equatorial segment, a bend and swelling of the mid-piece. Sperm in stage 2 had partial decondensation with pock marking and early expansion marked by larger head size (7-9 µm). Followed by stage 3, which includes decondensed sperm with a head size of 10 um or greater and sperm that had dispersing DNA, which was confirmed as DNA with Hoechst staining. This was done at hourly time points in no fewer than 8 repetitions.
Progesterone Treatment and Evaluation

Semen was thawed in a 37°C water bath for 30 seconds before separation using a discontinuous gradient (Irvine Scientific, Santa Ana, CA) which was centrifuged for 10 minutes at 400 x g. The pellet was then removed and diluted into a capacitating sperm-TALP medium containing heparin (10 µg/µl) for 4 hours at a concentration of 1 x 10^6 spermatozoa per mL with 5% CO² and 37°C. Dilutions were calculated after spermatozoa counting with a hemocytometer. Once capacitated, sperm were then introduced to progesterone (10 µM dissolved in ethanol) for a period of 15 minutes. Sperm were then either incubated further for 1 hour to allow for completion of the acrosome reaction prior to ICSI, or immediately stained with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA (5 µg/mL)) for 15 minutes for analysis through flow cytometry (Accuri C6 flow cytometer) as well as fluorescent microscopy. For flow cytometry, to observe a larger population, semen from 6 different bulls was used with at least 3 repetitions per bull. After thawing, semen was washed by centrifugation and 500 µl of sperm suspension was placed into a 1.5 mL centrifuge tube to allow for the determination of total acrosome reacting population. Progression of the acrosome reaction over 2 hours was monitored by observing changes in fluorescence. Pre-warmed slides were used containing 10 µL of an immobilized sperm suspension in mounting media under a cover slip. Loss of a fluorescing acrosome accompanied by a reminiscent halo on the sperm head was assumed to be characteristic of a completed acrosome reaction. Sperm were also assessed for hypermotility prior to staining by aliquoting 10 µL of treated sperm onto prewarmed slides. Samples were then randomized and viewed by a single observer, using integrated phase contrast.
microscopy at a magnification of 200x, to determine percentage of hyperactive sperm out of a total 50 motile sperm. This was repeated 3 times for a total of 150 sperm per slide, which was done in 3 replicates. Sperm were considered hyperactive if a curved, helical, or non-linear trajectory was present, along with highly exaggerated tail movements and distinct swimming pattern consistent with other observations of hyperactivated motility (Ho et al., 2002).

**Intracytoplasmic Sperm Injection (ICSI)**

Sperm injection was performed at 200x magnification (Nikon TE200) with Hoffman Modulated contrast on a heated stage maintained at 37°C. Mature metaphase II bovine oocytes were denuded in 1 mg/mL hyaluronidase with vortexing before being placed in prewarmed 5 µL droplets of hepes-TALP (Cassion, Smithfield, TN) under mineral oil in 50 X 9-mm polystyrene micromanipulation dishes (Falcon; Fisher Scientific, Atlanta, GA) for injection. Oocytes with no polar body were not included. Sperm were then washed twice in sperm-TALP before the addition of 2 µL of the sperm suspension to a 10 µL droplet of 7% PVP (SAGE In Vitro Fertilization Trumbull, CT) also in the injection dish. An injection pipette with an inner diameter of 8 µm (Origo Charlottesville, VA), connected to Narishige manipulators operated by Eppendorf CellTram oil syringe injectors, was used to select and immobilize sperm. Control, or untreated sperm, were chosen dependent on morphology and trajectory, while DTT treated sperm were selected based on characteristic abnormalities of the head after treatment (see Results) (Rho et al., 1998). In the case of progesterone treated sperm, selection was based upon presence of hyperactive characteristics, such as a curved trajectory with exaggerated and asymmetrical tail movements, as well as a missing or
hazy acrosome (see Results) around the acrosomal region of the head consistent with signs of an ongoing or completed acrosome reaction (Mortimer et al., 1987). Staining was not used for confirmation of sperm’s status during injection due to the possible toxicity of the stain. Once immobilized and the tail visibly kinked using the bottom edge of the injection pipette, the sperm was aspirated tail first into the injection pipette which was then moved into the 5 µl injection drops containing oocytes. Oocytes were orientated with the first extruded polar body at the 6 or 12 o’clock position and held with negative pressure of a holding pipette with an inner diameter of 30 µm (Origo Charlottesville, VA). A single spermatozoon is then brought close to the tip of the injection pipette as it was advanced forward into the oocyte. Once firm contact was made with the oolema, aspiration was used to break the oolema until free unimpaired movement of cytoplasm was seen. The aspirated cytoplasm and the sperm were then expelled past the central line of the oocyte. If any excess media was present, it was aspirated as the injection pipette was removed from the oocyte. Injected oocytes were then removed from the injection dishes and held in hepes-TALP at 37°C before activation.

**Oocyte Activation and Evaluation**

Both injected and non-injected oocytes were activated using an Ionomycin/DMAP protocol after ICSI. Non-injected oocytes were parthenogenetically activated to serve as quality control for each repetition. For activation, oocytes were introduced to 5 µm Ionomycin in hepes-TALP with 3 mg/mL BSA for 5 minutes followed by washing in hepes-TALP containing 30 mg/mL BSA for an additional 5 minutes, to stop the activation process. Oocytes were then washed twice in SOF media (Cassion,
Smithfield, TN) before being transferred to drops containing 5 mM DMAP in SOF to be cultured for 4 hours. After 4 hours, presumptive zygotes and parthenotes were washed and transferred to commercial culture media (BO-IVC IVF scientific) and incubated for 16 hours under a 6% CO² atmosphere at 38.5°C. After 18 hours of culture, oocytes which had degenerated after injection or were not successfully injected were removed and the remaining presumptive zygotes were washed in PBS and stained using Hoechst 33342 (1 µg/mL) in calcium and magnesium free PBS (Gibco) and incubated for 15 minutes. Presumptive zygotes were then placed on microscope slides (Thermo scientific) with excess media removed before 10 µl of gold anti-fade permount (Invitrogen) and a coverslip was applied. The slides were then viewed on a Nikon H600L at 400x to assess pronuclear formation. Parthenotes were considered activated if 1 or 2 pronuclei were present. Injected embryos were considered successfully fertilized if 2 pronuclei and 2 polar bodies were present without a sperm head. If a sperm head was present, the embryo was considered parthenogenetically activated but not successfully fertilized.

**Statistical Analysis**

In experiment 1, the mean estimated proportion of DTT treated sperm in each stage of decondensation over hourly time points was analyzed through ANOVA. Results from experiment 2 were also analyzed through ANOVA, to determine significance of progesterone induced acrosome reactions in treated versus non treated sperm. Flow cytometry results using multiple bulls were analyzed for bull variance and normality, and the data was determined to be normally distributed prior to ANOVA. All means are presented ± SEM. In experiments 3 and 4, differences for frequency of pronuclei
development after injection of DTT and progesterone treated sperm, respectively, compared to controls were analyzed using Yates’ corrected chi square. Differences were considered significant when p < 0.05.

3.3. Results

Experiment 1. Effects of DTT Treatment on Sperm Decondensation

As shown in table 1 and figures 1 and two, treatment with DTT elicits extensive progressive time dependent changes in spermatozoa as the outer membranes dissipate and allow for the dispersal of chromatin and DNA. Decondensation of sperm were separated into 3 different stages as seen in figure 1. These stages are defined by the progressive characteristics of decondensing sperm. With stage 1 including early characteristics, such as head folding at the equatorial segment or a bend in the mid-piece. Followed by stage 2 with partial decondensation seen by early expansion, marked by larger head size, and obvious pock-marking of the head. Finally, fully decondensed sperm heads measuring ≥10 µm in diameter and sperm with DNA dispersal, confirmed with Hoechst staining, were included in stage 3.
Table 1. Sperm undergoing decondensation following DTT treatment.

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Percent of Spermatozoa decondensation in Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage 1</td>
</tr>
<tr>
<td>Hour 1</td>
<td>80 ± 3.5</td>
</tr>
<tr>
<td>Hour 2</td>
<td>88 ± 4.4 *</td>
</tr>
<tr>
<td>Hour 3</td>
<td>44 ± 4.2</td>
</tr>
<tr>
<td>Hour 4</td>
<td>29 ± 7.4</td>
</tr>
<tr>
<td>Hour 5</td>
<td>18 ± 5.6</td>
</tr>
<tr>
<td>Hour 6</td>
<td>18 ± 7.2</td>
</tr>
<tr>
<td>Hour 7</td>
<td>12 ± 4.1</td>
</tr>
</tbody>
</table>

All values are ± SEM taken over at least 8 repetitions. * denotes significant maximum percent in time points for each stage. No such changes were observed in control sperm. (p < 0.05)

An estimated 88% of treated sperm displayed early changes in morphology, including a bend at the equatorial segment after the second hour of incubation. Sperm displaying partial decondensation, in stage 2, was estimated 42% by the third hour and increased to 62% 5 hours after treatment. Stage 3 expansion began at hour 3 and increased to approximately 64% by hour 7.
Figure 1. Morphological changes seen in spermatozoa following a 5mM treatment of DTT for 1 hour. (A) Control spermatozoon with normal morphology under phase-contrast. (B) Sperm after 1 hour of treatment with head folded at the equatorial segment. (C) Sperm at hour 3 displaying pock-marking consistent with partial decondensation and a characteristic bend at the mid-piece. (D) Spermatozoa, seen 3 hours after treatment, beginning to expand. (E) Sperm at hour 5 of incubation with continuing decondensation. (F) Fully decondensed nucleus of a spermatozoa 6 hours after treatment, approximately 12 µm in diameter. (G) Complete dispersal of the chromosomes after hour 7 of incubation. These morphological changes were not seen in control sperm. Scale bar is 5 µm.
Figure 2. Percent of the population of sperm treated with DTT in separate stages of decondensation demonstrating the relationship between stages at differing time points. (p < 0.01).

Experiment 2. Effects of Progesterone Treatment on Bovine Spermatozoa

Sperm were treated with progesterone after heparin capacitation and compared to controls with fluorescent microscopy and flow cytometry. Loss of the acrosome consistent with the acrosome reaction was measured using FITC-PNA staining, over a time period of 2 hours. Beginning with the percent of the population undergoing this reaction in the initial 15 minutes after treatment and continuing in 15-minute intervals. Hyperactivation was assessed immediately, prior to staining, in both progesterone treated and nontreated sperm. In treated sperm, there was a mean of 77.4% (± 6.5) of sperm considered hyper-motile, which decreased rapidly with time. In sperm not treated with progesterone, an estimated 25.3% (± 4.1) were hyperactive after capacitation alone (p < 0.05).
Immediately after treatment and staining, sperm were analyzed using flow cytometry to determine percentage of the population that had exposed acrosomal membranes and had initiated the acrosome reaction. Sperm with no staining were analyzed to set a baseline fluoresce level and compared to heparin control and progesterone treated sperm.

![Flow Cytometry Output](image)

Figure 3. Flow cytometry output for progesterone treated and nontreated sperm. (A) Forward versus side scatter plot of nontreated sperm to determine region of interest, P1. (B) FITC fluorescence seen in FL1 (gated for P1) for non treated and non stained sperm, as well as heparin capacitated and heparin and progesterone treated sperm stained with FITC-PNA.

By comparing the number of fluorescent sperm in each sample, the mean percentage of acrosome reacted sperm after progesterone treatment was found to be 83.7 (± 6.8), while the percent of acrosome reacted sperm after heparin only was 15 (±7.5). Showing a significant increase in FITC-PNA fluorescence and induction of the acrosome reaction following progesterone treatment (p < 0.05). This was consistent with results observed and manually counted by a blind observer under fluorescent microscopy. In which the mean percent of progesterone treated sperm which were positive for an acrosome reaction at 15 minutes was estimated to be 80.2% (± 4.2) and increased to 89.3% (± 3.9) after 2 hours. An observed 49.8 (± 5.1) and 62.5 (± 7.4)
percent of sperm had a completed acrosome reaction after 1 and 2 hours of incubation after treatment, respectively. A remaining 13% (± 2.7) of sperm were unaffected by treatment with no visible staining. In controls, sperm treated only with heparin had 28.9% (± 8.3) of sperm automatically acrosome react, which did not significantly increase with time and an estimated 14.2% (± 3.6) had a completed acrosome reaction or loss of acrosomal membranes after 2 hours (p < 0.05).

Figure 4. (A) FITC-PNA binding to the exposed acrosome 15 minutes after progesterone treatment under DIC microscopy. This was considered positive for an acrosome reaction. (B) Without fluorescence, a distorted acrosome can be seen as a haziness and lack of definition around the front edge of the head at 200-400x. (C) Indenting around the equatorial segment and a narrower head consistent with loss of acrosomal membranes, which is confirmed with FITC-PNA. (D) Staining remains only around binding regions of the acrosomal membranes creating a halo, which is indicative of a completed acrosome reaction and loss of acrosomal membranes.

Experiment 3. Use of DTT Sperm Treatment for Bovine ICSI

Semen treated with DTT as well as nontreated sperm were used for injection into mature metaphase II bovine oocytes. Oocytes were activated after injection and then cultured for 16 hours before cytological observations were made. Oocytes which were injected and developed 2 pronuclei and no visible sperm head were considered to be
successfully fertilized. Table 2 shows the rates of fertilization, as well as activation. Oocytes were considered activated, but not fertilized, if 1 or 2 pronuclei were present in conjunction with a visible sperm head. Based on these criteria, the proportion of oocytes successfully fertilized with DTT treated sperm (42 of 148: 28.3%) was significantly higher than controls (11 of 117: 9%), despite activation rates being significantly lower than both control injection and those parthenogenetically activated (82 out of 148: 55.4% vs. 84 of 117: 71.8% vs. 72 of 94 :76.5%; p < 0.001). In both injection groups, a proportion of oocytes remained at metaphase II with an intact sperm head present. There was no significant difference found between the DTT treated group which contained 24 of 148 (16.2%) and the control group that contained 22 of 117 (18.8%) of injected oocytes which did not activate. Cytological observations were also made to assess DNA fragmentation, seen as abnormalities in pronuclear structure and a scattering of visible DNA after staining. While differences in visible fragmentation were not significant between parthenote and control groups (13.8% vs 15.7%), the DTT treated group had a significantly higher rate at 35.4% than the other treatment groups.
Table 2. Effect of DTT treatment of sperm on fertilization rates of injected bovine oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of embryos</th>
<th>No. of activated (%)</th>
<th>No. of fertilized (%)</th>
<th>No. of DNA fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT treated sperm</td>
<td>148</td>
<td>82 (55.4%)</td>
<td>42 (28.3%) *</td>
<td>44 (35.4%) *</td>
</tr>
<tr>
<td>Control (no DTT)</td>
<td>117</td>
<td>84 (71.8%)</td>
<td>7 (6%)</td>
<td>15 (15.7%)</td>
</tr>
<tr>
<td>Parthenogenetically activated</td>
<td>94</td>
<td>72 (76.5%)</td>
<td>N/A</td>
<td>10 (13.8%)</td>
</tr>
</tbody>
</table>

Table 2. Differences in both fertilization and activation between ICSI with control and DTT treated sperm were found to be significant (p < 0.001). Oocytes were considered fertilized if they contained 2 pronuclei and no visible sperm, while oocytes which were considered activated had 1 or 2 PN with sperm or were parthenogenetically activated and not fertilized. DNA fragmentation was assessed in zygotes containing at least 1 pronuclei.

**Experiment 4. Use of Progesterone Treatment of Sperm for Bovine ICSI**

Sperm which were capacitated over a 4-hour period with heparin and treated, or not, with progesterone for 1 hour were used for injection into metaphase II bovine oocytes. Oocytes were then activated, cultured, and stained as described above, before pronuclear formation was assessed. The same criteria for activated and fertilized oocytes as described in experiment 3 are used for experiment 4 as well. Table 3 shows that the rate of fertilization based on these criteria were significantly higher for progesterone treated sperm than controls (42 of 110 (38.1%) vs 11 of 109 (10.1%)). While both injection groups contained oocytes that remained at metaphase II after injection, they did not differ significantly with 11.8% for the progesterone treated group and 12.8% for the control group.
Table 3. Effect of progesterone treatment of sperm on fertilization rates of injected bovine oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of embryos</th>
<th>No. of activated (%)</th>
<th>No. of fertilized (%)</th>
<th>DNA fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin + P4</td>
<td>110</td>
<td>55 (50%)</td>
<td>42 (38.1%) *</td>
<td>17 (17.5%)</td>
</tr>
<tr>
<td>Heparin only</td>
<td>109</td>
<td>84 (71.8%)</td>
<td>11 (10.1%)</td>
<td>15 (15%)</td>
</tr>
<tr>
<td>Parthenogenetically activated</td>
<td>76</td>
<td>53 (69.7%)</td>
<td>N/A</td>
<td>8 (15.1%)</td>
</tr>
</tbody>
</table>

Table 3. Differences in fertilization between ICSI with control and Progesterone (P4) treated sperm were found to be significant (p < 0.001). Oocytes were considered fertilized if they contained 2 pronuclei and no visible sperm, while oocytes which were considered activated had 1 or 2 PN with sperm or were parthenogenetically activated and not fertilized. Cytological observations were made for DNA fragmentation in zygotes containing at least 1 pronuclei and no significant differences were found between experimental groups.

Cytological observations were made, in a similar manner to experiment 3. After 16 hours post ICSI, embryos were stained and assessed. Interestingly, in a portion of the activated zygotes containing only 1 pronucleus and a sperm head (8 out of 55 activated oocytes; 14.5%), an abnormal staining pattern can be seen in the acrosomal region of the sperm head with a surrounding halo of stained DNA, seen in figure 5. This distinct staining pattern possibly signals on going decondensation and asynchronous fertilization.
Figure 5. Abnormal and normal staining patterns in fertilized zygotes using progesterone treated sperm. Shown is two embryos each injected with a sperm treated with progesterone. (A) An activated oocyte with 1 pronucleus with a sperm head present. Abnormal staining patterns can be seen in the acrosomal region of the head surrounded by partial development or indistinct halo of stained DNA, possibly signaling on going decondensation or asynchronous fertilization. No such abnormalities were seen in sperm not treated with progesterone. Compared to (B), which shows a 2PN embryo with 2 polar bodies created with progesterone treated sperm.

3.4. Discussion

Bovine ICSI is a potentially valuable tool and improvements upon this technique could benefit both research and cattle breeding. This study demonstrated the effects brought about by both DTT and progesterone treatment of sperm over time, as well as how these treatments can affect the efficacy of ICSI in cattle. The hyper-stabilized nature of bovine spermatozoa, which is especially resistant to physical and chemical disruption, is mostly due to the conversion of protamine sulfhydryl groups to disulfide bonds as the spermatozoa passes through the epididymis (Braun, 2001). These bonds, which are greater in number and strength compared to other species, result in bovine
spermatozoa having a more stabilized chromatin (Perreault et al., 1988). This produces a tightly condensed and highly stable nucleus that is resistant to decondensation (Hutchison et al., 2017). These features of bovine spermatozoa limit ICSI effectiveness in cattle. Seeking treatments which effect the sperm in a similar manner to that of fertilization may improve fertilization rates and embryo development following bovine ICSI.

In this study, it was shown that both DTT and progesterone using different mechanisms provide access to the male genome, allowing for decondensation and fertilization to occur after bovine ICSI. While the two treatments, DTT and progesterone, cannot be directly compared in this study, it is shown that both are capable of producing successfully fertilized embryos in comparable in vitro settings, with identical activation and culture protocols. These results, with some variations due to differences in semen preparation, maturation, activation, and culture protocols compare with results of other studies (Rho et al., 1998; Galli et al., 2003) However, limited publications could be found using progesterone as a pretreatment of bull sperm prior to ICSI. Similar results were observed with porcine ICSI (Katayama et al., 2002). This study focused on sperm decondensation and pronuclear development to elucidate the early processes of fertilization and ICSI in cattle. Further study should be done to assess later embryo development potential.

DTT is known to be a reducing agent, which once oxidized forms a stable ring structure with an internal disulfide bond. The redox reaction that occurs contains two sequential thiol-disulfide exchange reactions, which closes the ring structure, leaving behind a reduced disulfide bond (Ates et al., 2009). This reaction is what dissociates the
protamine, allowing for decondensation of bovine sperm, over at least a 4-7-hour period, followed by the replacement of protamine with maternal histones after injection. As DTT continues this reaction, the morphology of the head and midpiece is altered, causing bending or folding of the head at the equatorial segment, swollen or bent midpieces, followed by partial to complete decondensation during incubation in sperm-TALP (Rho et al., 1998). These morphological changes, also seen in this study, indicate a weakening and breaking down of the plasma and nuclear membranes, which then allow for decondensation. However, DTT has a high propensity to complete its’ ring structure and also catalyze undesired reactions with residues interacting with DNA and proteins to prevent or reduce other essential disulfide bonds. Contrastingly, DTT is also an oxidizing agent which may damage paternal chromosomes after treatment, as well as potentially interfere with the maternal genome creating an additional stressor to the already reduced capacity of the in vitro matured bovine oocyte to handle oxidative stress after ICSI (Szczygiel and Ward. 2002; Ashibe et al., 2019). While further study would be needed for verification, this may be the cause of the increased visible DNA fragmentation seen in oocytes injected with DTT treated sperm. Despite sperm being washed thoroughly after treatment, additional measures may need to fully remove DTT residue on the sperm.

Progesterone is known to play a role in vivo, in the bovine female reproductive tract to initiate hyperactivity and the acrosome reaction, both of which are crucial for fertilization to occur (Romero-Aguirregomezcorta et al., 2019). These steps may not be required in vitro with species, such as humans or mice, where high rates of successful embryo development after ICSI have been demonstrated with sperm permeabilized only
by a kinking or breaking of the tail at the midpiece (Tesari., 1998; Kimura Y. et al., 1995). However, in cattle and other species with low rates of fertilization after ICSI due to failure of the sperm to decondense, induction of the acrosome reaction and the subsequent increase in permeability of the outer membranes was shown to increase fertilization after ICSI. This has been shown with similar acrosome reaction inducing agents, such as lysophosphatidylcholine (LPC) and calcium ionophore A23187, which do not utilize the sperm’s natural endogenous pathway (Gómez-Martínez, 2019). Once capacitated, the spermatozoa’s cell surface receptors are able to interact with progesterone to immediately initiate a cascade of biochemical events, which result in the activation of intracellular pathways to cause an influx of extracellular calcium through the sperm-specific Catsper ion channel. Catsper has also been shown to play a crucial role in hyperactivation and motility (Tamburrino et al., 2014). Furthermore, the influx in calcium brought about by Catsper depolarizes the outer membranes and activates endogenous pathways which result in the dissociation of the acrosomal membranes and increase in cellular activity (Sun et al., 2017). Conjugated FITC-PNA was used to demonstrate this in vitro in this study, as it selectively binds to β-galactose sugars present only in the outer acrosomal membrane (Cheng et al., 1996). By observing the time at which a significant proportion of stained spermatozoa had a completed acrosome reaction, a 1-hour incubation time after treatment was deemed appropriate for injection. Even though 2-hours of incubation after treatment had a greater number of completed acrosome reacted sperm, motility was diminished, which is why 1-hour was found preferable for ICSI. By simulating physiological events in vitro with progesterone, it was shown that increased fertilization rates with ICSI in cattle can
be achieved without the toxicity that DTT, other reducing agents or calcium ionophores have, all of which have also been used increase success in bovine ICSI (Parinaud et al., 1992). However, different concentrations of progesterone, injection times, or other factors could assist in a more synchronous fertilization, resulting in a greater number of oocytes with 2 pronuclei, instead of 1 pronucleus with a potentially decondensing sperm. Further study would be needed to confirm if sperm presenting with abnormal staining patterns and the DNA distribution seen is actually a decondensing sperm in response to progesterone or another factor related to this treatment.
CHAPTER IV.
SUMMARY AND CONCLUSION

It has been shown that both DTT and progesterone treatments provide access to the male genome in bovine sperm by using different mechanisms for physically relaxing the chromatin versus physiologically initiating an acrosome reaction, both resulting in higher rates of fertilization with ICSI. DTT breaks disulfide bonds located within the sperm head to weaken membrane integrity, allowing for the expansion and subsequent decondensation of the nucleus. Progesterone, after capacitation with heparin, initiates the acrosome reaction, exposing the acrosomal membranes for vesiculation, and mimics \textit{in vivo} physiological processes prior to fertilization. With ICSI, these treatments have both been shown, in similar \textit{in vitro} settings with identical activation and culture protocols, to promote sperm decondensation and subsequent pronuclear development after injection into bovine oocytes, increasing rates of fertilization. However, oocytes injected with progesterone treated sperm experience similar rates of DNA fragmentation as controls, whereas oocytes injected with DTT treated sperm have significantly higher rates of DNA fragmentation compared to controls. This suggests progesterone may be a better treatment option for bovine ICSI and further research should be conducted to elucidate the sequential physiological processes the sperm undergo during fertilization and early embryo development to form more successful treatments for bovine ICSI to more readily reflect results seen with bovine IVF.

In the first experiment, the effects of 5mM DTT treatment on bovine spermatozoa were evaluated. Extensive progressive time dependent changes were seen in response to DTT treatment. With sperm going through stages of decondensation until reaching a
fully decondensed state, as DTT continues to reduce disulfide bonds within the outer membranes allowing for relaxation and expansion of the chromatin and sperm nucleus. These stages of decondensation were characterized and observed over a seven-hour time period. These results helped determine appropriate incubation times for further experiments with ICSI.

In the second experiment, the effect of heparin capacitation and progesterone treatment and its’ ability to induce an acrosome reaction was assessed in bovine spermatozoa. Using FITC-PNA to label exposed acrosomes of sperm undergoing an acrosome reaction and flow cytometry, it was determined that progesterone has a positive effect in inducing an acrosome reaction. As the acrosome reaction is a critical process prior to fertilization, it was determined that progesterone may be a viable treatment option for bovine ICSI.

The third experiment evaluated the effect of DTT pretreatment of sperm on fertilization rates in bovine ICSI. It was demonstrated that DTT significantly increased fertilization rates, or the rate of zygotes with 2 pronuclei and no visible sperm head, compared to nontreated sperm. However, cytological observations were made which demonstrated the presence of DNA fragmentation. The occurrence of these abnormalities in DNA staining was higher in zygotes following injection with DTT treated sperm. Possibly signaling a detrimental effect on early embryo development despite increased fertilization rates.

The final experiment studied the effect of progesterone treatment and capacitation of bovine sperm on fertilization rates with bovine ICSI. Using results from experiment 2, incubation time after treatment and morphology of affected sperm were
determined. Using these criteria, ICSI was preformed and fertilization rates were evaluated. It was shown that progesterone increased fertilization rates compared to controls. However, it was also seen after staining that an additional portion of 1PN zygotes which were injected with a progesterone treated sperm was still in the process of sperm decondensation and male pronuclear formation. This indicates asynchronous fertilization and further study and optimization of this technique may be needed. Yet this experiment shows that utilizing the endogenous fertilization pathways of the sperm, such as capacitation and progesterone induced acrosome reaction, can increase rates of fertilization in bovine ICSI.

We can conclude that both DTT and progesterone influence bovine sperm to assist in weakening the outer membranes and allowing accessibility of the male genome. In this study, the effects of both DTT and progesterone sperm treatments have been shown to positively influence fertilization rates in bovine ICSI, resulting in increased numbers of zygotes containing 2 pronuclei and no visible sperm head. However, zygotes injected with DTT treated sperm displayed significantly higher rates of abnormalities in DNA staining, consistent with DNA fragmentation. Such observations were not seen in zygotes injected with control, or progesterone treated sperm. This indicates that while both treatments lead to increased rates of fertilization, progesterone may be a better alternative for bovine ICSI. Further study is needed to assess fragmentation of injected zygotes and further optimization of progesterone treatment may be needed. However, this new protocol for bovine ICSI including the progesterone treatment of sperm has been demonstrated to increase fertilization rates. Utilizing the physiological processes of fertilization may be the key to successfully performing ICSI in
species where ICSI is not currently a viable or cost-effective option. As these processes of fertilization between sperm and oocyte are fairly ubiquitous between species, it is likely this new protocol can be adapted in other species or in situations where sperm decondensation does not readily occur.
APPENDIX.

MEDIA FORMULATIONS AND STOCK SOLUTIONS

**Sperm-TALP**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount/10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm- TL</td>
<td>Cassion IVL03</td>
<td>10 mL</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma 6003</td>
<td>0.03 g</td>
</tr>
<tr>
<td>Sodium Pyruvate 100x</td>
<td>Sigma P4562</td>
<td>100 µL</td>
</tr>
<tr>
<td>Pen/Strep 100x</td>
<td>Sigma P4333</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Prepare day of use, sterile filter (.2 µm) Equilibrate to 37°C and 5% CO\(^2\) before use.

**Hepes- TALP**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount/10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes- TL</td>
<td>Cassion IVL01</td>
<td>10 mL</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma 6003</td>
<td>0.03 g</td>
</tr>
<tr>
<td>Sodium Pyruvate 100x</td>
<td>Sigma P4562</td>
<td>100 µL</td>
</tr>
<tr>
<td>Pen/Strep 100x</td>
<td>Sigma P4333</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Prepare day of use, sterile filter (.2 µm) Equilibrate to 37°C before use.
**Hyaluronidase**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount/10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes- TALP</td>
<td>Cassion IVL01</td>
<td>10 mL</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>Sigma H4272</td>
<td>3 mg</td>
</tr>
</tbody>
</table>

Aliquot into 100 µL, store -20°C, dilute with additional 200 µL hepes-TALP before use.

**DTT 10X stock**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount/5 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm- TL</td>
<td>Cassion IVL03</td>
<td>5 mL</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Sigma D0632</td>
<td>0.0617 g</td>
</tr>
</tbody>
</table>

Aliquot and keep in -20°C 1 month

**5 mM DTT Swim-up solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm- TALP</td>
<td>stock</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>DTT 10x stock</td>
<td>stock</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Prepare day of use. Equilibrate at 37°C and 5%CO²
**Hoechst 10x stock**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (-ca, -mg)</td>
<td>Gibco 14190144</td>
<td>2 mL</td>
</tr>
<tr>
<td>Bisbenzimide</td>
<td>Sigma 14533</td>
<td>0.002 g</td>
</tr>
</tbody>
</table>

Aliquot and keep -20°C away from light for 1 month

**Hoechst staining solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (-ca, -mg)</td>
<td>Gibco 14190144</td>
<td>1 mL</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma 4503</td>
<td>0.03 g</td>
</tr>
<tr>
<td>Hoechst 10x stock</td>
<td>stock</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

Prepare day of use. For staining, add oocytes/embryos to solution for 15 minutes. Keep away from light.

**Heparin 100x Stock**

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (-ca, -mg)</td>
<td>Gibco 14190144</td>
<td>10 mL</td>
</tr>
<tr>
<td>Heparin</td>
<td>Sigma H3149</td>
<td>0.01 g</td>
</tr>
</tbody>
</table>

Filter (.2 μM) and aliquot. Store in -20°C
### Capacitating solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm-TALP</td>
<td>stock</td>
<td>10 mL</td>
</tr>
<tr>
<td>Heparin 100x stock</td>
<td>stock</td>
<td>.100 µL</td>
</tr>
</tbody>
</table>

Prepare day of use. Equilibrate at 37°C and 5% CO²

---

### Progesterone 10 mM Stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>Sigma P8783</td>
<td>0.0157 g</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>Sigma E7023</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

Do not filter. Store 4°C away from light for 1 week

---

### Progesterone 10 µM solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone Stock</td>
<td>stock</td>
<td>1 µL</td>
</tr>
<tr>
<td>Sperm-TALP</td>
<td>stock</td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Prepare day of use. Equilibrate 37°C at 5% CO²
FITC-PNA 100x Stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (-ca, -mg)</td>
<td>Gibco 14190144</td>
<td>4 mL</td>
</tr>
<tr>
<td>Lectin from Arachis hypogaea</td>
<td>Sigma L7381</td>
<td>2 mg</td>
</tr>
</tbody>
</table>

Filter (.2 µm). Store -20°C away from light

FITC-PNA staining solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm-TALP stock</td>
<td>stock</td>
<td>1 mL</td>
</tr>
<tr>
<td>FITC-PNA stock</td>
<td>stock</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Prepare day of use. Incubate 37°C at 5% CO² for 15 minutes for staining.

Ionomycin stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>Sigma D8418</td>
<td>268 µL</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Sigma 10634</td>
<td>1 mg</td>
</tr>
</tbody>
</table>

Aliquot into 5 µL and store -20°C
## Ionomycin step dilution

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heaps-TALP</td>
<td>stock</td>
<td>45 µL</td>
</tr>
<tr>
<td>Ionomycin stock</td>
<td>stock</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

Prepare day of use. Dilute further to working solution

## Ionomycin working solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepes-TALP</td>
<td>stock</td>
<td>990 mL</td>
</tr>
<tr>
<td>Ionomycin step dilution</td>
<td>stock</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Prepare day of use. Equilibrate 37°C

## SOF (synthetic oviductal fluid)

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOF</td>
<td>Cassion IVL05</td>
<td>9.4 mL</td>
</tr>
<tr>
<td>Sodium Pyruvate 100x</td>
<td>Sigma P4562</td>
<td>100 µL</td>
</tr>
<tr>
<td>Pen/Strep 100x</td>
<td>Sigma P4333</td>
<td>100 µL</td>
</tr>
<tr>
<td>BSA</td>
<td>Sigma 6003</td>
<td>0.04 g</td>
</tr>
<tr>
<td>BME 50x stock</td>
<td>Sigma B6766</td>
<td>200 µL</td>
</tr>
<tr>
<td>MEM 100x stock</td>
<td>Sigma M2279</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Prepare day of use. Equilibrate at 38.5°C at 5% CO\(^2\) before use.
### DMAP stock

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMAP</td>
<td>Sigma D2629</td>
<td>32.63 mg</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Heat to dissolve. Aliquot into 10 µL and store -20°C for 1 month.

### DMAP working solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMAP stock</td>
<td>stock</td>
<td>10 µL</td>
</tr>
<tr>
<td>SOF</td>
<td>stock</td>
<td>990 µL</td>
</tr>
</tbody>
</table>

Prepare day of. Equilibrate at 38.5°C at 5% CO₂ before use.


VITA

Lauren Nicole Gatenby was born in Houston, Texas in August 1994. She attended Lawrence E. Elkins High School in Missouri City, Texas, where she graduated in June 2012. She then went on to pursue a B.S. in Animal Science from Louisiana State University in the College of Agriculture. While at LSU, Lauren worked in the lab of Dr. Kenneth Bondioli and obtained a grant for undergraduate research during her Junior year. Lauren graduated LSU in May 2017. Before she continued her education and research.

Lauren began graduate school at Louisiana State University in 2017 under the tutelage of Dr. Kenneth Bondioli. She continued to study and research Reproductive Physiology and Embryo Biotechnology and collaborate on many projects. She is now a candidate for the degree of Master of Science, anticipating graduating August 2020, in Reproductive Physiology from the School of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.