THESIS

NEW METHODS FOR CRYOPRESERVING ROOSTER SPERMATOZOA

Submitted by

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ABSTRACT

NEW METHODS FOR CYROPRESERVING ROOSTER SPERMATOZOA

Rooster spermatozoa sustain permanent membrane damage during cooling, thawing and dilution due to inappropriate changes during the phase transition and osmotic intolerance. Glycerol is a common cryoprotectant used to protect cells during freezing, but its relatively large molecular weight and relatively low membrane permeability (compared to water) creates relatively long aniosmotic conditions when glycerol is added or removed. Glycerol-exposed rooster spermatozoa exhibit poor fertility, therefore, sperm must undergo an extensive dilution and sperm reconcentration process to remove glycerol and mitigate its negative effects on sperm. Alternative cryoprotectants with lower molecular weight than glycerol cross the membrane more quickly and induce less osmotic damage. Cryoprotectants that do not need to be removed from spermatozoa prior to insemination may eliminate the need for processing the sperm after thawing.

The first experiment determined the percentages of motile and membrane intact cells of frozen/thawed spermatozoa cryopreserved in different cryoprotectants. Samples exposed to glycerol exhibited higher percentages of total motile and membrane-intact sperm (54% and 58%, respectively) compared with sperm treated with methylacetamide (48% & 53%, respectively), dimethylacetamide (41% & 45%, respectively), ethylene glycol (38% and 39%, respectively), or methylformamide (28% and 37%, respectively; $P < 0.05$).

Exposure of sperm to a cryoprotectant for a prolonged period of time may have negative effects on the sperm. Rooster sperm, when thawed for insemination, may be kept undiluted and exposed to cryoprotectant for an extended period of time before used. The second experiment evaluated if exposing sperm to methylacetamide for a prolonged period of time affected sperm motility. Exposure of sperm to methylacetamide did not decrease the percentage of total motile sperm over a 1 hour period (> 50%, $P > 0.05$).

When sperm are cooled from 5°C to -196°C, membrane damage occurs due to membrane changes during the phase transition. This damage, for sperm from many species, can be mitigated by adding cholesterol or unsaturated phospholipids to the membrane. In the next set of experiments, cholesterol-loaded cyclodextrins (CLC) did not change rooster sperm osmotic tolerance limits or improve cryosurvival rates, even though cellular cholesterol levels were increased 2-fold. Similarly, treating sperm with lipid-loaded cyclodextrins (LLC) failed to improve sperm cryosurvival rates.

Diluent composition, cryoprotectant, sperm concentration and freezing procedure affect rooster spermatozoa cryosurvival, with each diluent having its own freezing protocol. Two different diluents, a glutamate-based and a trehalose-based diluent were used to cryopreserve rooster spermatozoa, since sperm in the latter diluent has been reported to have increased fertility when left undiluted with an methylacetamide.

The last set of experiments evaluated the percentages of total motile cells of frozen/thawed when glycerol or methylacetamide were used to cryopreserve rooster spermatozoa in trehalose-based or glutamate-based diluents packaged in 0.5-mL or 0.25-mL straws. Samples frozen in 0.5-mL straws exhibited higher percentages of motile cells (> 45%) than sperm frozen in 0.25-mL straws (< 45%; $P < 0.05$). Within the samples frozen in 0.5-mL straws, sperm cryopreserved in trehalose-based diluent diluent exhibited higher percentages of motile cells (> 60%) than sperm frozen in glutamate-based diluent with glycerol as the cryoprotectant (46%; $P < 0.05$).

The last experiment evaluated the percentages of motile and membrane-intact cells of frozen/thawed glycerol-exposed or methylacetamide-exposed rooster spermatozoa in trehalose-based or glutamate-based diluents. These sperm were frozen in 0.5-mL or 0.25-mL straws at two different sperm concentrations, 500 million cells/mL or 1 billion cells/mL. Sperm cryopreserved in trehalose-based medium and packaged in 0.5-mL straws can be frozen at 1 billion cells/mL or 500 million cells/mL, indicating that sperm can be effectively frozen at sufficiently high concentrations to permit freezing, thawing and inseminating sperm without having to concentrate the sperm prior to insemination. If

methylacetamide does not have to be removed from the sperm prior to insemination, this technique could be easily incorporated by breeders and researchers to use frozen sperm.

Rooster spermatozoa are unique cells that can tolerate a variety of environments when cooled, but after cryopreservation, many different changes occur to the sperm membrane that can cause infertility. Methylacetamide-exposed sperm elicit smaller osmotic changes during cooling, thawing and dilution, has been shown to increase sperm cryosurvival.

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CHAPTER I

REVIEW OF PUBLISHED LITERATURE

Introduction

Rooster spermatozoa cryopreservation, although successful, is associated with extremely variable fertility (Lake, 1986; Hammerstedt & Graham, 1992; Buss, 1993; Long, et al., 2010). Therefore, applying cryopreserved rooster spermatozoa outside of cryopreservation research has been unsuccessful (Fulton, 2006). Much of the unique poultry genetics were established and maintained at land-grant institutions. However, over the past few decades, as research funding has declined and as animal science and poultry science departments have merged, these unique poultry genetics are being lost due to increasing costs in maintaining them and to decreasing interest (Fulton, 2006). Approximately 40% (238 lines) of these unique poultry lines have been lost in the United States and Canada since 1984 (Pisenti, 1999). Greater fertility for cryopreserved rooster spermatozoa is needed, and if improved cryopreservation techniques are developed, they can be used to efficiently preserve these valuable poultry genetics and enable sharing these genetics around the world, through shipping cryopreserved spermatozoa.

Chickens were one of the first species to have sperm cryopreserved and offspring produced using artificial insemination of frozen sperm (Shaffner, et al., 1941). Polge, et al. (1949) successfully cryopreserved rooster sperm in 1949 using glycerol as the cryoprotectant. This cryoprotectant would eventually become the most commonly used cryoprotectant to preserve sperm from all livestock species (Davis, et al., 1963; Foote, 1998; Squires, et al., 2004; Purdy, et al., 2010; Malo, 2011). Glycerol, however, exhibits contraceptive effects to rooster spermatozoa when inseminated into the hen reproductive tract and, therefore, it must be reduced to a concentration of less than 1% (v:v) before the spermatozoa are artificially inseminated (Lake, 1986; Hammerstedt & Graham, 1992). There are currently three methods used to remove glycerol from the sperm prior to insemination: 1) gradient centrifugation; 2) dialysis; and 3) step-wise dilutions with centrifugation (Long, et al., 2004; Lake, 1986; Tajima, et al.,

1989; Phillips, et al., 1996). All of these methods can be detrimental to the rooster spermatozoa, and are labor intensive, making industry adaptation impractical (Lake, et al., 1984; Phillips, et al., 1996; Purdy, et al., 2009).

In cryopreserving sperm from other species, alternative cryoprotectants have been utilized to decrease the osmotic stresses the spermatozoa encounter during addition and dilution of the cryoprotectant (Squires, et al., 2004). Cryoprotectants with lower molecular weights than glycerol cross the spermatozoon membranes faster than glycerol and, therefore, do not induce the amount of osmotic damage that glycerol does, as the length of time sperm are exposed to osmotic imbalances is reduced, as well as the magnitude of the imbalance (Squires, et al., 2004). The objective of the first part of this thesis was to determine if utilizing alternative cryoprotectants could increase the cryosurvival of rooster spermatozoa.

Cooling spermatozoa induces a plasma membrane phase transition, causing irreversible membrane damage (Amann & Pickett, 1987). Spermatozoa that contain high levels of cholesterol and/or unsaturated lipids do not readily undergo this phase transition, and do not exhibit extensive membrane damage (Parks & Lynch, 1992). New technologies that incorporate cholesterol and/or unsaturated lipids into sperm membrane ultimately increase the cryosurvival of the spermatozoa (Purdy & Graham, 2004b; Moore, et al., 2005; Mocé, et al., 2010; Spizziri, et al., 2010). The objectives of part of this thesis (chapter 3 & 4) determined if incorporating cholesterol or unsaturated lipids into rooster spermatozoon plasma membranes could increase the cryosurvival of cryopreserved rooster spermatozoa.

The environment in which spermatozoa are maintained after ejaculation plays an important role in their survival (Wishart, 1989). Many different rooster sperm cryopreservation diluents have been developed, and each requires its own specialized protocol, utilizing different freezing rates, straw size, and sperm concentration (Lake & Stewart, 1978; Caudhuri & Lake, 1988; Tajima, et al., 1989; Sasaki, et al., 2010). Cooling rate has been correlated with sperm membrane damage, as it is partially responsible for the osmotic stresses placed on the spermatozoa (Oldenhof, et al., 2012). In order for a freezing protocol to be effective, the sperm must cool sufficiently slow enough to allow cryoprotectant and water

equilibration to occur (Hammerstedt, et al., 1990). This ultimately leads to less membrane damage due to smaller osmotic gradients created as the cryoprotectant equilibrates into the sperm cell (Amann & Pickett, 1987). The objective of the fourth part of this thesis was to determine if cooling rate, diluent and sperm concentration affect the cryosurvival of rooster spermatozoa.

Rooster Spermatozoon Structure

Spermatozoa are highly differentiated haploid cells that have highly condensed chromatin and limited capacity for biosynthesis and cell repair. Rooster spermatozoa are homogametic. The cells are approximately 0.5-μm at their widest point and approximately 100-μm in length, and an approximate volume of 10-μm^3 (Etches, 1996). A spermatozoon has four different regions: head, midpiece, principle piece and an endpiece. Each region of the spermatozoon plays an important role in fertilizing an oocyte.

The rooster spermatozoon head contains an acrosomal cap, acrosomal spine and nucleus. Both the acrosomal cap and acrosomal spine originate from the Golgi apparatus during spermatogenesis, and the acrosome contains the proteolytic enzymes needed during fertilization (Amann & Graham, 1993). The acrosomal cap sits atop the acrosomal spine and surrounds it. Just posterior to the acrosomal region is the nucleus, which contains the cell's highly condensed chromatin (Barth & Oko, 1989). The shape of the nucleus determines the shape of the spermatozoon, and the rooster spermatozoon's nucleus is thin, long and cylindrical, giving the head of the cell a bullet-like shape (Etches, 1996).

The midpiece, principle piece and endpiece contain the mitochondria and cytoskeleton of the cell to provide motility to the functional spermatozoa (Etches, 1996). The head and midpiece are attached by the proximal centriole, which is tightly fused to the distal centriole. The distal centriole makes up the core of the midpiece, and anchors the two central fibers (axoneme) that are part of the endpiece. The axoneme is surrounded by nine tubules that form the axial filament complex. This complex is surrounded by mitochondria, which provide the spermatozoon with energy metabolism capabilities through ATP production (Etches, 1996). The annulus at the end of mitochondrial ring discriminates the transition from caudal midpiece to principle piece. The axial filament complex extends into the principle piece, and the

rigidity of these fibers is needed for normal spermatozoon tail motion (Amann & Graham, 1993). The axial filament complex contains only axoneme and plasma membrane, which distinguishes the caudal principle piece from the end piece.

Plasma Membrane

The spermatozoal plasma membrane is the outermost membrane of the cell, and completely surrounds the spermatozoon (Barth & Oko, 1989). It consists of a lipid bilayer made up of polar phospholipids, cholesterol and proteins, a phospholipid-H_2O interface on the exterior of the cell and the glycocalyx, the outer-most region of the plasma membrane (Hammerstedt & Graham, 1992).

Individual phospholipids in the lipid bilayer are composed of a hydrophilic head piece and hydrophobic fatty acyl chains, leading to an arrangement of hydrophilic heads on external surfaces of the membrane and hydrophobic chains on the internal surface of the membrane (Amann & Graham, 1993). The phospholipid-H_2O interface is the primary site for all membrane transport, while the glycocalyx contains adsorbed proteins, and is the primary site for cell-cell contact recognition (Hammerstedt & Graham, 1992). The rooster spermatozoon membrane is composed of approximately 50% protein by weight, considered either integral (intrinsic) or peripheral (extrinsic) proteins (Amann & Graham, 1993). Integral proteins are tightly bound to the membrane, with some stretching across the lipid bilayer. Some integral proteins function as pores, channels or receptors, depending on their orientation in the membrane (Amann & Pickett, 1987). Peripheral proteins, in contrast, have little permanent binding to the membrane (Robertson, 1983), and can be easily removed from the membrane via mild salt or chemical treatments (Amann & Graham, 1993). The outer glycocalyx can change in different environments, as some proteins are only loosely bound by net negative charge attraction to the membrane. These adhered proteins are integral to the glycocalyx's response to its environment (Amann & Graham, 1993).

Spermatozoan plasma membranes are composed of heterogeneous membrane domains, and each part of the spermatozoon membrane has a unique lipid and protein composition that is needed during specific points during fertilization (Israelachvili, 1978; Hammerstedt & Graham, 1992). Within each

membrane domain, phospholipids are arranged randomly, forming a bilayer. This allows the non-polar region inside of the bilayer to act as a barrier, hindering the passage of water-soluble molecules. Membrane phospholipids can move laterally in the cell membrane within their particular bilayer domain (Amann & Graham, 1993). This lateral movement allows for the random arrangement of phospholipids at any given time.

Rooster sperm plasma membranes consist of many different phospholipid species, which are distinct from their mammalian counterparts. They have a lower protein to phospholipid ratios of 0.46 (w/w), compared to boar (1.26), bull (0.80), and stallion sperm (0.86), and therefore are more resistant to cold shock when reduced to temperatures around 5°C, than are mammalian spermatozoa (Wales, et al., 1959; Pickett, et al., 1967; Parks & Lynch, 1992). However, the rooster spermatozoal plasma membrane, while stable, is inelastic (Roberston, 1984), and when the sperm are exposed to hypotonic conditions, membranes can easily rupture.

Temperature plays a critical role in the behavior of the plasma membrane (Mazur, 1984). Membranes are fluid at body temperature, allowing lipids and proteins to move freely throughout their domains. As membranes are cooled to 5°C, their lipids undergo a phase transition, becoming rigid, with phospholipid and protein rearrangement (Hammerstedt & Graham, 1992). This is especially important because phospholipids that are associated with specific membrane proteins will dissociate from their associated proteins and congregate together, rendering many of these proteins nonfunctional (Amann & Graham, 1993). Once this phase transition occurs, the membrane becomes rigid, fragile, and more susceptible to damage. In addition, as cells are cooled, lipid/protein rearrangements occur, and upon warming they return to their more fluid state, but the phospholipids and proteins may not return to their original arrangements. This may lead to cell dysfunction and death (Hammerstedt, et al., 1990).

Membrane fluidity is dictated partially by the cholesterol:phospholipid ratio of the plasma membrane. Spermatozoon membranes with high cholesterol:phospholipid ratios (rabbit 0.88; human 0.99) have more rigid membranes at room temperature than at lower temperatures than sperm with low membrane cholesterol:phospholipid ratios, but as these cells are cooled during cryopreservation, the high

cholesterol content makes the membranes more fluid, and they incur less membrane damage than membranes from species that have low cholesterol:phospholipid ratios (boar: 0.26; bull: 0.45; rooster: 0.30; stallion: 0.23; Darin-Bennett & White, 1977; Parks & Lynch, 1992).

Recently, Morris, et al. (2011) has found that it is a combination of intracellular protein content and osmotic shrink which causes intracellular vitrification of spermatozoa during cooling, leading to osmotic shock when sperm are thawed and subsequent membrane damage. The sperm membrane is the first barrier for the sperm against the outside environment, and when this membrane is altered negatively, this can lead to an altering of the sperm's intracellular environment and cell dysfunction. Yet, when the sperm plasma membrane is altered positively, by increasing plasma membrane fluidity at lower temperatures, this in turn increases cell cryosurvival. Therefore, focusing research on plasma membrane manipulation and alternative cryoprotectants are the key to improving rooster spermatozoa cryosurvival. Altering membrane fluidity will be further discussed in subsequent sections.

Cryopreservation of Rooster Spermatozoa

As mentioned previously, although rooster sperm were cryopreserved in 1941, it was not until 1949 that Polge, et al., were able to produce live chicks from frozen/thawed rooster sperm. This work led to further research of rooster spermatozoa cryopreservation. It was also during that research that glycerol was discovered to be an effective cryoprotective agent, and that cooled rooster spermatozoa exposed to glycerol showed the same high motility as fresh rooster sperm controls (Polge, et al., 1949). However, cooled rooster sperm exposed to 10 to 15% glycerol did not survive being placed into the hen reproductive tract by artificial insemination even though they exhibited high percentages of motile cells (Smith & Polge, 1950). This contraceptive effect caused by glycerol on rooster sperm has since been well documented (Neville, et al., 1971; Westfall & Howarth, 1977; Hammerstedt & Graham, 1992). Polge (1951) developed a dialysis method to reduce the amount of glycerol of the sperm which improved the fertility of the frozen/thawed rooster sperm.

In 1964, Shaffner presented a new method to remove glycerol from the sperm by slowly diluting the cells and then reconcentrating the sperm by centrifugation. This method produced higher fertility rates than dialysis, but was time consuming and labor intensive. Further research demonstrated that the metabolic activity of glycerol-treated rooster sperm decreased when they were diluted, centrifuged and resuspended in fresh diluent (Clark & Shaffer, 1960). This dilution technique exposes the rooster sperm to a hypotonic environment; therefore, care must be taken to maintain the sperm membrane from rupture during osmotic swelling. In addition, sperm must be frozen at very high cell concentrations, since 500 to 700 million cryopreserved sperm are needed for insemination than that needed for fresh spermatozoa (50 to 100 million) to achieve similar fertility (Sexton, 1981; Lake, 1986). This increase in concentration has been shown to effect sperm cryosurvival, creating an environment that must be diluted prior to insemination for sperm to survive (Shannon, 1965).

Since 1970, almost all rooster spermatozoa cryopreservation protocols have utilized glycerol or dimethylacetamide as the cryoprotectant, and utilize a step-wise dilution procedure to reduce glycerol from the sperm prior to artificial insemination (Bacon, et al., 1986; Chalah, et al., 1999; Woelders, et al., 2006; Blesbois, et al., 2007). Replacing glycerol with dimethylacetmide eliminates the need for cryoprotectant removal, since dimethylacetamide readily crosses the rooster sperm membrane. High fertility rates for studies using this technique have been achieved by inseminating birds multiple times per week (Lake & Ravie, 1984; Lake, 1986; Purdy, et al., 2009). Yet with high fertility rates, this technique is complicated by the fact that dimethylacetamide is toxic to sperm and requires time sensitive processing. This problem makes this technique impractical for the commercial poultry industry.

Current Status of Cryopreservation

Currently, no artificial insemination using cryopreserved rooster spermatozoa is being used in the commercial poultry industry in the United States (Donoghue & Wishart, 2000). Even with the research that has been conducted over the years, little advancement has been made in rooster sperm cryopreservation. Researchers have focused on improving glycerol removal techniques using Percoll

gradients and a one-step dilution with centrifugation, instead of the classic step-wise dilution (Long & Kulkarni, 2004; Purdy, et al., 2009). However, these methods have shown that intramagnal fertility is optimal, requiring invasive techniques to inseminate hens. Compared to the classic step-wise dilution and centrifugation to remove glycerol, the Percoll method will expose rooster spermatozoa to a low osmotic environment almost instantaneously that will increase membrane damage due to swelling (Hossain, et al., 1999).

Recent studies have investigated low molecular weight cryoprotectants that would not need to be removed from the rooster spermatozoa prior to insemination (Van Voorst & Leenstra, 1995; Tselutin, 1999; Blesbois, et al., 2007; Sasaki, et al., 2010). These cryoprotectants show promise, Sasaki, et al. (2010) reported an 82% overall fertility rate, and 90% hatchability when rooster sperm were treated with methylacetamide and inseminated with the cryoprotectant left with the sperm. Sasaki et al. (2010) also reported a diluent for freezing rooster spermatozoa, developed previously by Hanzawa, et al. (2010), as well as a freezing method that utilizes 0.5-mL plastic straws. This method contrasts current freezing methods, which utilize 0.25-mL plastic straws (Lake, et al., 1978). This new method of freezing rooster spermatozoa could be easily incorporated into the industry, as straws would merely need to be thawed and the sperm inseminated.

Cryobanking valuable poultry genetics across the world has become a pertinent issue and there is a need for establishing genetic reservoirs of cryopreserved livestock gametes, embryos and other tissues (Boettcher, et al., 2005; Blackburn, 2006; Woelders, et al., 2006; Danchin-Burge, et al., 2011). Other technologies, including poultry gonadal tissue cryopreservation, are emerging as other methods to preserve valuable genetics from both male and female chickens (Song & Silversides, 2008). This technology is very labor-intensive and expensive, leaving improvement for adaption outside the research laboratory, but has high potential for use in cryobanking.

Principles of Cryopreservation

Research over the past half century has explored techniques and principles of low-temperature biology to effectively preserve gametes (Lake, 1986; Foote, 1998; Foote, 1999). As stated previously, rooster spermatozoa were the first sperm of any species to be frozen at low temperatures (-76°C), using a carbon dioxide-alcohol mixture (Shaffner, et al., 1941). Additional research has focused on increasing the cryosurvival of sperm by understanding the cryodamage caused by osmotic stress, intracellular and extracellular ice formation and plasma membrane responses to cooling throughout the freezing process (Tselutin, et al., 1999; Purdy & Graham, 2004a; Squires, et al., 2004).

The steps to cryopreserve rooster spermatozoa include: 1) cooling the sperm to 5°C; 2) adding the cryoprotectant to the sperm, 3) freezing the sperm; 4) storing the sperm in liquid nitrogen; and 5) thawing and diluting the sperm (Buss, 1993). In step 1, freshly collected sperm are immediately diluted with diluent (Burrows and Quinn, 1937; Lake, et al., 1978; Sasaki, et al., 2010). This dilution keeps sperm alive for long periods of time and the sperm are cooled from body temperature (41.5°C) to 5°C over ice (Mazur, 1984). Temperature-induced plasma membrane phase transition changes occur to sperm from many species during this step, and these changes can result in membrane damage (Hammerstedt, et al., 1990). This is the point where most membrane damage occurs to many types of sperm cells, although in rooster sperm, this is not the case since they do not undergo cold shock (Steponkus, et al., 1983; Parks & Lynch, 1992). Cold shock is defined as the insult a sperm undergoes when cooled too quickly prior to freezing (Pickett & Komarek, 1967).

During step 2, the cryoprotectant is added to the cells, and the sperm will experience osmotic stress as the cryoprotectant creates an extracellular hyperosmotic environment. Water will move out of the sperm, causing the cell to shrink (Hammerstedt, et al., 1990). It is important during this time to make sure there is adequate time for cryoprotectant equilibration. Cryoprotectants do not cross the membrane as fast as water, and therefore time must be allowed for water and cryoprotectant equilibration (Amann &

Pickett, 1987). Equilibration is completed in approximately 2 minutes, and the sperm packaged into either 0.25-mL or 0.5-mL polyvinylchloride straws, depending on the method being utilized (Hammerstedt, et al., 1976, Lake, et al., 1978 & Sasaki, et al., 2010).

During step 3, the rooster sperm are frozen in liquid nitrogen vapor (Lake, et al., 1978). Cooling between -6°C to -15°C is the most critical part of freezing, as this is when water in the diluent is transitioning to the crystalline state (Mazur & Cole, 1989; Hammerstedt, 1995). Unfrozen channels remain between the ice crystals which contain high salt concentrations. Spermatozoa that are able to tolerate the high salt environment survive in these unfrozen channels. Since the spermatozoa that will survive freezing are all found in these unfrozen channels, its volume is critical to survival of the sperm (Amann & Pickett, 1987; Mazur & Cole, 1989). The volume of the cell must be large enough to support cell function intracellularly, but small enough to keep the membrane phospholipids in a bilayer configuration (Amann & Graham, 1993). High salt concentrations in the unfrozen channels damage the sperm (Quinn, 1989; Hammerstedt, 1990). Cryoprotectants lower the freezing point of the diluent, increase the volume of the unfrozen fraction, decrease high salt concentrations in this fraction and alter the formation of ice crystals (Amann & Pickett, 1987). This ultimately increases the cryosurvival of sperm residing in this high salt environment.

Once the spermatozoa reach -50°C, the unfrozen fraction and the sperm vitrify and become inert. Therefore, sperm can be plunged into liquid nitrogen after reaching -50°C, and can be held in storage at -196°C indefinitely (Step 4; Graham, 1996). When samples are ready to be used, rooster sperm are thawed in 5°C water for most methods (Lake, et al., 1978; Sasaki, et al., 2010).

Step 5 is one of the most critical steps in the cryopreservation process, due to the large volume changes the sperm will experience during dilution (Griffiths, et al., 1979). In this step, sperm will experience osmotic stress as well as membrane damage. As the sperm are thawed, their membranes will leave their vitrification state, as well as undergo the membrane phase transition to a more fluid state. Here, membrane damage from previous steps will be manifest when the plasma membrane reverts back to its normal state (Hammerstedt, et al., 1990).

After thawing, the sperm will be diluted. This extracellular hypoosmotic environment causes water influx, and the sperm swell (Griffiths, et al., 1979). Depending on the cryoprotectant used, water can enter the cell beyond the point of membrane volume expansion tolerance (membrane tolerance), and the sperm cell will lyse. This is due to unequal movement of the cryoprotectant and water across the plasma membrane (Amann & Pickett, 1987). Sperm that are improperly diluted, with more water being added to the sperm's extracellular environment, can also cause membrane volume expansion past tolerance level to the point of lyse.

Osmotic Stress

Adding cryoprotectants to or removing cryoprotectants from the sperm induces osmotic gradients across sperm membranes, which cause the sperm to shrink or swell, respectively, which can ultimately irreversibly damage the cell and possibly cause cell death (Blanco, et al., 2008). When cryoprotectants are added to the cells, a hyperosmotic environment is created, and since water crosses the membrane faster than does the cryoprotectant, water quickly exits the spermatozoon due to osmosis (Amann & Graham, 1993). As the cryoprotectant reaches equilibrium, water equilibrates with it, and cell volume is restored.

When a cryoprotectant-loaded spermatozoon is diluted without glycerol in the diluent or exposed to the female reproductive tract, it is exposed to a hypoosomotic environment. As described above, because cryoprotectant crosses the membrane slower than water, water will enter the spermatozoon, causing the sperm to swell. Spermatozoa will return to their normal size once the cryoprotectant leaves the cell (Amann & Graham, 1993). However, if membrane tolerance is exceeded, the spermatozoon plasma membrane will rupture, causing cell lysis. Not only can irreversible damage from cellular swelling and shrinking affect the plasma membrane, but internal cell organelles maybe similarly affected (Willoughby, et al., 1996).

Rooster sperm have a lower-limit of osmotic tolerance of 17 mOsm, although its upper osmotic limit has not been characterized (Watson, et al., 1992). This critical osmolality of 17 mOsm for rooster sperm is defined as the osmolality at which 50% of the cells have swollen to the point of rupture of the

plasma membrane. The critical osmolality of sperm differs between species, and the ability of sperm to survive these osmotic stresses and volume changes that occur affect their ability to survive the freezing process (Hammerstedt, et al., 1990).

In theory, the rooster spermatozoon's cylindrical-tapered shape should accommodate larger amounts of cell volume change to a sphere-shape without rupturing (Watson, et al., 1992). In contrast, it has been observed that a rooster plasma membrane swells over both the head and tail (Bakst, 1980), and when one part of the membrane does not accommodate to larger amounts of cell volume, this leads to the plasma membrane bursting sooner than theorized (Watson, et al., 1992). Since the upper limit of osmotic tolerance for rooster sperm is unknown, both theories could prove true until this limit is uncovered.

Cryoprotectants

As sperm are cooled, both extracellular and intracellular ice form. Although intracellular ice is a major cause of cell death during freezing for most cells, does not have the same effect on sperm (Mazur, 1984; Morris, et al., 2007). Two types of cryoprotectants, penetrating and non-penetrating, are added to cells to protect than from freezing damage (Doebbler, 1966; Meryman, 1971). Non-penetrating cryoprotectants, including sugars such as lactose and sucrose, proteins, and polymers such as methylcellulose, do not cross the sperm plasma membrane, and therefore increase the extracellular hyperosmotic gradient and dehydrate the cells, limiting the chance of intracellular ice formation (Morris, et al., 2007). Penetrating cryoprotectants, (glycerol, dimethylacetamide, dimethyl sulfoxide) cross the plasma membrane and enter the cell. These penetrating cryoprotectants protect the sperm by displacing intracellular water thus minimizing the formation of intracellular ice formation. Furthermore, penetrating cryoprotectants also increase the unfrozen fraction of the solution, and reduce salt concentration in this fraction (Amann & Pickett, 1987). Non-penetrating cryoprotectants are usually used in high concentrations when freezing rooster sperm to lower the freezing point of the diluent and increase the

plasma membrane temperature resistance (Phillips, et al., 1996). These cryoprotectants displace water in the cell, preventing the formation of intracellular ice. However, the mechanisms by which these occur are not fully understood (Amann & Pickett, 1987).

Glycerol is a commonly used cryoprotectant to freeze rooster spermatozoa (Hammerstedt & Graham, 1992; Buss, 1993; Gill, et al., 1996; Sasaki, et al., 2010). Even though rooster sperm diluted in solutions containing glycerol have motility and membrane integrity that are comparable to unfrozen rooster sperm, glycerol severely reduces sperm fertilizing potential (Tajima, et al., 1989; Hammerstedt & Graham, 1992; Phillips, et al., 1996). Glycerol has a molecular weight of 92, and because it is much larger than water, it crosses the plasma membrane more slowly than water (Gao, et al., 1995). This causes cells during glycerol addition and removal to experience osmotic stress that leads to membrane damage by cell shrinkage and swelling (Hammerstedt & Graham, 1992). In contrast, cryoprotectants that are smaller than glycerol cross the plasma membrane more rapidly than glycerol and subsequently cause less osmotic excursions to occur (Squires, et al., 2004; Purdy, et al., 2010; Sasaki, et al., 2010). These low molecular weight cryoprotectants, with molecular weights of less than 90, can increase mammalian sperm cryosurvival rates and fertility compared to glycerol, as well as eliminate the need for post-thaw processing and cryoprotectant removal (Nagase, et al., 1972; Hanada & Nagase, 1980; Schmehl, et al., 1986; Dalimata & Graham, 1997; Squires, et al., 2004).

Alternative cryoprotectants have been used to cryopreserve rooster spermatozoa, with limited success (Lake & Ravie, 1984; Mitchell & Buckland, 1975; Westfall & Harris, 1975; Graham, et al., 1982; Hammerstedt & Graham, 1992, Phillips, et al., 1996). Most alternative cryoprotectants do not protect the rooster sperm equal to that of glycerol during freezing, with few exceptions. Recently, Sasaki, et al. (2010) utilized methylacetamide to cryopreserve rooster sperm, and achieved relatively high fertility rates of greater than 80%. Further benefits of the use of alternative cryoprotectants will be discussed further in succeeding sections.

Cholesterol & Unsaturated Lipids

When membranes are cooled, they not only undergo a membrane phase transition, but they also lose lipids from the membrane (Pickett & Komarek, 1967). This lipid loss can cause membrane disruption, lipid and protein rearrangement within the membrane and possible cell death (Chakrabarty, et al., 2007). Much of the ability of sperm to withstand this cooling damage and osmotic damage is dictated by composition of cholesterol and phospholipids in the plasma membrane (Amann & Pickett, 1987; Hammerstedt, et al., 1990; Watson, 2000).The membrane cholesterol:phospholipid ratio plays an important role in the ability of sperm to withstand cooling and freezing damage (Darin-Bennett & White, 1977; Watson, 1981). Cholesterol decreases the membrane transition temperature, which helps maintain a fluid membrane at low temperatures, thereby increasing plasma membrane stability and decreasing cold-induced membrane damage (Quinn, 1989).

Human spermatozoa have a high cholesterol:phospholipid ratio of 0.99 (w/w), and therefore do not easily cold shock (Darrin-Bennett & White, 1977; Davis, 1981). Spermatozoa from most of our domestic species, however, have much lower cholesterol:phospholipid ratios (bull: 0.45, boar: 0.26, stallion: 0.36), and these sperm are much more sensitive to cold shock (Watson, 1981; Parks & Lynch, 1992; White, 1993). It has been shown that increasing the cholesterol content of these plasma membranes that contain low cholesterol levels, the membranes become more tolerant to osmotic damage during cooling, increasing cryosurvival rates (Purdy & Graham, 2004a; Moore, et al., 2005; Mocé & Graham, 2006; Peris, et al., 2007; Mocé, et al., 2010; Spizziri, et al., 2010). Rooster sperm have a low cholesterol:phospholipid ratio (0.30), but are also resistant to cold shock. The major glycolipid portion of rooster sperm are different than that of mammalian sperm, which could account for the cold shock resistance (Parks & Lynch, 1992).

Cholesterol-Loaded Cyclodextrins (CLC)

Cyclodextrins are cyclic oligosaccharides formed from starch through cyclodextrin glycosyltransferase action. Glycosyltransferases are enzymes that form glycosidic bonds by transferring

specific parts of a sugar from a donor molecule to a specific acceptor molecule (Jouni, et al., 2000). They come in 3 different structures based on the number of D-glycopyranose units make up the cyclic oligosaccharide units; α cyclodextrins contain 6 units, β cyclodextrins contain 7 units and γ cyclodextrins contain 8 units. Different side chains can be attached to the cyclodextrins which affect their solubility and their ability to interact with the glycocalyx of cell membranes (Kilsdonk, et al., 1995). Cyclodextrins have a hydrophilic surface and a hydrophobic core; this allows the molecule to be soluble in aqueous solutions but allows hydrophobic molecules to solublize in the cyclodextrin. Hydrophobic molecules in the cyclodextrin can be "solublized" in an aqueous solution, without structural change (Hu, et al., 2005).

Cyclodextrins have been used extensively in the pharmaceutical industry to deliver insoluble or biodegradable compounds to cells (Ortiz Mellet, et al., 2011). Methyl-β-cyclodextrin has been used in the laboratory to remove cholesterol from cell plasma membranes (Christian, et al., 1997). While all three cyclodextrin sizes can hold cholesterol and lipids, only α-cyclodextrin and β-cyclodextrin are soluble in methanol, which facilitates loading hydrophobic molecules into the cyclodextrin easily. However, γ-cyclodextrin is soluble only in water, making incorporation of lipids and cholesterol into this cyclodextrin more difficult (Graham, unpublished).

The β-cyclodextrins have greater affinity to transport sterols down a concentration gradient than α-cyclodextrins or γ-cyclodextrins (Yancey, et al., 1996; Christian, et al., 1997; Ohvo, et al., 1997; Choi & Toyoda, 1998; Cross, 1999). In addition, methyl-β-cyclodextrin has the greatest capacity to transport cholesterol, and can incorporate greater amounts of cholesterol than other cyclodextrins (Christian, et al., 1999). Cyclodextrins can be pre-loaded with cholesterol (cholesterol-loaded cyclodextrins; CLC) or other lipids and can transport these lipids down a concentration gradient into plasma membranes, increasing the level of these lipids within membranes (Klein, et al., 1995; Purdy & Graham, 2004a;b; Moore, et al., 2005; Mocé & Graham, 2006; Amidi, et al., 2010; Mocé, et al., 2010; Spizziri, et al., 2010).

In 2000, CLCs were first used to increase the cholesterol content of stallion spermatozoa membranes, resulting in higher percentages of motile and viable cells after cryopreservation (Combes, et al., 2000). CLCs have been subsequently used to cryopreserve bull (Purdy & Graham, 2004a), stallion

(Moore, et al., 2005), ram (Mocé, et al., 2010), rat (Graham, unpublished) and most recently boar (Blanch, et al., 2012) sperm. As stated, CLCs have been shown to improve cryosurvival rates for a variety of sperm that have low cholesterol:phospholipid ratios. It is not known if CLCs will benefit rooster spermatozoa during cryopreservation.

Lipid-Loaded Cyclodextrins (LLC)

Phospholipid, more specifically their fatty acids, and cholesterol concentration of a membrane affects the fluidity and osmotic tolerance of the membrane (Darrin-Bennett & White, 1977). Rooster spermatozoa have a different plasma membrane composition than mammalian species. Rooster sperm have a low protein:phospholipid ratio, as well as a low cholesterol:phospholipid ratio, but also a high level of unsaturated fatty acids in their phospholipid composition (Parks & Lynch, 1992). Since cyclodextrins can solublize and transport lipids, as well as cholesterol, by preloading them with lipids containing unsaturated fatty acids one can attempt to alter the sperm plasma membrane fluidity. Increasing the level of unsaturated fatty acids should cause the membrane to remain fluid at lower temperatures. The lipids used in the experiments presented in this thesis were the synthetic lipids 1,2-dilinolenoyl-sn-glycero-3-phosphocholine: (PC 18:3), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine: (PC 18:2), and 1,2-dilinoleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt): (PG 18:2). All three phospholipids have two fatty acid chains that are 18 carbons in length with either 2 or 3 unsaturated bonds per chain. The unsaturated bonds located on the fatty acid chains provide a kink to decrease membrane compaction during cooling, unlike saturated fats, which contain no unsaturated bonds, allowing for membrane compaction during cooling (Haines, 1978). These unsaturated lipids should increase membrane fluidity at low temperatures, and this has been shown to increase cell cryosurvival (Quinn, 1989).

Sperm Quality Evaluation Methods

Over the past millenia, methods have been developed to evaluate sperm function in vitro and correlate these data with the fertilizing capability of sperm (Williams & Savage, 1927; Moench & Holt, 1931; Graham, et al., 1990; Graham & Mocé, 2005). Although the only way to truly assess sperm fertilizing potential is to perform a fertility trial, this assay is often inaccessible due to time or cost constraints. Therefore, in vitro sperm quality evaluation methods have been developed to measure the quality parameters in vitro. Spermatozoa can be evaluated for motility, morphology, cell characteristics by flow cytometry, perivitelline and oocyte membrane binding and penetration assays, and in vitro fertilization (Graham, et al., 1990; Bramwell & Howarth, 1992; Carrell, 2000; Graham & Mocé, 2005). These assays can estimate the overall effects that treatments have on the in vitro quality of the rooster sperm, conforming to inaccessibility to perform a fertility trial.

Motility

A spermatozoon must display normal motility in order to fertilize an oocyte in vivo (Graham, 2001). However, motility analyses are not highly correlated with fertility, as many other factors contribute to a spermatozoon's ability to fertilize an oocyte besides motility (Graham, et al., 1980).

There are two types of motility measurements commonly accessed: 1) total motility; and 2) progressive motility. The percentage of all motile sperm (total) in a sample is most commonly used to evaluate the quality of rooster spermatozoa. Motility should be assessed at 37°C and can be assessed visually through a microscope or with a Computer Assisted Sperm Analysis (CASA) system (Hamilton Thorne Motility Analyzer; IVOS, Beverly, MA). CASA has the ability to assess many different parameters that make up motility, such as sperm straightness and sperm velocity. However, many of these parameters are not highly correlated with fertility. Yet evaluating motility can easily uncover sperm that are dead versus alive relatively quickly and effortlessly. The CASA system has the ability to assess relatively large numbers of spermatozoa in a reasonable time and is non-biased (Graham & Mocé, 2005). To evaluate sperm using CASA, sperm must be diluted to between 20 and 30 million sperm/mL

(Vantman, 1988). At this dilution, rooster sperm quickly become immotile, so care must be taken to dilute rooster sperm in diluents containing 1 mg/mL bovine serum albumen (BSA), which mitigates this problem (Harrison, et al., 1982; White, et al., 1984).

Flow Cytometric Evaluation

Flow cytometry can evaluate multiple parameters of individual cells in a sperm population that are unrelated to one another, such as membrane viability, mitochondrial function and intracellular calcium levels. By simultaneously measuring multiple sperm attributes in a population, the researcher can better evaluate the spermatozoa in that sample without having to fertilize oocytes (Graham, 2001; Gillan, et al., 2005; Graham & Mocé, 2005). To analyze cells using flow cytometry, sperm are stained with fluorescent dyes, allowed to incubate and the samples injected into a chamber through which fluid is flowing. The fluid flows through the nozzle that breaks the fluid stream into individual droplets, each containing one cell. These droplets pass through a laser beam, which excites the dyes in the sperm. Photomultiplier tubes, with filters allowing only specific wavelengths of light to pass them, detect whether each sperm cell has a specific dye(s) associated with it (Garner, et al., 1986; Graham, 2001; Gillan, et al., 2005). The power of this technology is that multiple sperm attributes can be measured simultaneously and several thousand cells can be evaluated accurately and precisely in less than a minute (Graham, et al., 1990; Gillan, et al., 2005). In addition, current rooster spermatozoa cryodiluents contain no egg yolk, milk proteins and other particulate matter that are approximately the same size as a sperm, which can create problems evaluating sperm by flow cytometry (Lake & Stewart, 1978; Caudhuri & Lake, 1988; Tajima, et al., 1989; Sasaki, et al., 2010). This makes flow cytometry a better assay to evaluate rooster sperm if a fertility trial cannot be accomplished.

Flow cytometry can be used to assess spermatozoa's plasma membrane integrity. Damage to the spermatozoon's plasma membrane decreases its integrity, and these cells are more likely to be non-functional or dead. Membrane integrity can be evaluated using fluorescent dyes that can enter the cytoplasm and nucleus of cells that have compromised plasma membranes. Propidium iodide intercalates

into the cell's DNA, binding to its nucleic acids, but is impervious to the intact plasma membranes (Garner, et al., 1986, Graham, et al., 1990). Therefore, propidium iodide will only enter cells that have plasma membrane damage, will bind to the DNA and when excited by laser light and will emit red fluorescence that is greater than 610nm (Grogan & Collins, 1990; Harrison & Vickers, 1990).

Although a high correlation (r=0.68) has been reported between the percentages of live stallion sperm and the fertility of inseminated mares using the same samples, using flow cytometery (Wilhelm, et al. 1998), this is not the case with rooster spermatozoa. A large difference is seen between membrane integrity measurements and observed fertility in the hen, and an accurate measurement for fertilizing capabilities would be to perform a fertility trial (Hammerstedt & Graham, 1992).

Insemination Protocols

Rooster spermatozoa are artificially inseminated through the everted cloaca of the hen, and sperm are placed inside the vagina near the sperm storage tubules. Approximately 50-µL of sperm can be inseminated without having large portions of the inseminate efflux from the hen's reproductive tract (Amann, et al., 1997). An insemination dose should contain 100 million fresh sperm or 200 million frozen/thawed sperm (Phillips, et al., 1996). Sperm can be inseminated every other day or even multiple times a day, but maximum fertility can be achieved using fresh semen, by inseminating hens once per week (Amann, et al., 1997). Technologies that require inseminating hens more than once a week will preclude them being used in the industry (Sasaki, et al., 2010).

Effect of Glycerol on Sperm inside Hen's Reproductive Tract

A small percentage of fertility for rooster sperm was reported by Polge (1951) when sperm were exposed to glycerol. However, if the glycerol was diluted and removed from the sperm, fertility was restored. Shaffner (1964) reported that glycerol must be slowly removed from the sperm to avoid osmotically-induced damage and reduce its contraceptive effect. Shaffner (1964) developed a step-wise dilution method to remove glycerol, followed by centrifugation, to reconcentrate the sperm. However, a

step-wise method to dilute sperm after thawing requires the need to freeze rooster sperm at very high concentrations to maintain adequate sperm numbers for artificial insemination (Lake, 1986). Also, this is a very time-consuming and laborious process (Hammerstedt & Graham, 1992).

When rooster sperm exposed to glycerol are placed in the hen reproductive tract, they are transferred from an environment of 1300 mOsm to one of 300 mOsm which causes immediate cell swelling and possible damage, rendering the sperm infertile (Hammerstedt, et al., 1990). In contrast, when rooster sperm in the presence of glycerol are deposited into the hen's shell gland using surgical or intramagnal insemination, there is less contraceptive effect and fertility is achieved (Allen & Bobr, 1955). This technique, however, is very challenging, time consuming and expensive, making it impractical outside of a laboratory setting.

In order to be used in the industry, rooster spermatozoa must remain viable and fertilize oocytes for at least 7 days post-insemination (Amann, et al., 1997). This puts added pressure on having an excellent cryopreservation technique for rooster sperm as compared to mammalian sperm that need to fertilize a single oocyte in approximately 72 hours (Lake, 1986; Hammerstedt & Graham, 1992). The hen's reproductive tract, compared to mammalian species, is smaller, with only one ovary being functional. Essentially, the hen's reproductive tract is a single long tube containing an infundibulum, magnum, isthmus, shell gland and vagina (Jacob, et al., 2011). A chicken can store sperm in host glands, located near the junction of the vagina and the shell gland. Stored sperm are released over a period of 10 to 21 days, allowing for sequential fertilization of ova (Gilbert, et al., 1968).

Fertility Trials

The only test to accurately measure rooster spermatozoa fertility is to perform a fertility trial. Although in vitro assays can tell us much about sperm physiology, they are unable to capture the whole spermatozoon fertilizing ability (Graham, 2005). Fertility measurements for poultry include percent fertile eggs laid and percent hatchability. The percentage of fertile eggs laid does not necessarily reflect the

number of chicks produced, as some eggs do not develop past the embryo stage (early-stage dead embryos). Hatchability is calculated as the number of chicks that develop full term and hatch compared to the number of fertile eggs (Wilson, 1997).

Fertility trials can be conducted at any time if hens are kept under regulated lighting (Wilson, 1997). Spermatozoa should be inseminated once every 18 days to assess the longevity of the cryopreserved sperm in the hen reproductive tract, and eggs collected daily (Blackburn, et al., 2009). Chicken eggs should be incubated between 37°C to 38°C for 7 days and then candled to determine fertility and embryonic growth (Wilson, 1991). Eggs that do not show embryo development should be cracked to check for early dead embryos (Phillips, et al., 1996). At this time, fertile eggs should be incubated to hatch for 21 to 22 days at 50% humidity until day 18 and humidity raised to 70% to keep the membrane surrounding the chick when the egg is cracked from drying on the chick, or disposed of properly.

Conclusions

Since the first experiments of cryopreserving rooster spermatozoa, there has been a large amount of inconsistency with fertility of cryopreserved sperm using various techniques. Glycerol elicits contraceptive effects on rooster spermatozoa in the hen reproductive tract, and therefore must be removed from the diluent prior to insemination when semen is placed intravaginal. This process is both labor-intensive and increases osmotic stress on the rooster sperm during the dilution and reconcentration process (Hammerstedt & Graham, 1992). Recent studies show promise using alternative cryoprotectants that do not need to be removed from cryopreserved sperm prior to insemination (Woelders, et al., 2006; Sasaki, et al., 2010). Modifying cryopreservation techniques by packaging in different straws, using different diluents or freezing sperm at different concentrations, along with using alternative cryoprotectants, may increase cryosurvival of rooster spermatozoa sufficiently to make frozen semen a viable option to preserve poultry genetics efficiently by increasing cryosurvival and subsequent fertility. Altering plasma membrane composition by adding cholesterol or unsaturated phospholipids may increase

membrane fluidity at lower temperatures and decrease membrane injury during cryopreservation. This is seen in many different other species, including the stallion, bull, ram and boar (Moore, et al., 2005; Purdy & Graham, 2004a; Mocé, et al., 2010; Blanch, et al., 2012).

The studies in the experiments described in this thesis investigate using alternative cryoprotectants, cholesterol-loaded cyclodextrins, lipid-loaded cyclodextrins and modifying cryopreservation techniques by packaging in different sized straws, using different diluents or freezing sperm at different concentrations, in an effort to improve rooster sperm cryosurvival.

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CHAPTER II

EFFECTS OF ALTERNATIVE CRYOPROTECTANTS ON CRYOSURVIVAL OF ROOSTER SPERMATOZOA

Introduction

The dairy industry has extensively used artificial insemination using cryopreserved sperm for reproduction management for decades since artificial insemination was first introduced in the 1930's (Foote, et al., 2002). In contrast, the poultry industry has not integrated large scale use of cryopreserved rooster spermatozoa due to the sperm's poor fertility after cryopreservation (Fulton, 2006). Therefore, new techniques need to be developed to improve rooster sperm cryopreservation.

Rooster sperm possess a unique shape, and membrane lipid/protein composition that make these sperm particularly susceptible to cryopreservation damage (Bakst, 1980; Watson, 1990; Parks & Lynch, 1992; Etches, 1996). Their cylindrically-shaped head, as well as their unique lipid composition, restricts their membranes from undergoing the large volume changes needed to survive cryopreservation efficiently (Parks & Lynch, 1992; Blanco, et al., 2008), as the cell experiences different osmotic environments (Amann & Pickett, 1987; Blanco, et al., 2008).

Prior to being cryopreserved, cryoprotectant is added to cells and when the cells are thawed and then diluted in a solution containing no cryoprotectant, anisosmotic conditions occur, causing the cells to swell which can damage the membrane (Hammerstedt, et al., 1990; Gao, et al., 1995).

Cryoprotectants with lower molecular weights than glycerol (MW = 92), the most commonly used cryoprotectant, cross membranes more rapidly than glycerol, equilibrate more rapidly than glycerol and therefore do not induce as much cell volume change (Hammerstedt & Graham, 1992; Buss, 1993; Gill, et al., 1996; Sasaki, et al., 2010). If cell volume increases can be kept below that which induces rupture, and if these cryoprotectants protect the cells from cryopreservation damage, they could be very useful for preserving cells that normally do not survive well using glycerol as the cryoprotectant (Lake &

Ravie, 1984). Alternative cryoprotectants have been used to cryopreserve stallion sperm, which also have narrow osmotic tolerances, and these alternative cryoprotectants induce less osmotic excursions, inducing less cell damage due to volume changes (Squires, et al., 2004).

As a solution freezes, ice is formed from pure water, with unfrozen channels between the ice crystals and all the salts in the solution become concentrated in the unfrozen channels. In addition, the sperm reside in the unfrozen channels to survive cryopreservation (Mazur & Cole, 1989; Hammerstedt, 1995). Effective cryoprotectants must be non-toxic to sperm, while providing protection against freezing damage. Penetrating cryoprotectants are used exclusively to cryopreserve rooster sperm. Penetrating cryoprotectants work by increasing the extracellular unfrozen channel volume and decreasing the salt concentration of the unfrozen channels (Amann & Graham, 1993).

Alternative cryoprotectants have been used with limited success for cryopreserving rooster sperm (Westfall & Harris, 1975; Mitchell & Buckland, 1976; Graham, et al., 1980; Lake & Ravie, 1984; Hammerstedt & Graham, 1992; Phillips, et al., 1996). Dimethylacetamide (MW = 73) has been used and does not need to be removed from the sperm prior to insemination (Purdy, et al., 2009). However, rooster sperm cryopreserved using dimethylacetamide as the cryoprotectant do not have high fertility and hens must be inseminated multiple times a week due to the extensive process of diluting the cryoprotectant, which limits its use outside laboratory settings (Lake & Ravie, 1984; Lake, 1986; Purdy, et al., 2009).

Ethylene glycol and methylformamide are other cryoprotectants that have been used to freeze stallion sperm, as well as bovine embryos and oocytes (Squires, et al., 2004). Both of these cryoprotectants have lower molecular weights than glycerol (ethylene glycol = MW 62, methylformamide = MW 59), and equilibrate across the cell membrane more quickly than glycerol (Hammerstedt & Graham, 1992). However, neither of these cryoprotectants have been studied extensively for cryopreserving rooster sperm (Hammerstedt & Graham, 1992). Ethylene glycol and methylformamide do not need to be diluted from stallion sperm prior to inseminating to mares, and have resulted in higher

fertility rates than sperm frozen with glycerol as the cryoprotectant (Squires, et al., 2004). This method could be employed to allow higher fertility rates when inseminating hens with rooster sperm that are frozen using lower molecular weight cryoprotectants.

In two recent studies, methylacetamide has been used as a cryoprotectant to cryopreserve rooster sperm; and the sperm were inseminated after thawing without removing the cryoprotectant, resulting in 82% fertility, and 90% hatchability (Hanzawa, et al., 2010; Sasaki, et al., 2010). These fertility rates are sufficiently high to be adapted by the commercial poultry industry to allow for genetic preservation of important genetic lines (Sasaki, et al., 2010).

Several different cryoprotectants, methylacetamide, dimethylformamide, methylformamide and ethylene glycol, may protect rooster sperm from cryopreservation damage without having to be removed from the cells prior to insemination. The objectives of these experiments were to determine if alternative cryoprotectants protect rooster spermatozoa during cryopreservation.

Materials & Methods

Semen Collection, Processing and Cryopreservation

Ten Rhode Island Red, Black Sumatra, Bantam Silkie, Phoenix and Naked Neck breed roosters were individually housed at the United States Department of Agriculture's National Animal Germplasm Program facility. The birds were exposed to 16 hours of light and 8 hours of dark per day and were provided water and Flock Raiser® SunFresh® Recipe Crumbles complete feed (Land O' Lakes Purina Mills LLC, Grey Summit, MO) *ad libitum*. All animal care and procedures used to collect semen were approved by the Animal Care and Use Committee of the USDA. All chemicals were reagent-grade purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Roosters were collected (3 times/week) by abdominal massage (Burrows, et al., 1937) into conical sample cups (4-mL; Thermo Fisher Scientific, Waltham, MA). Immediately after collection, ejaculates were pooled, diluted 1:1 (v:v) with a glutamate-based diluent (LLT; Lake, et al., 1978), and transported at 5°C over ice to the laboratory within 30 min. Spermatozoal concentrations were determined using a Spectronic 20D spectrophotometer

(Milton Roy Company, Rochester, NY) at 550-nm and initial sperm motility was determined using a Leitz Dialux 20 phase-contrast microscope (Leitz, West Germany), using a motility scale from 1 to 4, with 1 having little wave action and 4 having vigorous wave action (Tajima, et al., 1989). Ejaculates were discarded if motility was evaluated below 3.

Ejaculates were diluted to 1 billion cells/mL with LLT at 5°C and divided into 5 equal aliquots. These sperm aliquots were diluted 1:1 (v:v) in LLT containing 18% cryoprotectant (glycerol, methylformamide, dimethylformamide, ethlyene glycol and methylformamide) to obtain a final cryoprotectant concentration of 9% (Phillips, et al., 1996). The spermatozoa were loaded into 0.25-mL polyvinylchloride straws (IMV Technologies, Minneapolis, MN), frozen in liquid nitrogen vapor, 6-cm above the level of liquid nitrogen for 15 minutes (Tajima, et al., 1989) and then plunged into liquid nitrogen for storage at -196°C until analysis. This experiment was replicated 24 times.

Motility Analysis

The percentage of motile spermatozoa in each sample was determined using a computer-assisted sperm analysis system (CASA; HTM IVOS; Hamilton-Thorne Biosciences, Beverly, MA). System parameters for motility analysis using the CASA were as follows: 40 frames acquired at 60 frames per sec; minimum contrast 40; minimum cell size 2 pixels; lower VAP cut-off 20-µm/sec; lower VSL cut-off 10-µm/sec; VAP cut-off for progressive cells 50-µm/sec and straightness 75%. Each sample was mixed with LLT plus BSA (1%) and an 8-µL drop of spermatozoa from each sample was placed on a 37°C preheated CELL-VU counting slide (Thermo Fisher Scientific, Waltham, MA) and a minimum of 5 fields and at least 250 spermatozoa per sample were analyzed.

Flow Cytometric Analysis

The plasma membrane integrity of spermatozoa was determined using flow cytometry. Spermatozoa (10,000 cells/sample) were analyzed using a CyAN ADP flow cytometer (Beckman Coulter,

Miami, FL) and data analyzed using HyperCyt Software (Beckman Coulter, Miami, FL). The fluorescent dye propidium iodide (PI) was excited using a 488-nm argon laser at 20mW power. The fluorescence of PI was detected using a filter setup combination of a 530-nm band pass filter (FL-1 detector) and a 613-nm band pass filter (FL3 detector) to detect PI (Purdy, et al., 2009).

Experiment 2.1: Effects of Alternative Cryoprotectants on Frozen/Thawed Rooster Spermatozoa Motility and Membrane Integrity

For motility evaluation, two straws from each treatment were thawed in a 5°C water bath for 5 minutes. The contents from straws were diluted 1:10 (v:v) with LLT containing 1% BSA to give a final spermatozoa concentration of 45.5 million cells/mL. Each sample was immediately assessed for motility by CASA.

For membrane integrity evaluation, two straws were thawed as described above, and the contents of straws were diluted 1:100 (v:v) with LLT with 1% BSA to give a final spermatozoa concentration of 4.95 million cells/mL. Each sample was stained using 5-µL of propidium iodide (PI; 1 mg/mL in H_2O) and incubated for 5 min at 21°C to allow for stain incorporation (Krishan, 1975). Each sample was mixed after incubation and immediately analyzed on the flow cytometer. The percent of PI negative cells were recorded as the percentage of membrane-intact cells.

Experiment 2.2: Effects of Methylacetamide Exposure on Frozen/Thawed Rooster Spermatozoa Motility

Rooster spermatozoa were collected, diluted in LLT and extended in LLT containing 18% methylacetamide (9% final concentration) and frozen as previously described.

Two straws were thawed in a 5°C water bath for 5 min and the motility was assessed at 0 min, 5 min, 10 min, 15 min, 20 min, 30 min, 45 min and 60 min using CASA, and undiluted samples were kept over ice for the duration of analysis. The contents from straws were diluted 1:10 (v:v) with a trehalose-based diluent with 1% BSA to give a final spermatozoa concentration of 50 million cells/mL.

Statistical Analysis

Data were analyzed by analysis of variance and treatment means were separated by Student-Newman Keuls mean separation technique (SAS Institute Inc., 1985), for differences in sperm motility and plasma membrane integrity between CPA treatments. Treatments were considered different if $P < 0.05$.

Results

Experiment 2.1: Effects of Alternative Cryoprotectants on Frozen/Thawed Rooster Spermatozoa Motility and Membrane Integrity

Sperm cryopreserved with glycerol as the cryoprotectant exhibited higher percentages of motile cells (54%) than sperm cryopreserved with methylacetamide (48%), dimethylformamide (41%) or ethylene glycol (38%), while sperm frozen with methylformamide had the lowest percentages of motile sperm (28%; $P < 0.05$; Figure 2.1;).

Sperm cryopreserved with glycerol as the cryoprotectant exhibited higher percentages of membrane-intact cells (58%) than sperm cryopreserved with methylacetamide (53%) or dimethylformamide (45%), while sperm frozen with either ethylene glycol or methylformamide had the lowest percentages of membrane intact sperm (39% and 37%, respectively; $P < 0.05$; Figure 2.2).

Experiment 2.2: Effects of Methylacetamide Exposure on Frozen/Thawed Rooster Spermatozoa Motility

Sperm cryopreserved with methylacetamide exhibited > 50% motile cells after thawing. Incubating sperm with methylacetamide for up to 60 min did not affect the percentage of motile sperm ($P > 0.05$; Figure 2.3).

Discussion

Membrane damage from osmotic fluctuation during cryoprotectant addition and removal, and during the freezing process itself is a major cause of cell death during cryopreservation (Hammerstedt & Graham, 1992). After thawing, when sperm are diluted from the cryoprotectant, the longer it takes a cryoprotectant to cross a sperm membrane, the greater the potential for cells to swell to point that the sperm membrane exceeds its critical osmotic tolerance and will lyse (Amann & Pickett, 1987). Using low molecular weight cryoprotectants that cross the membrane more quickly than glycerol may inhibit cell volumes from reaching their critical osmotic tolerances (Squires, et al., 2004).

This study indicates that methylacetamide protects rooster sperm from cryopreservation damage similar to glycerol. The other cryoprotectants studied, methylformamide, dimethylformamide and ethylene glycol, did not maintain similar percentages of motile cells. These results are similar to previous studies, which reported that dimethylformamide did not protected rooster sperm similarly to glycerol (Krivtsova & Otpushchennikov, 1984; Lake, 1986). These differences could be due to the specific rooster breeds utilized in this study, heritage and exotic chicken breeds, which are genetically different from the commercial chicken strains, used in other studies. These genetic lines of chickens are highly inbred, and could be causing changes to the physiological and chemical changes to the sperm cell itself (Long & Kulkarni, 2004). Changes to these physical and chemical properties could explain the difference in breeds, as well as the difference in CPA interaction with the sperm cells. Different responses to cryopreservation have been reported for sperm from different poultry strains (Bacon, et al., 1986; Froman & Bernier, 1987; Tajima, et al., 1990; Alexander, et al., 1993; Long, et al., 2010).

Many cryoprotectants can be toxic to sperm at the levels used to be effective cryoprotectants. Therefore, it was important to determine if relatively long-term exposure of sperm to methylacetamide affected sperm survival. Exposure of methylacetamide up to 1 h did not affect sperm motility.

Methylacetamide and glycerol were effective for cryopreserving rooster sperm. Sasaka, et al.

(2010) reported high fertility rates with methylacetamide (82%), but did not compare methylacetamide-exposed sperm fertility to glycerol-exposed sperm after dilution and centrifugation. This comparison for fertilizing capacity needs to be evaluated.

Conclusions

Methylacetamide can be an effective alternative to glycerol as a cryoprotectant for freezing rooster spermatozoa. Methylacetamide passes through the sperm membrane more quickly than glycerol and reduces damage caused by excessive cell swelling when the cryoprotectant is removed, and is not toxic to sperm over an extended period of time. If rooster sperm can withstand the volume changes experienced by sperm during freezing and insemination without having to be removed in a step-wise fashion followed by reconcentration, this would enable cryopreserved sperm to be effectively incorporated into the commercial poultry industry. A fertility trial is necessary to compare the fertilizing capacities of sperm frozen in methylacetamide and inseminated without dilution and reconcentration, compared to sperm frozen in glycerol that has dilutions and then is centrifuged prior to insemination.

Figures

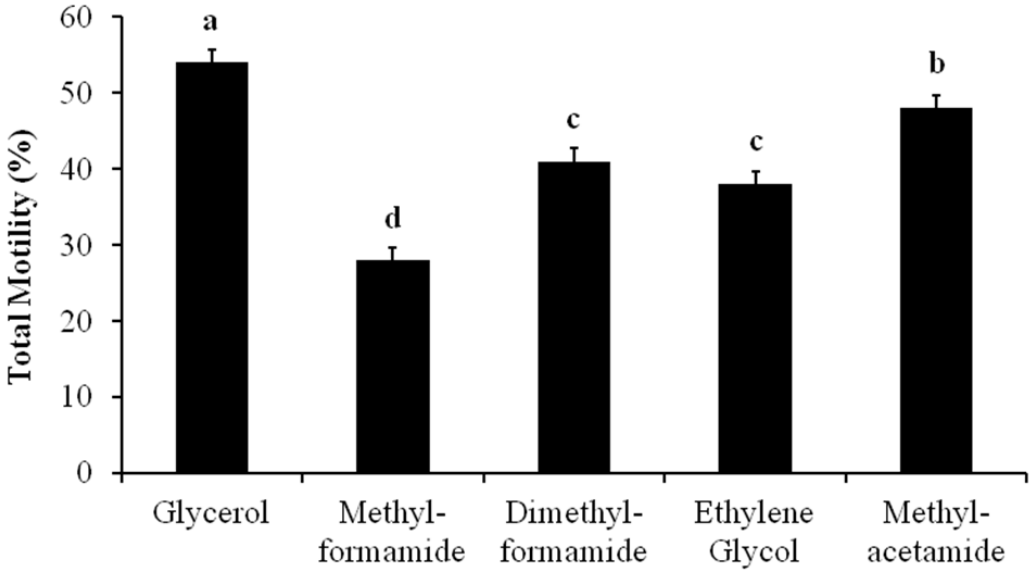

Figure 2.1. The percentage of total motile frozen/thawed rooster spermatozoa treated with 5 different cryoprotectants (glycerol, methylformamide, dimethylformamide, ethylene glycol and methylacetamide) each at 9% of the diluent volume. N=24
Different superscripts (a, b, c & d) signify significance between groups ($P < 0.05$).

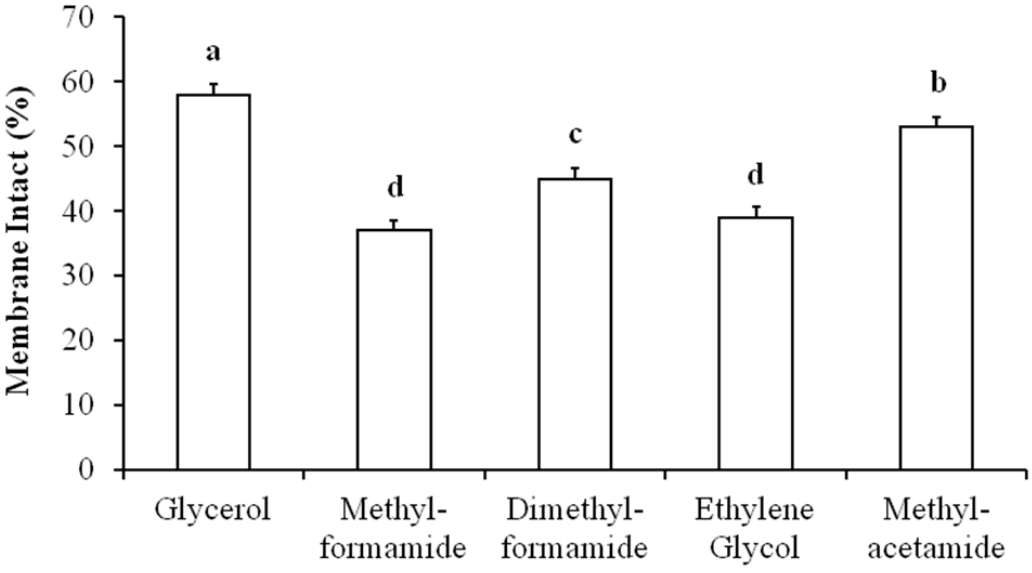

Figure 2.2. The percentage of total membrane intact frozen/thawed rooster spermatozoa treated with 5 different cryoprotectants (glycerol, methylformamide, dimethylformamide, ethylene glycol and methylacetamide) each at 9% of the diluent volume. N=24
Different (a, b, c & d) signify significance between groups ($P < 0.05$).

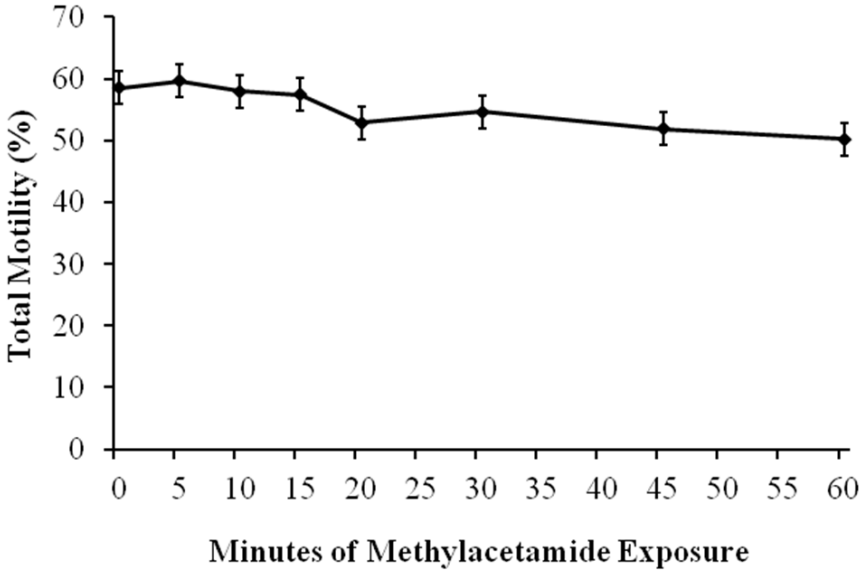

Figure 2.3. The percentage of total motile frozen/thawed rooster spermatozoa exposed to methylacetamide at 8 different time points (0, 5, 10, 15, 20, 30, 45 & 60 min) at 9% of the diluent volume. N=9

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CHAPTER III

EFFECTS OF CHOLESTEROL ON CRYOSURVIVAL OF ROOSTER SPERMATOZOA

Introduction

Membrane damage during cooling is a major cause for sperm death during cryopreservation (Morris, et al., 2007; Morris, et al., 2012). Membrane lipid composition plays a significant role in a sperm's response to cooling and warming during cryopreservation (Amann & Graham, 1993). As described previously, sperm membranes are composed of lipids, cholesterol and proteins in specific arrangements and the cholesterol:phospholipid ratio plays a major role in membrane fluidity (Darin-Bennett & White, 1977; Israelachvili, 1978; Hammerstedt & Graham, 1992). Membranes with high cholesterol:phospholipid ratios exhibit lower transition temperatures, which allow the membrane to remain fluid through the majority of the freezing process and this decreases the amount of membrane damage during cooling and warming (Rottem, et al., 1973; Darin-Bennett & White, 1977; Parks & Lynch, 1992).

Attempts have been made to alter the lipid composition of sperm by feeding the roosters unique diets without success (Bongalhardo, et al., 2009; Castellano, et al., 2010). However, cyclodextrin technology quickly alters membrane lipid composition in vitro, effectively transferring sterols into and out of the sperm membrane (Klein, et al., 1995; Christian, et al., 1997). Cyclodextrins have a hydrophilic exterior and a hydrophobic center. This allows them to solubilize lipids, and act as a lipid exchanger and transport lipids into or out of the membrane down a concentration gradient (Yancey, et al., 1996). Certain cyclodextrins (β-cyclodextrins) are effective in encapsulating cholesterol into its core (Hu, et al., 2005). Side chains can be added to cyclodextrins that change the ability of the cyclodextrin to interact with cells depending upon the composition of the cell's glycocalyx allowing the cyclodextrin to transfer lipids into or out of its core (Kilsdonk, et al., 1995).

Cyclodextrins can be preloaded with cholesterol prior to adding them to cells (Mocé, et al., 2010a). Cholesterol-loaded methyl-β-cyclodextrins (CLC) (Purdy & Graham, 2004a) transport sterols

into some cell types more efficiently than either α-cyclodextrins or γ-cyclodextrins (Yancey, et al., 1996; Christian, et al., 1997; Ohvo, et al. 1997; Choi & Toyoda, 1998; Cross, 1999). Methyl-β cyclodextrin has great capacity to transport cholesterol than β-cyclodextrin for a number of cells and species of sperm, as the side chain interacts more efficiently with the cell's glycocalyx (Christian, et al., 1999).

CLCs have been used to effectively add cholesterol to sperm membranes, prior to cryopreservation, for bull (Purdy & Graham, 2004a), stallion (Moore, et al., 2005), ram (Mocé, et al., 2010b), rat (Graham, unpublished) and boar (Tomás, et al., 2011) sperm, increasing the membrane cholesterol:phospholipid ratio and the osmotic tolerance of the cells, both of which increase sperm cryosurvival rates (Purdy & Graham, 2004a;b; Moore, et al., 2005; Mocé & Graham, 2006; Mocé, et al., 2010b; Spizziri, et al., 2010). Rooster sperm have a low cholesterol:phospholipid ratio (0.30; Parks & Lynch, 1992), and CLCs may increase this ratio, and subsequently increase their cryosurvival.

The objectives of the following experiments were to determine if adding cholesterol-loaded cyclodextrins to rooster sperm, prior to cryopreservation, increase sperm motility and membrane integrity after cryopreservation and dilution.

Materials & Methods

Semen Collection, Processing & Cryopreservation

Ten Rhode Island Red, Black Sumatra, Bantam Silkie, Phoenix and Naked Neck breed roosters were individually housed at the United States Department of Agriculture's National Animal Germplasm Program facility. The birds were exposed to 16 hours of light and 8 hours of dark per day and were provided water and Flock Raiser® SunFresh® Recipe Crumbles complete feed (Land O' Lakes Purina Mills LLC, Grey Summit, MO) *ad libitum*. All animal care and procedures used to collect semen were approved by the Animal Care and Use Committee of the USDA. All chemicals were reagent-grade purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Roosters were collected (3 times/week) by abdominal massage (Burrows, et al., 1937) into conical sample cups (4-mL; Thermo Fisher Scientific, Waltham, MA). Immediately after collection, ejaculates were pooled, diluted 1:1 (v:v)

with a glutamate-based diluent (LLT; Lake, et al., 1978), and transported at 5°C over ice to the laboratory within 30 min. Spermatozoal concentrations were determined using a Spectronic 20D spectrophotometer (Milton Roy Company, Rochester, NY) at 550-nm and initial sperm motility was determined using a Leitz Dialux 20 phase-contrast microscope (Leitz, West Germany), using a motility scale from 1 to 4; with 1 having little wave action and 4 having vigorous wave action (Tajima, et al., 1989). Ejaculates were discarded if motility was evaluated below 3.

Cholesterol-Loaded Cyclodextrins (CLCs)

Cyclodextrins were pre-loaded with cholesterol, as described by Purdy & Graham (2004a). Briefly, 1-g of cyclodextrin was dissolved into 2-mL of methanol (MeOH) in a glass tube. In a second glass tube, 200-mg of cholesterol was dissolved into 1-mL of chloroform. The cyclodextrin/MeOH solution was added to the cholesterol/chloroform solution, and the two were mixed thoroughly. This was poured into a 100-mm glass petri dish and placed on a heated aluminum block and flushed with nitrogen gas until crystals formed on the bottom of the petri dish. These crystals were removed and the CLCs stored at 22°C in a glass vial until use. A working solution was made by dissolving 0.050-g of CLCs into 1-mL of glutamate-based diluent, and mixing thoroughly. This CLC working solution was stored at -20°C.

Motility Analysis

The percentage of motile spermatozoa in each sample was determined using a computer-assisted sperm analysis system (CASA) (HTM IVOS; Hamilton-Thorne Biosciences, Beverly, MA). System parameters for motility analysis using the CASA were as follows: 40 frames acquired at 60 frames per sec; minimum contrast 40; minimum cell size 2 pixels; lower VAP cut-off 20-µm/sec; lower VSL cut-off 10-µm/sec; VAP cut-off for progressive cells 50-µm/sec and straightness 75%. Each sample was mixed

with LLT plus BSA (1%) and an 8-µL drop of spermatozoa from each sample was placed on a 37°C preheated CELL-VU counting slide (Thermo Fisher Scientific, Waltham, MA) and a minimum of 5 fields and at least 250 spermatozoa per sample were analyzed.

Flow Cytometric Analysis

The plasma membrane integrity of spermatozoa was determined using flow cytometry. Spermatozoa were analyzed using a CyAN ADP flow cytometer (Beckman Coulter, Miami, FL); a total of 10,000 cells/sample were assessed and data were analyzed using HyperCyt Software (Beckman Coulter, Miami, FL). The fluorescent dye propidium iodide (PI) used was excited using a 488-nm argon laser at 20mW power. The fluorescence of PI was detected using a filter setup combination of a 530-nm band pass filter (FL-1 detector) and a 613-nm band pass filter (FL3 detector) to detect PI (Purdy, et al., 2009).

Total Lipid Extraction

Total lipid extraction was performed following procedures by Folch, et al. (1957), and modified by Bligh & Dyer (1959). Briefly, sperm samples were dried following procedures stated in experiment 3.3 and stored in a dessicator. Dry samples removed from dessicator and the contents of each weighed and added to 4-mL of a chloroform/methanol solution (2:1; v:v). Each sample was homogenized using a Tissue-Tearor homogenizer (Model 985-370, BioSpec Products, Inc., Bartlesville, OK) at 5,000 RPM for 30 sec. Tubes were placed onto an agitator at 100 RPM (IKA-Vibrax-VXR, Janke & Kunkel GMBH & Co., Staufen, Germany) for 20 min, after which they were filtered through filter paper (Thermo Fisher Scientific, Waltham, MA, Hardened Ashless Circles, coarse porosity, >20-25-µm particle retention) into a second 12x75-mm borosilicate glass test tube. A total of 0.8-mL of 0.59% NaCl solution was added to each sample and the tubes were covered with tinfoil and stored overnight (5°C).

Samples were returned to 23°C, with the sample now partitioned into 2 phases. The bottom phase (containing the lipid) was removed using a 9-inch glass Pasteur capillary pipet into pre-weighed 20-mL glass scintillation vials and the vials placed into a heated aluminum block and flushed with nitrogen gas until dried. The samples were placed into an oven (100°C) for 24 h after which they were placed into a dessicator for 3 h to cool. Each sample was weighed and the total dry sample weight was calculated by subtracting the weight of the glass tube without sample from the glass tube weight with sample. Total dry sample weight was recorded to 0.001 grams. Total percent lipid of the sample was calculated by dividing the total lipid weight by the total dry sample weight.

Cholesterol Extraction and Purification

The 20-mL glass scintillation vials with lipid-extracted sample were taken from the dessicator and 4-mL of absolute ethanol was added, along with 1-mL of 33% KOH. The samples were mixed with the caps tightly closed for 15 sec using a vortex mixer and placed into an 80°C water bath for 30 min. Each sample was mixed 3 times during the first 10 min, 2 times during the second 10 min and 1 time during the final 10 min of incubation. Vials were removed from the water bath and placed into ice water to cool. Each vial was wiped dry and 3-mL of nanopure water was added to each sample, along with 2.25-mL of hexanes and 0.75-mL of 0.066% stigmasterol in hexanes solution, used as an internal control. The samples were mixed for 15 sec and held undisturbed for 1 h for the two phases to separate. The top layer, containing purified cholesterol, was removed using a 9-inch glass Pasteur capillary pipet into a gas chromatograph vial with a 300-µL glass insert. Samples were immediately analyzed on the gas chromatograph. The percent total cholesterol was calculated by dividing the corrected total peak areas into the cholesterol peak area, and multiplying by 100.

Gas Chromatography Analysis

A Hewlett-Packard model 5890 series II Gas-Chromatographer (Palo Alto, CA), fixed with a series 7683 injector and flame-ionization detector was used to analyze the cholesterol content of the

sample. The instrument was equipped with a 30-m × 530-µm ID x 0.1-µm film fused silica capillary column (J& W Scientific DB-1, Agilent Technologies, Santa Clara, CA). The carrier gas was helium at 50-psi, with hydrogen gas at 22 psi and air at 23 psi. Column oven temperature was increased from 150°C to 210°C at 10°C/min, held for 2 min, and temperature increased from 210°C to 250°C at 25°C/min, and then held at 250°C for 1 min. The injector and detector were maintained at 300°C. Total run time was 11 min.

Experiment 3.1: Cholesterol-Loaded Cyclodextrin Effects on Rooster Spermatozoa Osmotic Tolerance

Diluted rooster spermatozoa at 5°C were divided into 4 aliquots and further diluted 1:1 with a LLT. Sperm aliquots were then treated with 0, 1, 2 or 5 mg/mL of CLC, the samples held at 22°C for 30 min to allow cholesterol incorporation, and then cooled to 5°C prior to experimentation.

A 20-µL volume of treated sperm was placed into each of 13 test tubes containing a LLT at 0, 50, 100, 150, 225, 270, 300, 350, 370, 425, 600, 1200 and 2400 mOsm to which 1% BSA had been added. Sperm were maintained in anisosmotic conditions for 5 min after which they were returned to isosmotic conditions at approximately 300 mOsm. Motility determined immediately after returning the sperm cells to isosmotic conditions, using CASA. This experiment was replicated 8 times.

Experiment 3.2: Effects of Cholesterol-Loaded Cyclodextrins on Rooster Spermatozoa Cryopreservation

Spermatozoa were diluted to 800 million cells/mL with LLT at 5°C and were divided into 19 equal aliquots. Sperm were treated with 6 different cyclodextrin structures (2-hydroxypropyl-β, α, α-hydrate, β, β-hydrate and methyl-β) loaded with cholesterol at 3 different concentrations (0.5, 1 and 2-mg/mL). Sperm were allowed to incubate for 30 min at 5°C to allow for cholesterol incorporation. The sperm were then diluted 1:1 (v:v) in LLT containing 18% dimethylformamide to obtain a final CPA concentration of 9% (Phillips, et al., 1996). Dimethlyformamide was used as the cryoprotectant as CLC effects would more easily be detected, as dimethylformamide was not the best treatment observed in the

alternative cryoprotectant experimentation (Chapter II). The spermatozoa were loaded into 0.25-mL polyvinylchloride straws (IMV Technologies, Minneapolis, MN), frozen in liquid nitrogen vapor 6-cm above the level of liquid nitrogen for 15 min (Tajima, et al., 1989) and plunged into liquid nitrogen for storage until further use. This experiment was replicated 8 times.

Two straws from each treatment were thawed in a 5°C water bath for 5 min and the motility was assessed. The contents from straws were diluted 1:10 (v:v) with LLT with 1% BSA to give a final spermatozoa concentration of 40 million cells/mL. Each sample was immediately assessed for motile sperm using CASA.

To evaluate membrane integrity, two straws were thawed as described above, and the contents diluted 1:100 (v:v) with LLT with 1% BSA, to give a final spermatozoa concentration of 4 million cells/mL. Each sample was stained using 5-µL of PI (1 mg/mL in H_2O) incubated for 5 min at 23°C to allow for stain incorporation (Krishan, 1975) and analyzed on the flow cytometer, as described previously.

Experiment 3.3: Incorporation of Cholesterol into Rooster Spermatozoa Plasma Membrane using Cholesterol-Loaded Cyclodextrins

Fresh rooster spermatozoa were diluted to a total volume of 7.5-mL using LLT, divided into five 1.4-mL aliquots and different levels of LLT and CLCs added as follows:

Tube #	*LLT*	*CLC*	*CLC Concentration*
1	100-µL	0-µL	0-mg/mL
2 and 4	70-µL	30-µL	1-mg/mL
3 and 5	0-µL	120-µL	4-mg/mL

Each tube was mixed thoroughly and the sperm were allowed to incubate with CLCs at 5°C for 30 min.

Following incubation, the sperm were removed from extra CLCs in the sample, by layering the tube contents over 1-mL of 30% Percoll in PBS and centrifuging at 400 g for 10 min. Two layers were observed following centrifugation. The top clear layer was discarded and the bottom layer was resuspended, in the new tube, with 2-mL of PBS. The samples were washed twice by centrifugation at 800 g for 10 min. And each sample resuspended using 1.5-mL of PBS and placed into preweighed tubes. The sperm concentration of each sample was determined using a hemocytometer (Foote, 1979).

The glass tubes were placed into a 100°C oven for 48 h to dry. After drying, the tubes were placed into a glass dessicator for 30 min to cool and each tube was weighed after cooling. Samples were stored in glass dessicator at 27°C until Total Lipid Extraction and Cholesterol Extraction and Purification was conducted. This experiment was replicated 4 times.

Statistical Analysis

Data were analyzed by analysis of variance and treatment means were separated by Student-Newman Keuls mean separation technique (SAS Institute Inc., 1985), for differences in sperm motility, plasma membrane integrity and total cholesterol between CLC treatments. Treatments were considered significantly different if $P < 0.05$.

Results

Experiment 3.1: Cholesterol-Loaded Cyclodextrin Effects on Rooster Spermatozoa Osmotic Tolerance

Sperm at isosmotic conditions of 300 mOsm exhibited percentages of motile sperm (>60%) that decreased when exposed to hypoosmotic conditions of 0 mOsm (< 31%) and hyperosmotic conditions of 2400 mOsm (< 18%) . Sperm samples incubated with CLCs was not a significant source of variation at any of the osmotic concentrations tested ($P > 0.05$; Figure 3.1).

Experiment 3.2: Effects of Cholesterol-Loaded Cyclodextrins on Rooster Spermatozoa Cryopreservation

Control samples and samples incubating sperm with CLCs contained similar percentages of motile (51% versus 50%, respectively) and membrane-intact sperm (47% versus 49%, respectively; $P > 0.05$; Tables 3.1 & 3.2).

Experiment 3.3: Incorporation of Cholesterol into Rooster Spermatozoa Plasma Membrane using Cholesterol-Loaded Cyclodextrins

CLC addition of 1 mg/mL and 4 mg/mL resulted in an increase in rooster sperm cholesterol content (11% and 13% total area, respectively) versus untreated rooster sperm (5% total area; $P < 0.05$), as shown in Figure 3.2.

Discussion

The cryopreservation of rooster sperm would allow for indefinite storage of vital genetics and for the global sharing of these genetics (Fulton, 2006). Yet, the fertilizing ability of cryopreserved rooster sperm is compromised due to membrane damage during freezing (Lake, 1986; Hammerstedt & Graham, 1992; Buss, 1993; Long, 2010). Increasing the cryosurvival rate of rooster sperm is vital to allow for wide-spread adaption of cryopreserved rooster sperm in insemination protocols in laboratories and commercial poultry industry (Blesbois, 2007).

Membrane composition affects cell cryosurvival rates, and increasing cholesterol content of sperm from several mammalian species improved their cryosurvival rates. However, treating rooster sperm with CLC did not improve the motility or membrane integrity of the cells, regardless of cyclodextrin structure. Additionally, CLC did not widen the osmotic tolerance of rooster sperm as it does for stallion, ram and boar sperm (Glazer, et al., 2009; Mocé, et al., 2010b; Tomás, et al., 2011)

Even though CLC treatments did not improve rooster sperm cryosurvival rate or osmotic

tolerance, CLC treatment did increase the cholesterol content of rooster sperm 2-fold, similar to cholesterol increases seen for mammalian sperm (Purdy & Graham, 2004a; Moore, et al., 2005; Mocé, et al., 2010b).

Although the cholesterol:phospholipid ratio of rooster sperm is only 0.30, rooster sperm do not cold shock when cooled to 5°C as do many mammalian sperm (Parks & Lynch, 1992). This unique characteristic is likely due to the phospholipid composition of rooster sperm plasma membrane, which contains a high percentage of serine and inositol phosphoglyceride fraction, as well as a high proportion of shorter, more saturated choline phosphoglycerides (16:0, 18:0, 18:1), with a wider distribution of chain length and degree of unsaturation than mammalian sperm. Along with this unique lipid composition, mammalian sperm contain both high melting point glycolipids and low melting point glycolipids, while high melting glycolipids are absent from rooster sperm (Parks & Lynch, 1992). These glycolipids have been shown to have a different phase behavior, and are the first lipids in the membrane to undergo a phase transition when cooled. With the lack of high-melting point glycolipids in rooster sperm, the membrane stays in more of a homogeneous phase transition state during cooling. This homogeneous state decreases the amount of membrane damage sustained by rooster sperm during cooling. This unique lipid composition might explain why CLC did not benefit rooster sperm cryopreservation since rooster sperm membranes naturally remain fluid at low temperatures, adding cholesterol did not help.

Previous studies also indicate that membrane protein content is correlated with sperm cold shock susceptibility (Barrios, et al., 2005, Parks & Lynch, 1992).. Rooster sperm, which do not cold shock, contain a low membrane protein:phospholipid ratio (0.46) compared to bull (0.80) and stallion sperm (0.86) which are susceptible to cold shock (Parks & Lynch, 1992). Rooster sperm's low protein content, coupled with the absence of high melting point glycolipids may be reasons why rooster sperm are able to withstand lower temperatures and not cold shock.

Conclusions

CLC treatment induces cholesterol incorporation into rooster sperm. However, cholesterol addition failed to widen the osmotic tolerance of treated sperm, or improve the percentage of motile or membrane intact sperm after cryopreservation. This could be due to the unique lipids that compose rooster sperm, which are not commonly found in high levels in mammalian sperm that show improved cryosurvival following CLC treatment.

Figures & Tables

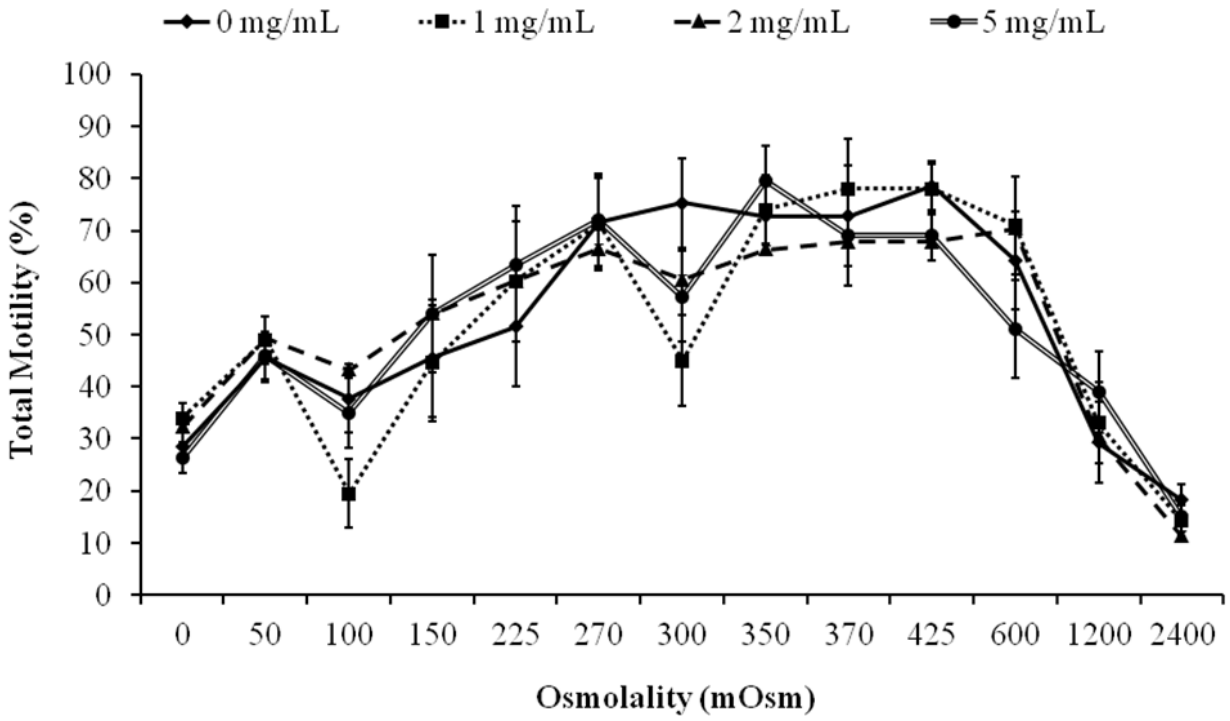

Figure 3.1. The percentages of total motile rooster spermatozoa treated when spermatozoa are treated with either 0 ( ), 1 ( ), 2 ( ) or 5 ( ) mg CLC/500 million sperm and placed into anisotonic conditions. N=8

Table 3.1. The percentage of total motile frozen/thawed rooster spermatozoa treated with 6 different cyclodextrin structures (2-hydroxypropyl-β, α, α-hydrate, β, β-hydrate and methyl-β) at 3 CLC concentrations (0.5 mg/mL, 1 mg/mL and 2 mg/mL) prior to cryopreservation. N=8

	CLC Concentration			
CLC Structure	0.5 mg/mL	1 mg/mL	2 mg/mL	SEM
2-Hydroxypropyl-β	51	48	53	3.3
α	50	54	50	3.9
α-hydrate	49	51	55	2.5
β	48	55	48	4.1
β-hydrate	50	51	52	3.8
methyl-β	51	52	46	4.2
Control	51	51	51	----
SEM	3.9	3.6	3.4	----

Table 3.2. The percentage of membrane intact frozen/thawed rooster spermatozoa treated with 6 different cyclodextrin structures (2-hydroxypropyl-β, α, α-hydrate, β, β-hydrate and methyl-β) at 3 CLC concentrations (0.5 mg/mL, 1 mg/mL and 2 mg/mL) prior to cryopreservation. N=8

	CLC Concentration			
CLC Structure	0.5 mg/mL	1 mg/mL	2 mg/mL	SEM
2-Hydroxypropyl-β	53	49	50	2.4
α	49	48	49	2.0
α-hydrate	48	49	48	1.9
β	50	52	42	2.0
β-hydrate	52	49	43	1.8
methyl-β	47	51	48	1.6
Control	47	47	47	----
SEM	2.1	2.1	1.8	----

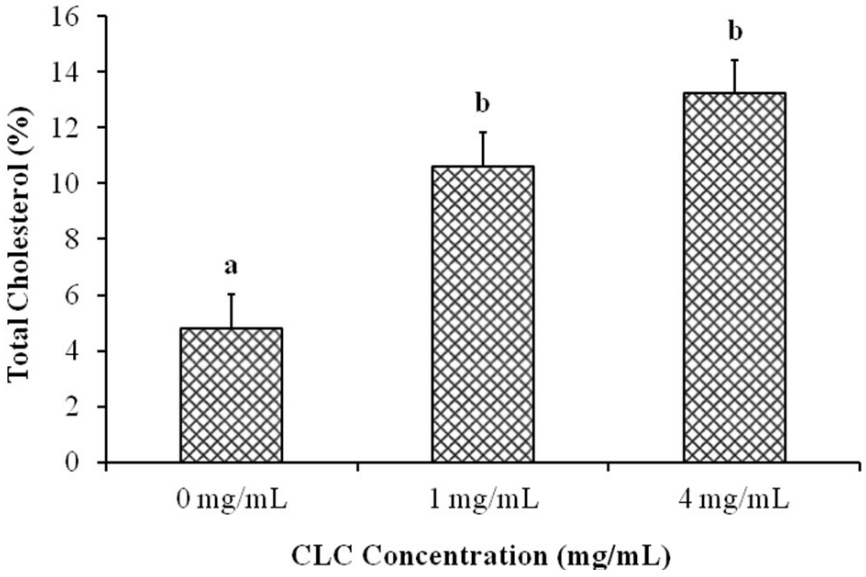

Figure 3.2. The total cholesterol in rooster spermatozoa treated at 3 CLC concentrations.
Bars with different superscripts (a, b, c & d) signify significance between groups ($P < 0.05$). N=4

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CHAPTER IV

EFFECTS OF LIPID ADDITION ON CRYOSURVIVAL OF ROOSTER SPERMATOZOA

Introduction

The poultry industry is one of the most technologically-advanced commercial animal production sectors in agriculture (Pardue, 2010). However, cryopreserved rooster sperm is not used to inseminate hens, due to its poor fertility and current cumbersome and technical methodology that must be used (Donoghue & Wishart, 2000). If cryopreserved rooster sperm were available that possessed high fertility and simple methods, it could be easily be adapted by the commercial poultry industry and by research laboratories into current artificial insemination schemes when implementing a streamlined cryopreservation process to preserve genetics for future use. Therefore, there is a need to improve rooster sperm cryopreservation.

Sperm plasma membranes are composed of proteins, cholesterol and phospholipids (Israelachvili, 1978; Hammerstedt & Graham, 1992; Parks & Lynch, 1992). These lipids, along with proteins, form the membrane bilayer, protecting and providing structure to the sperm cell. The ratio of phospholipids to cholesterol and proteins has been shown to be involved in how the cell responses to its surrounding environment during cryopreservation (Darin-Bennett & White, 1977; Parks & Lynch, 1992). Changing the phospholipid composition of rooster sperm membranes can decrease fertility without decreasing motility (Froman & Thurston, 1984). Therefore, both lipid composition and the arrangement of the lipids in membrane play a role in the ability of sperm to fertilize an oocyte (Hammerstedt, et al., 1990).

Sperm become fragile during freezing, and can be damaged during cooling, prior to and after freezing (Mazur, 1984). This damage is due in part to the membrane transitioning from a fluid to a gel state as the sperm membrane is cooled (Benson, et al., 1967). As the lipids in the membrane undergo their phase transition, the different lipids rearrange in the membrane This rearrangement induces the formation

of new microdomains of specific lipids which these can cause weak spots in the membrane, increasing the chance for membrane dysfunction and damage (Amann & Pickett, 1987; Hammerstedt, et al., 1990; Watson, 2000).

Rooster sperm membranes contain a unique composition of lipids that prevents the sperm from undergoing cold shock just before and at 5°C, as do mammalian sperm; suggesting that these membranes do not undergo significant phase transition at this temperature, as do mammalian sperm (Wales, et al., 1959; Pickett, et al., 1967; Watson, 1981; Parks and Lynch, 1992 White, 1993). This could be due to the prominence of glycolipids in the rooster sperm membrane, which are dispersed throughout the entire sperm plasma membrane, and keep the membrane fluid at low temperatures (Quinn, 1989; Parks & Lynch, 1992; Bongalhardo, et al., 2002). However, it is speculated that the membranes undergo phase transition at some temperature below 5°C, which alters membrane function, reducing the fertilizing potential of frozen-thawed sperm (Parks & Lynch, 1992).

Cyclodextrins can be used to transport hydrophobic molecules into or out of membranes (Christian, et al., 1997). They contain a hydrophilic exterior allowing it to "dissolve" in water and a lipophilic core that can be loaded with hydrophobic molecules (Loftsson & Duchêne, 2007; Mendez-Ardoy, et al., 2012). Cyclodextrins also act as transfer molecules that can transport hydrophobic molecules from their core down a concentration gradient into or out of a cell membrane efficiently (Yancey, et al., 1996). Cholesterol can easily be incorporated into cyclodextrins which interact with sperm membranes and increase the cholesterol content of sperm membranes (Purdy & Graham, 2004a;b; Moore, et al., 2005; Mocé & Graham, 2006; Amidi, et al., 2010; Mocé, et al., 2010; Spizziri, et al., 2010). However, there has been little research involved with altering the phospholipid content of sperm membranes even though cyclodextrins can encapsulate fatty acids and transfer them to cell membranes (Pallai, et al., 2009; Brunaldi, et al., 2010).

Although altering the cholesterol content of rooster sperm did not affect sperm cryosurvival rate, altering rooster sperm membrane lipid composition could alter membrane transition temperatures, but could also alter membrane osmotic tolerance during thawing and dilution (Kiso, et al., 2012). Increasing

the amount of lipids with unsaturated fatty acids decreases the temperature at which the membrane undergoes the phase transition and by keeping the membrane in a fluid state at low temperatures should inhibit cooling-induced membrane rearrangements (Haines, 1978; Holt & North, 1985; Quinn, 1989.) This should decrease membrane dysfunction upon warming (Bongalhardo, et al., 2002).

The objective of this study was to determine if incorporating phospholipids containing fatty acids with unsaturated bonds into rooster sperm membranes would improve sperm cryopreservation success.

Materials & Methods

Semen Collection, Processing & Cryopreservation

Ten Rhode Island Red, Black Sumatra, Silkie Bantam, Phoenix and Naked Neck breed roosters were individually housed at the United States Department of Agriculture's National Animal Germplasm Program facility. The birds were exposed to 16 hours of light and 8 hours of dark per day and were provided water and Flock Raiser® SunFresh® Recipe Crumbles complete feed (Land O' Lakes Purina Mills LLC, Grey Summit, MO) *ad libitum*. All animal care and procedures used to collect semen were approved by the Animal Care and Use Committee of the USDA. All chemicals were reagent-grade purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Roosters were collected (3 times/week) by abdominal massage (Burrows, et al., 1937) into conical sample cups (4-mL; Thermo Fisher Scientific, Waltham, MA). Immediately after collection, ejaculates were pooled, diluted 1:1 (v:v) with a glutamate-based diluent (LLT; Lake, et al., 1978), and transported at 5°C over ice to the laboratory within 30 min. Spermatozoal concentrations were determined using a Spectronic 20D spectrophotometer (Milton Roy Company, Rochester, NY) at 550-nm and initial sperm motility was determined using a Leitz Dialux 20 phase-contrast microscope (Leitz, West Germany), using a motility scale from 1 to 4; with 1 having little wave action and 4 having vigorous wave action (Tajima, et al., 1989). Ejaculates were discarded if motility was evaluated below 3.

Lipid-Loaded Cyclodextrins (LLCs)

Cyclodextrins were loaded according to procedures stated by Purdy & Graham (2004a). Briefly, 1-g of methyl-β cyclodextrin was dissolved into 2-mL of methanol (MeOH) in a glass tube. A total of 100-mg of each lipid was dissolved in 1-mL of chloroform. The dissolved cyclodextrins were added to the lipid/chloroform solution, and the two were mixed thoroughly. This was poured into a 100-mm glass petri dish and placed on a heated aluminum block and flushed with nitrogen gas until crystals formed on the bottom of the petri dish. These crystals were scraped off the petri dish when fully dried and the LLCs were stored at -80°C in a glass vial.

A working solution was made by dissolving 0.050-g of LLC into 1-mL of LLT diluent, and mixing thoroughly. Each LLC working solution was stored at -20°C.

Motility Analysis

The percentage of motile spermatozoa in each sample was determined using a computer-assisted sperm analysis system (CASA; HTM IVOS; Hamilton-Thorne Biosciences, Beverly, MA). System parameters for motility analysis using the CASA were as follows: 40 frames acquired at 60 frames per sec; minimum contrast 40; minimum cell size 2 pixels; lower VAP cut-off 20-µm/sec; lower VSL cut-off 10-µm/sec; VAP cut-off for progressive cells 50-µm/sec and straightness 75%. Each sample was mixed with LLT plus BSA (1%) and an 8-µL drop of spermatozoa from each sample was placed on a 37°C preheated CELL-VU counting slide (Thermo Fisher Scientific, Waltham, MA) and a minimum of 5 fields and at least 250 spermatozoa per sample were analyzed.

Total Lipid Extraction

Total lipid extraction was performed following procedures by Folch, et al. (1957), and modified by Bligh & Dyer (1959). Briefly, sperm samples were dried following procedures stated in experiment 4.2 and stored in a dessicator. Dry samples removed from dessicator and the contents of each weighed and

added to 4-mL of a chloroform/methanol solution (2:1; v:v). Each sample was homogenized using a Tissue-Tearor homogenizer (Model 985-370, BioSpec Products, Inc., Bartlesville, OK) at 5,000 RPM for 30 sec. Tubes were placed onto an agitator at 100 RPM (IKA-Vibrax-VXR, Janke & Kunkel GMBH & Co., Staufen, Germany) for 20 min, after which they were filtered through filter paper (Thermo Fisher Scientific, Waltham, MA, Hardened Ashless Circles, coarse porosity, >20-25-µm particle retention) into a second 12x75-mm borosilicate glass test tube. A total of 0.8-mL of 0.59% NaCl solution was added to each sample and the tubes were covered with tinfoil and stored overnight (5°C).

Samples were returned to 27°C (room temperature), with the solvent now in 2 phases. The bottom phase (containing the lipid) was removed using a 9-inch glass Pasteur capillary pipet into the pre-weighed 20-mL glass scintillation vials and the vials placed into a heated aluminum block and flushed with nitrogen gas until dried. The samples were placed into an oven (100°C) for 24 h after which they were placed into a dessicator for 3 h to cool. Each sample was weighed and the total dry sample weight was calculated by subtracting the weight of the glass tube without sample from the glass tube weight with sample. Total dry sample weight was recorded to 0.001 grams. Total percent lipid of the sample was calculated by dividing the total lipid weight by the total dry sample weight.

Saponification and Methylation of Lipids

Saponification and methylation of lipids was performed following the procedures of Park & Goins (1994), as modified by Phillips, et al. (2010). Briefly, 20-mL glass scintillation vials with lipid-extracted samples were taken from the dessicator and 1-mL of 0.5 N KOH in MeOH was added. The vials were tightly capped and placed into a 70°C water bath for 10 min to saponify by hydrolysis. Samples were removed from water bath and 1-mL of 14% Boron Trifluoride (BF_3) in MeOH was added to each vial, to derivatize fatty acids into methyl esters. Samples were tightly capped and placed into a 70°C water bath for 30 min. Vials were removed from water bath and allowed to cool to room temperature. After reaching 22°C, 2-mL of HPLC-grade hexanes and 2-mL of saturated NaCl were added to each sample and the sample mixed for 1 min using a vortex mixer. The samples were allowed to separate into

two phases, the upper layer containing the methyl esters in hexanes was removed, placed into a glass tube containing 800-mg of Na_2SO_4 and 1-mL of hexanes was added and mixed briefly. The sample was transferred to a new 20-mL glass vial, placed onto a warm aluminum block and flushed with nitrogen gas until the hexanes was completely removed. Each sample was reconstituted by adding 500-µL of hexanes and 200-µL of sample was put into a 300-µL glass vial and immediately analyzed using gas chromatography. The percent total lipid was calculated by dividing the corrected total peak areas into the lipid peak area, and multiplying by 100.

Gas Chromatography Analysis

An Agilent Technologies model 6890 series Gas-Chromatographer (Santa Clara, CA), fixed with a series 7683 injector and flame-ionization detector was used to evaluate samples for lipid incorporation. The instrument was equipped with a 100-m × 250-µm ID x 0.2-µm film fused silica capillary column (SP-2560, Supelco Inc., Bellefonte, PA). The front injector was 1-µL run on splitless inlet. Total flow was maintained at 54.1-mL/min and purge flow to split vent was set at 50-mL/min at 2 min. The carrier gas was helium with a flow rate of 1.0-mL/min at 33.39 psi with an average velocity of 21-cm/sec. Column oven temperature was increased from 130°C to 200°C at 4°C/min, and then held at 200°C for 72.5 min. Detector gas flows were maintained at 40-mL/min for hydrogen, 450-mL/min for air and 45-mL/min for makeup helium, with let offset at 2.0. The injector and detector were maintained at 250°C. Data rate was 10 Hz and minimum peak width was set at 0.02 min. Total run time was 90 min.

Experiment 4.1: Effects of Lipid-Loaded Cyclodextrins on Frozen/Thawed Rooster Spermatozoa Motility

Three different lipids: 1) 1,2-Dilinolenoyl-sn-glycero-3-phosphocholine (PC 18:3), 2) 1,2-Dilinoleoyl-rac-glycerol (PG 18:2) and 3) 1,2-Dilinoleoyl-sn-glycero-3-phosphocholine (PC 18:2; Avanti Polar Lipids, Inc., Alabaster, AL) were loaded into methyl-β cyclodextrins (LLCs) as described, and 50-mg/mL working solutions made. Spermatozoa were diluted to 800 million cells/mL with LLT at 5°C, and

divided into 24 equal aliquots. Sperm were treated with each of the three LLCs at different concentrations (0, 0.25, 0.5, 1, 1.5, 2, 4 and 6 mg/mL). Samples incubated with LLCs for 30 min at 5°C to allow lipid transfer to occur. Sperm aliquots were then diluted 1:1 (v:v) with LLT containing 18% dimethylformamide to obtain a final cryoprotectant concentration of 9% (Phillips, et al., 1996). Dimethlyformamide was used as the cryoprotectant as LLC effects would more easily be detected, as dimethylformamide was not the best treatment observed in the alternative cryoprotectant experimentation (Chapter II). The spermatozoa were loaded into 0.25-mL polyvinylchloride straws (IMV Technologies, Minneapolis, MN), frozen in liquid nitrogen vapor 6-cm above the level of liquid nitrogen for 15 min (Tajima, et al., 1989) and stored in liquid nitrogen until analysis. This experiment was replicated 8 times.

Two straws from each treatment were thawed in a 5°C water bath for 5 min and the motility was assessed. The contents from straws were diluted 1:10 (v:v) with LLT with 1% BSA to give a final spermatozoa concentration of 40 million cells/mL. Each sample was immediately assessed for total motile sperm by CASA.

Experiment 4.2: Incorporation of Lipids into Rooster Spermatozoa Plasma Membrane using Lipid-Loaded Cyclodextrins

Fresh rooster spermatozoa ejaculates were collected, pooled and diluted to a total volume of 7.5-mL using LLT, and mixed. The samples were split into 5x 1.4-mL aliquots, and different concentrations of one lipid per day were added to the aliquots. Each lipid LLC was replicated 3 or 4 times. The amounts of LLT and LLC added to each test tube on a specific day was:

Tube	*LLT*	*LLC*	*LLC Concentration*
1	100-µL	0-µL	0-mg/mL
2 and 4	70-µL	30-µL	1-mg/mL
3 and 5	0-µL	120-µL	4-mg/mL

Each tube was mixed thoroughly after the LLCs were added and the sperm incubated at 5°C for 30 min. After incubation, each tube was mixed.

Following incubation, the sperm were removed from extra LLCs in the sample, by layering the tube contents over 1-mL of 30% Percoll in PBS and centrifuging at 400 g for 10 min. Two layers were observed following centrifugation. The top clear layer was discarded and the bottom layer was resuspended, in the new tube, with 2-mL of PBS. The samples were washed twice by centrifugation at 800 g for 10 min. And each sample resuspended using 1.5-mL of PBS and placed into preweighed tubes. The sperm concentration of each sample was determined using a hemocytometer (Foote, 1979).

The glass tubes were placed into a 100°C oven for 48 h to dry. After drying, the tubes were placed into a glass dessicator for 30 min to cool and each tube was weighed after cooling. Samples were stored in glass dessicator at 27°C until Total Lipid Extraction, Saponification and Methylation was conducted.

Statistical Analysis

Data were analyzed by analysis of variance and treatment means were separated by Student-Newman Keuls mean separation technique (SAS Institute Inc., 1985), for differences in sperm motility and total lipid between LLC treatments. Treatments were considered different if $P < 0.05$.

Results

Experiment 4.1: Effects of Lipid-Loaded Cyclodextrins on Frozen/Thawed Rooster Spermatozoa Motility

Control samples and samples incubating sperm with LLCs resulted in no difference in percentages of motile sperm for PC 18:2, PG 18:2 and PC 18:3 ($P > 0.05$; Figure 4.1).

Experiment 4.2: Incorporation of Lipids into Rooster Spermatozoa Plasma Membranes using Lipid-Loaded Cyclodextrins

Control samples and samples incubating sperm with LLCs contained similar percentages of lipid addition for sperm incubated with PC 18:2 (0.00003107% versus 0.00004645%, respectively) and PC 18:3 (0.00003107% versus 0.00002925%, respectively; $P > 0.05$; Figures 4.2 & 4.3). Samples incubated with PC 18:3 LLCs exhibited a trend of lipid incorporation, with higher total lipid percentages (0.0004405%) over control sperm (0.0001935%; $P > 0.05$; Figure 4.4).

Discussion

Low fertility rates achieved with current cryopreservation methods prohibits cryopreserved rooster sperm from being used to artificially inseminate hens in the industry (Hammerstedt & Graham, 1992; Fulton, 2006; Purdy, et al., 2009). Membrane damage caused during freezing and during dilution of the sperm when they are exposed to hypotonic environments during cryoprotectant removal are likely the cause for reduced fertilizing capacity (Griffiths, et al., 1979; Hammerstedt, et al., 1990; Hammerstedt & Graham, 1992; Hammerstedt, 1995). Increasing membrane fluidity at lower temperatures, decreases membrane damage and increases sperm cryosurvival. Increasing the amount of PC 18:2, PG 18:2 and PC 18:3 were tested in these experiments, but these additions did not improve sperm motility. Further experimentation should be investigated using flow cytometry to demonstrate the fluidity changes that might be seen when using these particular lipids when cryopreserving rooster sperm.

As LLC concentration increased, there was a trend of decreasing percentages of motile sperm. The reason for this is not known, particularly since PC 18:2 and PG 18:2 did not incorporate into the sperm. This may be a result of some residual component from loading the LLC's, but similar effects were not seen when cholesterol-loaded cyclodextrins were added to sperm using a similar methodology. This could be due to the saturation levels of the lipids used in the experimentation. Cyclodextrins are able to transfer sterols into and out of cell membrane, therefore, it is possible that the cyclodextrins were not fully loaded with lipid, and may have extracted cholesterol from the membrane during incubation.

Conclusions

The addition of lipids containing unsaturated fatty acids using lipid-loaded cyclodextrins did not improve rooster sperm motility after cryopreservation. Using methyl-β cyclodextrins to change the phospholipid composition of rooster sperm was not effective.

Figures

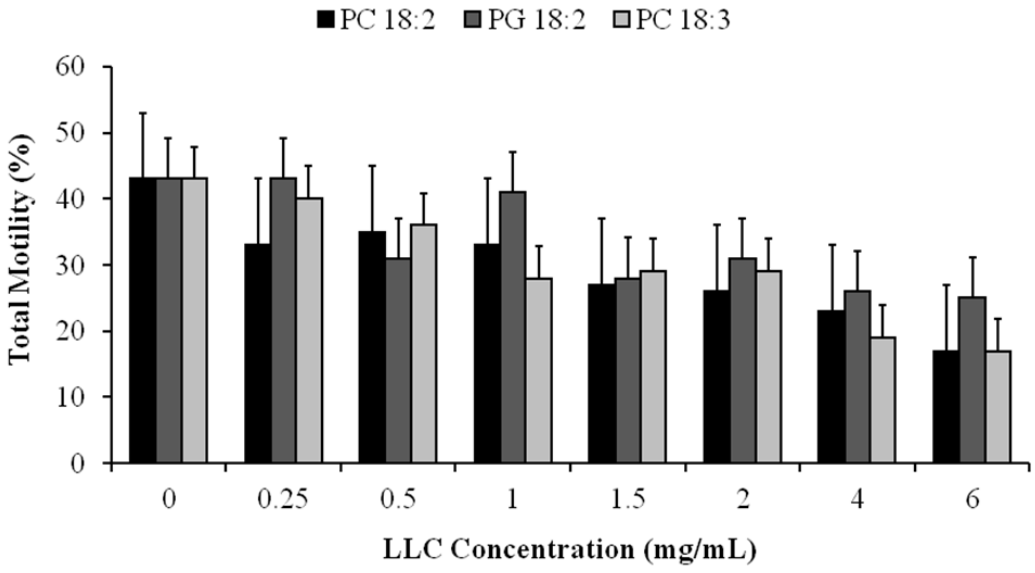

Figure 4.1. The percentages of total motile rooster spermatozoa after cryopreservation when treated with 3 different lipids (1,2-Dilinoleoyl-sn-glycero-3-phosphocholine [PC 18:2], 1,2-Dilinoleoyl-rac-glycerol [PG 18:2] and 1,2-Dilinolenoyl-sn-glycero-3-phosphocholine [PC 18:3]) at 8 different levels of LLC concentration (0, 0.25, 0.5, 1, 1.5, 2, 4 and 6 mg/mL) prior to cryopreservation. N=8. Bars signify standard error.

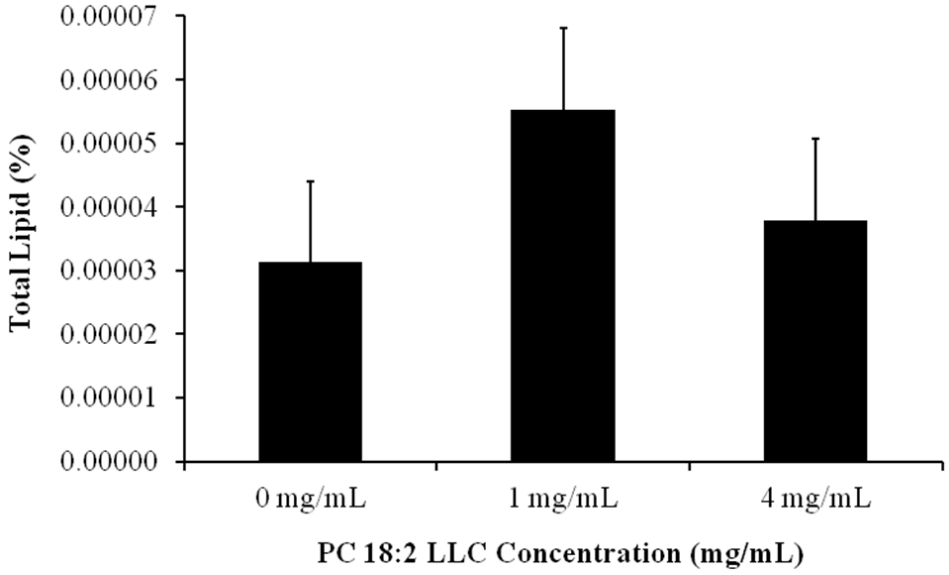

Figure 4.2. The percentages of total lipid of PC 18:2 (1,2-Dilinoleoyl-sn-glycero-3-phosphocholine) in rooster spermatozoa treated with 1 mg/mL and 4 mg/mL LLC. N=4. Bars signify standard error

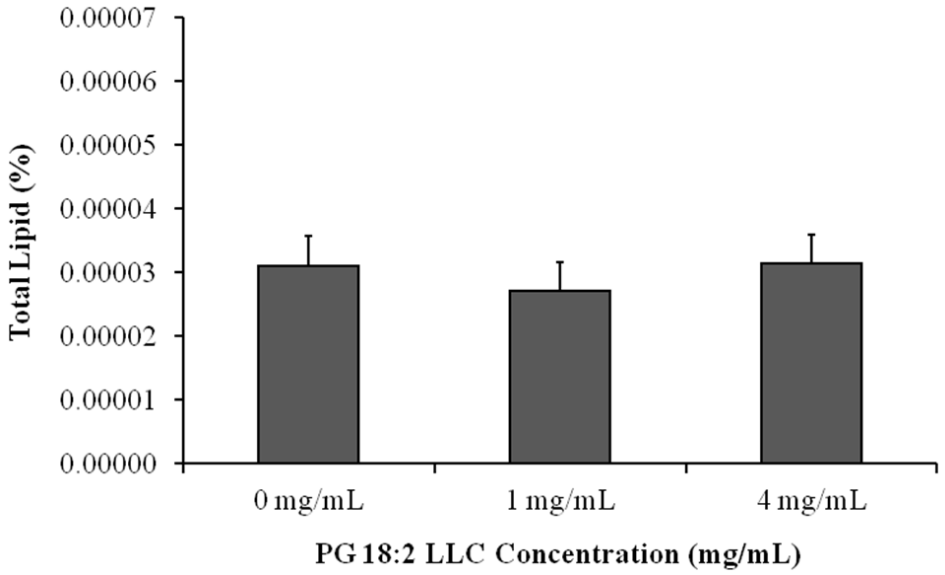

Figure 4.3. The percentages of total lipid of PG 18:2 (1,2-Dilinoleoyl-rac-glycerol) in rooster spermatozoa treated with 1 mg/mL and 4 mg/mL LLC. N=4. Bars signify standard error.

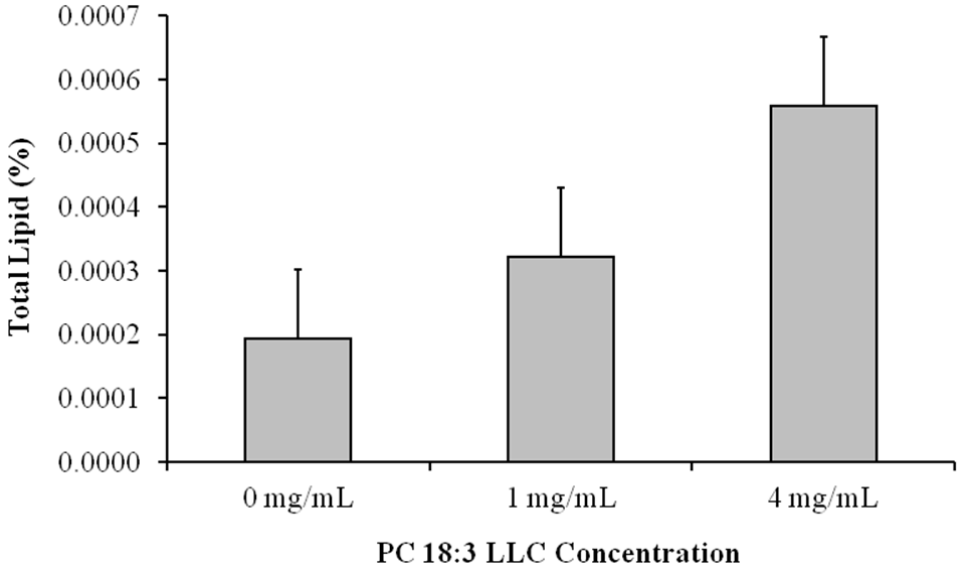

Figure 4.4. The percentages of total lipid of PC 18:3 (1,2-Dilinolenoyl-sn-glycero-3-phosphocholine) in rooster spermatozoa treated with 1 mg/mL and 4 mg/mL LLC. N=3. Bars signify standard error.

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CHAPTER V

EFFECTS OF DILUENTS, STRAW SIZE, SPERMATOZOA CONCENTRATION AND CRYOPROTECTANT ON ROOSTER SPERMATOZOA CRYOPRESERVATION

Introduction

Being able to cryopreserve sperm permits the transport of unique genetics; it can decrease genetic drift in populations, bank genetics for years, be used to restore lost populations, and can be used to inseminate at any time (Blesbois, 2007). The poultry industry has not been able to capitalize on these benefits as cryopreserved rooster sperm possess low fertility and require extensive and expensive methods to achieve that fertility (Lake, 1986; Hammerstedt & Graham, 1992; Buss, 1993; Long, 2010). Many methods to cryopreserve rooster sperm have been developed over the years to improve the cryosurvival rates and fertility of rooster sperm, but these have had only limited success (Lake, 1986; Hammerstedt & Graham, 1992; Buss, 1993; Long, et al., 2010).

Researchers have investigated diluent composition, cooling rate, straw volume and different cryoprotectants to optimize rooster sperm cryosurvival (Lake, 1986; Hammerstedt & Graham, 1992; Buss, 1993). A glutamate-based diluent (LLT; Lake, et al., 1978) has been one of the most commonly used diluents to freeze rooster sperm. However, a trehalose-based diluent was recently developed to cryopreserve rooster sperm using methylacetamide, an alternative cryoprotectant (SJD; Hanawa, et al., 2010; Sasaki, et al., 2010). Methylacetamide does not need to be diluted from the sperm in SJD before insemination. However, no studies have compared freezing rooster sperm in LLT and SJD diluents.

The use of low molecular weight cryoprotectants has been shown to induce less membrane damage during cryopreservation and insemination, and potentially could eliminate the need to slowly dilute the sperm from the cryoprotectant prior to insemination, as has been shown in many different species (Lake & Ravie, 1984; Mitchell & Buckland, 1975; Westfall & Harris, 1975; Graham, et al., 1982; Hammerstedt & Graham, 1992, Phillips, et al., 1996; Squires, et al., 2004; Purdy, et al., 2010; Sasaki, et al., 2010).

Sperm concentration has both physiological and practical effects on sperm during freezing and on insemination. If sperm are not frozen at a sufficiently high cell concentration using glycerol as the cryoprotectant, after thawing, dilution and centrifugation, there will not be sufficient sperm for insemination when using only one straw of semen (Sexton, 1981; Lake, 1986). However, if sperm are frozen at high concentrations, cryosurvival rates are affected (Shannon, 1965; Alvarez, et al., 2012). If rooster sperm can be frozen using cryoprotectants that do not need to be slowly diluted from the sperm prior to insemination, these sperm can be frozen at sperm concentrations needed for inseminating (Sasaki, et al., 2010). In addition, not having to remove the cryoprotectant prior to insemination simplifies insemination to the point that frozen semen could be incorporated into the industry.

This study compared the effects of two different rooster sperm freezing diluents (LLT and SJD), two cryoprotectants and two different straw volumes to cryopreserve rooster sperm.

Materials & Methods

Semen Collection, Processing & Cryopreservation

Ten Rhode Island Red, Black Sumatra, Bantam Silkie, Phoenix and Naked Neck breed roosters were individually housed at the United States Department of Agriculture's National Animal Germplasm Program facility. The birds were exposed to 16 hours of light and 8 hours of dark and were provided water and Flock Raiser® SunFresh® Recipe Crumbles complete feed (Land O' Lakes Purina Mills LLC, Grey Summit, MO) *ad libitum*. All animal care and procedures used to collect semen were approved by the Animal Care and Use Committee of the USDA. All chemicals were reagent-grade purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Roosters were collected (3 times/week) by abdominal massage (Burrows, et al., 1937) into conical sample cups (4-mL; Thermo Fisher Scientific, Waltham, MA). Immediately after collection, ejaculates were pooled, split into 2 aliquots, and each diluted either 1:1 (v:v) with LLT or 1:1 (v:v) with SJD and transported at 5°C over ice to the laboratory within 30 minutes. Spermatozoal concentrations were determined using a Spectronic 20D spectrophotometer (Milton Roy Company, Rochester, NY) at 550-nm and initial sperm motility was

determined using a Leitz Dialux 20 phase-contrast microscope (Leitz, West Germany), using a motility scale from 1 to 4; with 1 having little wave action and 4 having vigorous wave action (Tajima, et al., 1989). Ejaculates were discarded if motility was evaluated below 3.

Motility Analysis

The percentage of motile spermatozoa in each sample was determined using a computer-assisted sperm analysis system (CASA) (HTM IVOS; Hamilton-Thorne Biosciences, Beverly, MA). System parameters for motility analysis using the CASA were as follows: 40 frames acquired at 60 frames per sec; minimum contrast 40; minimum cell size 2 pixels; lower VAP cut-off 20-µm/sec; lower VSL cut-off 10-µm/sec; VAP cut-off for progressive cells 50-µm/sec and straightness 75%. Each sample was mixed with LLT plus BSA (1%) and an 8-µL drop of spermatozoa from each sample was placed on a 37°C preheated CELL-VU counting slide (Thermo Fisher Scientific, Waltham, MA) and a minimum of 5 fields and at least 250 spermatozoa per sample were analyzed.

Flow Cytometric Analysis

The plasma membrane integrity of spermatozoa was determined using flow cytometry. Spermatozoa were analyzed using a CyAN ADP flow cytometer (Beckman Coulter, Miami, FL) and a total of 10,000 cells/sample assayed, and data were analyzed using HyperCyt Software (Beckman Coulter, Miami, FL). The fluorescent dye propidium iodide (PI) used was excited using a 488-nm argon laser at 20mW power. The fluorescence of PI was detected using a filter setup combination of a 530-nm band pass filter (FL-1 detector) and a 613-nm band pass filter (FL3 detector) to detect PI (Purdy, et al., 2009).

Experiment 5.1: Effects of Diluent, Straw Size and Cryoprotectant on Frozen/Thawed Rooster Spermatozoa Motility

Sperm initially diluted 1:1 in LLT and SJD were diluted to 800 million cells/mL with their respective diluent at 5°C after the sperm concentration was determined, Each sample was then divided into 4 equal aliquots and diluted 1:1 (v:v) in their respective diluent containing 18% glycerol or methylacetamide to obtain a final CPA concentration of 9% (Phillips, et al., 1996). The spermatozoa were loaded into either 0.5-mL or 0.25-mL polyvinylchloride straws (IMV Technologies, Minneapolis, MN) based on previous experiments (Sasaki, et al., 2010), and frozen in liquid nitrogen vapor. Sperm packaged in 0.5-mL straws were frozen 4-cm above the level of liquid nitrogen for 30 min (Sasaki, et al., 2010), while sperm packaged in 0.25-mL straws were frozen 6-cm above the level of liquid nitrogen for 15 min (Tajima, et al., 1989). The straws were plunged into liquid nitrogen and stored at -196°C until analysis. This experiment was replicated 10 times.

Two straws from each treatment were thawed in a 5°C water bath, 25-mL straws 5 min and 0.5-mL straws for 1 min and 40 sec. The contents of the straws were diluted 1:10 (v:v) with their respective diluent, containing 1% BSA providing a final spermatozoa concentration of 40 million cells/mL and motility assessed using CASA.

Experiment 5.2: Effects of Sperm Concentration, Diluent, Straw Size and Cryoprotectant on Frozen/Thawed Rooster Spermatozoa Motility and Membrane Integrity

Sperm initially diluted 1:1 in LLT and SJD were diluted to 2 billion cells/mL with their respective diluent at 5°C after the sperm concentration was determined. Each sample was divided into 8 equal aliquots, and half were diluted 1:1 (v:v) to 1 billion cells/mL. Samples were diluted 1:1 (v:v) in their respective diluent containing 18% glycerol or methylacetamide to obtain a final cryoprotectant concentration of 9% and final spermatozoa concentration of 1 billion cells/mL resulted for high concentration treatments, and 500 million cells/mL for low concentration treatments (Phillips, et al.,

1996). The spermatozoa were loaded into either 0.5-mL or 0.25-mL polyvinylchloride straws (IMV Technologies, Minneapolis, MN) and frozen as described in experiment 5.1. This experiment was replicated 10 times.

Two straws from each treatment were thawed and diluted, as described in experiment 5.1, providing a final sperm concentration of 100 million cells/mL for high sperm concentration treatments and 50 million cells/mL for low sperm concentration treatments, and motility assessed using CASA.

To evaluate membrane integrity, two straws were thawed as described above, and the contents of straws were diluted 1:100 (v:v) with either LLT or SJD diluent containing 1% BSA, according to treatment, resulting in a final spermatozoa concentration of 10 million cells/mL for higher concentration treatments, and 5 million cells/mL for lower concentration treatments. Each sample was stained with 5-µL of PI (1 mg/mL in H_2O) for 5 min at 21°C to allow for stain incorporation (Krishan, 1975) after which each sample was mixed and analyzed by flow cytometery.

Statistical Analysis

Data were analyzed by analysis of variance and treatment means were separated by Student-Newman Keuls mean separation technique (SAS Institute Inc., 1985), for differences in sperm motility and plasma membrane integrity between diluents, CPA, straw size and sperm concentration treatments. Treatments were considered different if $P < 0.05$.

Results

Experiment 5.1: Effects of Diluent, Straw Size and Cryoprotectant on Frozen/Thawed Rooster Spermatozoa Motility

Sperm cryopreserved in 0.5-mL straws exhibited higher percentages of motile cells (> 45%) than sperm frozen in 0.25-mL straws (< 45%; $P < 0.05$) For samples frozen in 0.5-mL straws, sperm cryopreserved in SJD diluent exhibited higher percentages of motile cells (> 60%) than sperm frozen in LLT with glycerol as the cryoprotectant (46%; $P < 0.05$; Figure 5.1).

Experiment 5.2: Effects of Sperm Concentration, Diluent, Straw Size and Cryoprotectant on Frozen/Thawed Rooster Spermatozoa Motility and Membrane Integrity

Sperm cryopreserved in SJD medium and packaged in 0.5-mL straws at 1 billion cells/mL resulted in the highest percentage of motile cells, regardless of cryoprotectant used (> 53%), while sperm frozen in LLT and packaged in 0.25-mL straws at 500 million cells/mL exhibited the lowest percentage of motile sperm (< 34%; Table 5.1). When sperm were diluted in LLT with glycerol, the lowest percentages of motile sperm were observed when the cells were frozen in 0.25-mL straws at 500 million cells/mL (34%; Table 5.1).

Sperm cryopreserved in SJD with glycerol at 500 million cells/mL in 0.5-mL straws resulted in the highest percentage of membrane intact cells (83%), while sperm cryopreserved in LLT with glycerol at 1 billion cells/mL in 0.25-mL straws resulted in the lowest percentage of membrane intact cells (61%; Table 5.2).

In general, sperm cryopreserved in SJD exhibited higher motility and membrane-intact cells than LLT, and sperm frozen in 0.5-mL straws exhibited higher motility and membrane-intact cells than sperm frozen in 0.25-mL straws ($P < 0.05$).

Discussion

Methylacetamide appears to be an effective cryoprotectant for cryopreserving rooster sperm. This cryoprotectant does not appear to harm cells when incubated with sperm at 8% over an hour, and sperm left undiluted from methylacetamide exhibit similar motility and membrane integrity as sperm cryopreserved with glycerol. Sasaki, et al. (2010) reported sperm cryopreserved using methylacetamide fertilized 82% of eggs when the sperm were inseminated with the cryoprotectant left with the sperm. Other cryoprotectants, when inseminated with sperm, do not result in sufficiently high fertility to be used in the industry. Sperm frozen with methylacetamide as the cryoprotectant exhibited high percentages of motile sperm in both experiments, especially for sperm frozen in the SJD diluent.

The effect that straw size has on sperm cryosurvival depends on the diluent being used and the cooling rate used (Christensen & Tiersch, 1997; Nöthling & Shuttleworth, 2005; Mocé, et al., 2010). Sperm frozen in 0.5-mL straws exhibited higher percentages of both motile sperm in both experiments, especially for sperm frozen in the SJD diluent.

LLT contains five ingredients: sodium glutamate, fructose, magnesium acetate, potassium citrate and sodium acetate. Fructose is used as the sperm's nutritional energy source in LLT, while the other components increase the osmotic pressure of the diluent to physiological levels. Sasaki, et al. (2010) has recently developed a diluent containing trehalose, sodium glutamate, glucose, magnesium acetate, potassium citrate, sodium acetate, potassium acetate, BES and Bis-Tris. Diluent components have a well documented relationship between sperm cryosurvival and fertility (Sexton & Fewlass, 1978; Sexton, 1980; Salamon & Ritar, 1982). Overall, sperm survived cryopreservation better in SJD than LLT, possibly due to the higher buffering capacity of the diluent. Both diluents had the same osmotic pressure (360 mOsm) but SJD had a lower pH (6.8) compared to LLT (7.5). This slight difference in pH could explain the difference in sperm cryosurvival in these diluents.

Rooster sperm must be frozen at a relatively high sperm concentration in order to have sufficient viable sperm after dilution to remove the cryoprotectant and reconcentration to inseminate with reasonable sperm numbers (Lake, 1986; Hammerstedt & Graham, 1992; Purdy, et al., 2009). However, sperm do not need to be diluted and reconcentrated if cryopreserved with methylacetamide, therefore, the sperm can be frozen at lower concentrations and still have sufficiently high sperm concentrations for insemination. Sperm frozen at 1 billion cells/mL in the SJD diluent exhibited high motility and membrane integrity when frozen in 0.5-mL straws, regardless of cryoprotectant. If sperm concentration is too high, there is simply not sufficient space in unfrozen channels to accommodate all the sperm and sperm become trapped in the ice crystals leading to cell death (Amann & Pickett, 1987; Mazur & Cole, 1989).

Conclusions

Rooster sperm frozen in 0.5-mL straws in the SJD diluent cryopreserve more effectively than sperm frozen in the LLT diluent in 0.25-mL straws, the classic method used today. In addition, since methylacetamide passes through the sperm membrane more quickly than glycerol, causing less volume change damage to the cells, and it may not need to be removed from the sperm after thawing prior to insemination.

Figures & Tables

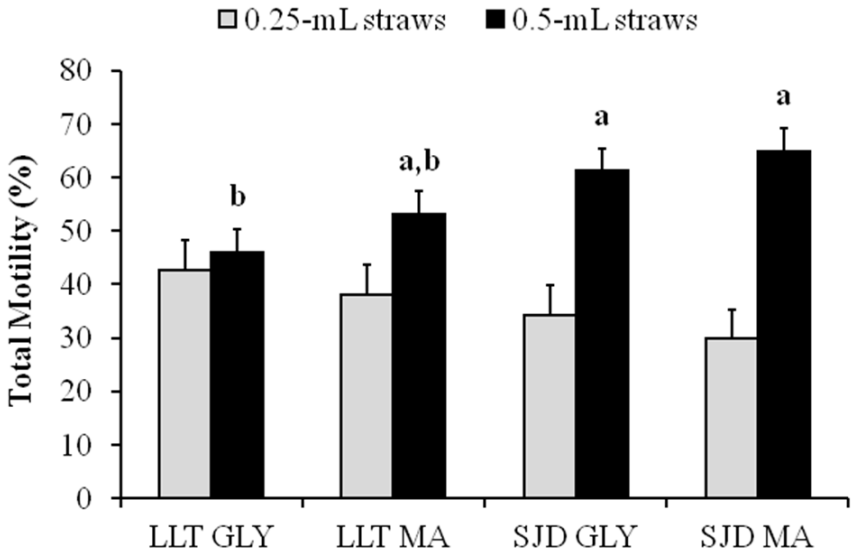

Figure 5.1. The percentage of total motile frozen/thawed rooster spermatozoa diluted in LLT or SJD and frozen using either glycerol (GLY) or methylacetamide (MA) in 2 different straw sizes (0.25-mL or 0.5-mL). N=10
Different superscripts within the same column color signify significance between columns of the same color ($P < 0.05$).

Table 5.1. The percentage of total membrane-intact frozen/thawed rooster spermatozoa diluted in LLT or SJD and frozen using either glycerol (GLY) or methylacetamide (MA) at either at 2 concentrations 500 million cells/mL (low) or 1 billion cells/mL (high) in 2 different straw sizes (0.25-mL or 0.5-mL). N=10
Different superscripts signify significance ($P < 0.05$).

Diluent & CPA	Straw Size	0.25-mL		0.5-mL		
	Concentration	Low	High	Low	High	SEM
LLT GLY		34[b]	44[ab]	40[ab]	49[a]	3.5
LLT MA		29	42	43	46	4.8
SJD GLY		38	41	41	53	4.9
SJD MA		38	41	45	55	5.6
SEM		4.9	4.8	4.7	4.5	----

Table 5.2. The percentage of total motile frozen/thawed rooster spermatozoa diluted in LLT or SJD and frozen using either glycerol (GLY) or methylacetamide (MA) at either at 2 concentrations 500 million cells/mL (low) or 1 billion cells/mL (high) in 2 different straw sizes (0.25-mL or 0.5-mL). N=10 Different alpha superscripts signify significance within row; different symbol superscripts signify significance with column ($P < 0.05$).

Diluent & CPA	Straw Size	0.25-mL		0.5-mL		
	Concentration	Low	High	Low	High	SEM
LLT GLY		$68^{*\dagger}$	$61^{*\dagger}$	$70^{\dagger}$	69	2.8
LLT MA		$63^{\dagger}$	$57^{\dagger}$	$68^{\dagger}$	65	3.6
SJD GLY		77^{ab*}	71^{b*}	83^{a*}	77^{ab}	2.5
SJD MA		74^{a*}	$63^{b*\dagger}$	$76^{a*\dagger}$	70^{ab}	3.0
SEM		2.6	3.3	2.7	3.4	----

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CHAPTER VI

SUMMARY OF THESIS

Conclusions

Rooster spermatozoa sustain irreversible damage during cryopreservation (Lake, 1986; Hammerstedt & Graham, 1992; Buss, 1993; Long, et al., 2010). This is due in part to membrane damage caused by osmotic shock, as well as lipid and protein rearrangement within the membrane when the membrane undergoes the phase transition, from a fluid state to a crystalline state (Hammerstedt, et al., 1990). Low molecular weight cryoprotectants have been shown to decrease osmotic shock and membrane damage, because they cross the plasma membrane quickly, decreasing overall cell volume changes during aniosmotic conditions thereby reducing membrane damage (Hammerstedt & Graham, 1992; Buss, 1993; Gill, et al., 1996; Squires, et al., 2004; Sasaki, et al., 2010). In this study, several alternative cryoprotectants were tested in freezing diluents, and methylacetamide, shows promise in protecting sperm motility and membrane intact sperm cells after freezing, while methylformamide, ethylene glycol and dimethylformamide did not.

Cholesterol is an important component in sperm membranes and the cholesterol:phospholipid ratio of the plasma membrane has an effect on whether sperm are susceptible to cold shock or not (Watson, 1981; Parks & Lynch, 1992; White, 1993). As the cholesterol content of a membrane increases, temperature in which the membrane undergoes the phase transition decreases and a fluid membrane incurs less damage than a crystalline one (Hammerstedt, et al., 1990). Therefore, increasing cholesterol content of a membrane decreases its damage when cooled (Quinn, 1989). Increasing the cholesterol content of mammalian sperm using cholesterol-loaded cyclodextrins (CLCs) increases the motility and membrane intact cells, after cryopreservation (Purdy & Graham, 2004a;b; Moore, et al., 2005; Mocé & Graham, 2006; Amidi, et al., 2010; Mocé, et al., 2010; Spizziri, et al., 2010). In the present study, cholesterol added to the plasma membrane did not improve sperm motility or increase membrane

integrity. Although cholesterol was increased greater than 2-fold in rooster sperm, neither sperm motility nor membrane integrity were increased after cryopreservation, compared to untreated sperm.

Fatty acids have also been shown to affect membrane fluidity (Pallai, et al., 2009; Brunaldi, et al., 2010). Addition of LLC containing different lipid species and different unsaturated fatty acids failed to improve rooster sperm cryosurvival rates. Although part of this lack of effect was due to the lipids not transferring into the membrane, the treatments seemed to be detrimental to rooster sperm.

Freezing protocols for rooster sperm have explored different diluents, cryoprotectants, sperm concentrations, cooling rates and packaging sizes on sperm cryosurvival (Lake & Ravie, 1984; Mitchell & Buckland, 1975; Westfall & Harris, 1975; Graham, et al., 1982; Hammerstedt & Graham, 1992, Phillips, et al., 1996). We found that sperm frozen in a trehalose-based diluent (SJD, Sasaki, et al., 2010) had higher percentages of motile and membrane intact cells than the more commonly used rooster sperm diluent, LLT (Lake, et al., 1978). In addition, when SJD is used to cryopreserve sperm using methylacetamide as the cryoprotectant, high percentages of motile and membrane intact cells can be achieved when the sperm are packaged in 0.5-mL straws. A sperm concentration of 500 million cell/mL appears to provide better cryosurvival rates than freezing the sperm at 1 billion cells/mL. These results need to be further verified in a fertility trial.

Finally, sperm frozen in methylacetamide can be left with the cryoprotectant for over a one hour and if sperm frozen in methylacetamide eliminates the need for dilution prior to insemination, this cryopreservation procedure could be adapted for both research and commercial poultry industry use (Hanawa, et al., 2010; Sasaki, et al., 2010).

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APPENDIX I

MEDIA & STAIN RECIPES

Media Recipes

Glutamate-Based Diluent (Lake's Low Temp., LLT)	g/L
Na Glutamate	19.2
Fructose	6.0
Mg acetate * $4H_2O$	0.8
K citrate * H_2O	1.2
Na acetate (anhydrous)	5.1

Adjust osmolality (360-370 mOsm) and pH (7.5)
Add cryoprotectant by volume

Trehalose-Based Diluent (Japanese)	g/L
Glucose	2.0
Trehalose *$2H_2O$	38.0
Na Glutamate	12.0
K acetate	3.0
Mg acetate * $4H_2O$	0.8
K citrate * H_2O	0.5
BES	4.0
Bis-Tris	4.0

Adjust osmolality (360-370 mOsm) and pH (6.8)
Add cryoprotectant by volume

Lake's High Temp. Diluent (LHT)	g/L
TES	13.78
NaCl	8.00
Glucose	6.00
NaOH	1.10

Adjust osmolality (380 mOsm) and pH (7.4)

Low Potassium (Low K+ PBS)	g/L
NaCl	5.85
KCl	0.2
NaH_2PO_4 * H_2O	1.19
Na_2HPO_4	1.80
Glucose	10.0

Adjust osmolality (300-310 mOsm) and pH (7.4)

30% Percoll Gradient	For 100 mL
10x Low K+ PBS	3.3 mL
Percoll	29.7 mL
Low K+ PBS	67 mL

Stain Recipes

Propidium Iodide (PI) Fluorescent Stain
Working solution at 1 mg/mL
Stain 4 to 5 million cells with 5 μL PI for 5 min
Staining is consistent in any spermatozoa diluent
Stain stored at 5ºC

APPENDIX II

GAS CHROMATOGRAPHY SOLUTION RECIPES

Folch Recipes

2:1 Chloroform:MeOH Mixture	For 1 L
Chloroform	667 mL
Methanol	333 mL

0.59% NaCl Solution	For 100 mL
NaCl	0.9 g

Saponification and Methylation Recipes

0.5 N KOH in MeOH	For 100 mL in MeOH
KOH	2.81 g

Saturated NaCl Solution	For 100 mL
NaCl	31.7 g

Cholesterol Purification Recipes

33% KOH Solution	For 670 L
KOH	330 g

0.066% Stigmasterol in Hexane	For 250 mL in Hexanes
Stigmasterol	0.165 g