Evaluation of Different Concentrations of Egg Yolk in Canine Frozen Semen Extender

Stephanie W. Trout

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Beverly J. Purswell, Chair
Sherrie G. Clark
William H. Eyestone

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Abstract

This study tested different concentrations of egg yolk in canine freezing extender void of glycerol, a commonly used cryoprotectant, by examining the motility and morphology throughout the freezing process: initial (baseline after extender added), post-cool (after three hours at 5°C) and post-thaw (after freezing.) Initial values of pH, osmolarity, motility and morphology were obtained for comparison of the samples. Spermatozoa from six normal dogs as determined by progressive linear motility > 70% and normal morphology > 60% was used. Semen was collected and pooled for five freezing trials. The concentrations of egg yolk used in the extender were: 0%, 10%, 20%, 30% and 40%. Assessment of each sample was blinded to the treatments until all results were obtained and statistics had been analyzed. Based on this study a 20% egg yolk concentration is slightly superior to a 30% egg yolk concentration when assessing post-thaw motility, morphology and longevity and significantly superior to a 0%, 10% or 40% egg yolk concentration. The study also showed motility and normal post-cool and post-thaw sperm morphology did not always correlate. Utilization of 0% and 10% concentrations of egg yolk has negative effects on semen quality as measured by the motility and/or morphology. Results confirm freezing does not affect secondary sperm abnormalities, abnormalities of the tail and distal section of the middle piece, during cooling or freezing. Primary abnormalities, abnormalities of the head and midpiece, increased in the 0% extender during cooling and all extenders during freezing. The pH of the extenders before the addition of sperm was significantly different. Once sperm was added to the extenders, there was no longer a significant difference in pH. There was a positive correlation for both motility and normal morphology percentages post-cool and post-thaw for the extenders with similar osmolarity to the semen.

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Abbreviations

ATP	Adenosine-5'-triphosphate
°C	Degrees Celsius
ADP	Adenosine diphosphate
BSP	Bovine seminal plasma
cAMP	Cyclic adenosine monophosphate
EY	Egg yolk
GOT	Glutamic-oxaloacetic transaminase
HDL	High-density lipoprotein
LDF	Low-density fraction
LDL	Low-density lipoprotein
mOsm	Osmole
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
SP	Seminal plasma

Chapter 1: Literature Review

It has been shown that there are not only species differences in the response to cryopreservation but also differences between the individuals of a single species. Canine spermatozoa are no different with this intraspecies variability. Though canine semen cryopreservation has been described and utilized for decades, only recently has popularity for the technique grown. Semen cryopreservation reduces disease transmission and injury to the dog and bitch. It also allows for international shipment of semen and long-term preservation of valuable genetics lines. During the years of canine sperm cryopreservation the optimal extender has yet to be developed. There is a need for work in the various components of freezing extenders as well as freezing techniques. The purpose of the present study was to evaluate one cryoprotectant, egg yolk, in the cryopreservation of canine semen.

1.1 Spermatozoal anatomy and physiology

The normal anatomy of the sperm is broken down into head, midpiece and tail. The head contains the male genetic material whose combination with the female genetic material during fertilization forms a viable embryo. The head is surrounded by an acrosome containing enzymes used for penetrating the female ova. The midpiece houses the mitochondrial sheath used for Adenosine-5'-triphosphate (ATP) production that provides energy for the tail. The tail, also known as the flagellum, propels the spermatozoa. [23]

All of these sperm components work together and can be affected adversely by the cryopreservation process. Projects have been performed using viability testing of the head, midpiece and tail post-cryopreservation, but are difficult to perform in a private practice setting. Sperm motility and morphology are the tests classically used to assess post-thaw viability. Martins-Bessa et al. [46] corroborated previous work that significant correlations were found between flow cytometry as compared to eosin-nigrosin staining using light microscopy evaluation and visual versus computer assisted motility evaluation. [13] [60] Although the subjective assessments of sperm motility and morphology require experience and expertise, these tests provide simple and quick methods of sperm evaluation.

Oettle created a morphology chart for canine semen using minor and major classifications, now referred to as primary and secondary abnormalities. [52] Primary abnormalities occur during spermatogenesis and are defects mainly of the head or the midpiece. Secondary abnormalities occur during epididymal storage or handling and preparation that affect the midpiece and tail. [53] The percent normal morphology below which fertility is adversely affected was found to be 60% in one study done by Oettle. The conception rate of semen from dogs with >60% normal morphology was 61% whereas the conception rate of semen of dogs with <60% normal morphology was 13%. [52] Johnston found the average percentage of progressive motility of canine spermatozoa to be 70% or greater. [37] No correlation between speed of progression of spermatozoa or percentage of progressively motile spermatozoa and fertilizing capability of canine spermatozoa has been reported.[38] [40,87]

A plasma membrane covers the entire spermatozoon serving as the main physical barrier to the external environment. This membrane maintains the cellular integrity of the spermatozoa and participates in membrane fusion events during fertilization. [30] The junction between the sperm head and tail prevents the movement of proteins and lipid between these two sections. [20] The plasma membrane is often considered to be the site at which freezing-induced injury to cells is initiated. [49] Cryoprotectants such as egg yolk protect plasma membranes during any cooling process by preventing or restoring the loss of phospholipids from the membrane, thus stabilizing the membrane. [21]

Factors that can affect canine spermatozoa include age, genetics, health and environment. Canine spermatozoa may become adversely altered at any age. Prepubertal dogs exhibit abnormal or dead sperm usually in low concentrations. [83] An older dog may exhibit similar abnormalities due testicular degeneration and the decreased production of testosterone. Genetics play a role in certain family lines that exhibit a premature onset of sterility. [44] The health of the animal through disease or diet plays a roll in spermatogenesis. Diseases that are known to cause infertility include hypopituitarism, hypothyroidism, hyperadrenocorticism, neoplasia, infection, fever and immune-mediated orchits. [23] Elevated environmental temperatures affect spermatogenesis, this heat stress impacts the ejaculate ~62 days after insult. Yu et al. [100]discussed four factors that may explain male-to-male variability in canine sperm quality. These factors are differences in sperm membrane properties determined genetically, membrane permeability characteristics, morphologic abnormalities during sperm maturation, and sperm maturation in dogs of varied ages.

1.2 Osmolarity effect on the spermatozoon

The osmolarity of dog semen is ~310 mOsm. [28] Songsasen et al. [79] evaluated the osmotic sensitivity of spermatozoa by exposing them to hypertonic solution of various osmolarities from 290 to 1500 mOsm followed by dilution into isotonic medium. The study showed during exposure to NaCl and monosaccharides solutions with 290 mOsm the motility of spermatozoa remained constant, osmolarity ≥500 mOsm caused a significant decrease in motility and total motility ceased at >700 mOsm. Exposure to glycerol and ethylene glycol were tolerated despite spermatozoa's sensitivity to osmotic stress and the shrinking and swelling caused by permeating cryoprotectants. Another study [33] evaluated three extenders during semen cooling with the following osmolarities: 392 mOsm, 1156 mOsm and 408 mOsm. The extender with an osmolarity of 1156 mOsm exhibited a significant decrease in motility in comparison to the other two extenders with lower osmolarities. Both of these studies suggest spermatozoa tolerate osmolarities within 290-400 mOsm.

1.3 Cryopreservation effect on the spermatozoon

Cryopreservation alters the structure and physiology of the spermatozoon through a halt in cellular activity upon completion of freezing, which returns upon thawing. [47] Alterations may be present in the post-thaw motile population and are mainly ultra structural, biochemical and functional changes affecting interaction with the female tract, and fertilization. [7] The etiology of such alterations may represent a combination of factors, such as inherited fragility of the spermatozoa to withstand the cryopreservation process and the semen dilution. Damage to the plasma and acrosome membranes, mitochondrial sheath and axoneme have also been described. [72]

The cryopreservation process involves the steps of temperature reduction, cellular dehydration, freezing, and thawing. Varying degrees of cellular damage may occur at each of the cryopreservation phases. The functional state of the frozen-thawed cells is affected by the injuries accumulated throughout the freezing process.[48] Spermatozoal motility preservation post-thaw is superior to morphological integrity. [7] Salamon and Maxwell stated plasma and outer acrosome membranes are the most cryosensitive but biochemical changes have also been detected. These include the release of glutamic-oxaloacetic transaminase (GOT), loss of lipoproteins and amino acids, decrease in phosphatase activity, decrease in loosely bound cholesterol protein, increase in

sodium and decrease in potassium content, inactivation of hyaluronidase and acrosin enzyme, loss of prostaglandins, reduction of ATP and adenosine diphosphate (ADP) synthesis and decrease in acrosomal proteolytic activity. [71]

Cold shock occurs when spermatozoa are cooled below physiologic temperature. [94] This etiology involves damage to the cellular membranes and alteration in metabolic function through changes in the arrangement of membrane constituents. [58] The spermatozoa from horse, cat, dog, and humans are relatively insensitive to cold shock, whereas spermatozoa from cattle, sheep and goat show medium sensitivity, and boar spermatozoa are extremely sensitive [96]. Cold shock occurs when a decrease in temperature causes a thermotropic phase transition in the membrane phospholipids from a liquid-crystalline to a gel phase, resulting in more rigid membrane structure. The specific phase transition temperatures for the different phospholipids in the membrane result in lateral migration with rearrangement of membrane components and lipid phase separations within the plane of the membrane. The lateral migration may create microdomains of non-bilayer-forming lipids and may modify surrounding environments. Upon thawing, these alterations predispose opposing membranes to fuse and affect protein activity, leading to overall altered membrane permeability to water and solutes. [48] The damaging effect of cooling or the extent of lateral phase separation depends on a combination of membrane elements such as cholesterol/phospholipid ratio, content of non-bilayer-preferring lipids, degree of hydrocarbon chain saturation, and protein/phospholipid ratio in the membrane.[58]

After cooling, membrane permeability is increased and this may be a consequence of increased membrane disturbances in specific protein channels. Channel regulation of calcium uptake is affected by cooling causing a reduction in cellular function and death. The uptake of calcium during cooling influences capacitation changes and fusion events between plasma membrane and acrosomal membrane. [7] Cold shock reduces membrane permeability to water and solutes and injures acrosomal membranes. [65] Irreversible alterations in the sperm membrane which include disturbances in the protein lipid bilayer structure, decreased membrane fluidity, increased membrane permeability, acrosome damage, dehydration, enzyme and phospholipid liberation, reduced metabolic activity and diminished consumption of ATP are all consequences of cooling and freezing. These effects may compromise fertility. [27]

When the temperature within the cells falls below the freezing point, ice forms. Water crystallizes outside the cell, leaving the remaining solutes at a higher concentration altering the pH.

The sperm membrane prevents the extension of ice crystals into the cell causing the intracellular water to become super cooled and an osmotic gradient across the plasma membrane is established resulting in loss of water from the spermatozoa. Irreversible damage to the spermatozoon may occur if the freezing rate is too rapid, plasma and acrosome membranes being the most cryosensitive. Water has inadequate time to leave the cell thus causing the intracellular water to become super cooled forming intracellular ice, which result in cell death. [47] [24] [7]

In summary, the cryopreservation protocol causes damage to spermatozoa by the influence of several factors, namely the dramatic changes in temperatures, submission to osmotic and toxic stresses derived from exposure to molar concentrations of cryoprotectants and finally the formation and dissolution of ice in the intracellular and extracellular environment. The damaging effects of cooling and freezing upon sperm membrane varies among domestic species and is influenced by several elements namely cholesterol/phospholipids ratio, content of lipids in the bilayer, degree of hydrocarbon chain saturation and protein/phospholipid ratio. [48]

1.4 Brief Overview of canine semen cryopreservation

Sperm from dairy bulls were first frozen during the 1950's and the first live offspring from canine frozen semen was in 1969. [73] The American Kennel Club approved the use of frozen semen in 1981. Protocols used in canine semen cryopreservation are usually adaptations of bovine semen cryopreservation protocols. [74] The standard ingredients needed for a freezing extender include a buffer, sugars, salts, penetrating and non-penetrating cryoprotectants and antibiotics.

Different penetrating cryoprotectants have been investigated such as glycerol, dimethylsulphoxide (DMSO), ethylene glycol and dimethyl-formamide. Glycerol is a commonly used penetrating cryoprotectant but reported concentrations vary from 1-16%. [55,78,80] [54,59,68]

A variety of buffers have been utilized in canine freezing extender, where as the most commonly reported buffer is a Tris or Tris-Citrate. Common sugars used include fructose, glucose, sucrose, lactose, raffinose and lactose. Many studies have been reported examining different combinations of all the previously mentioned buffers and sugars. [8,19,33,42,55,61,70,78,87,99]

Dog semen has been successfully stored in pellets and straws ranging in size from 0.25 ml to 2.5 ml. [8,51,73,87] Farstad [22] discussed three factors that make straws superior to pellets. These factors include ease of identification of semen donor, reduction of contamination and the lack of consistency in a freezing rate for pellets to compare to straws during analysis of results.

Several studies have analyzed the cooling rates of sperm during freezing. [51,54,68,100] Rota et al. concluded that a wide range of freezing rates may be applied to dog spermatozoa. [68]

Reports have shown post-thaw quality of dog semen is improved when thawed at fast rate, 7-9 seconds, in higher temperatures, 70-75°C and 55°C as compared to 37°C. [34,51,55,68] Nothling found [51] the 5-8 seconds thaw rate at 70°C superior compared to a 2 minute thaw rate at 37°C, but did not compare reciprocal rates and temperatures. Dobrinski found the optimal thawing temperature to be 37°C. [19]

1.5 The effect of egg yolk on semen cryopreservation

Cryoprotectants are classified as two types: penetrating and non-penetrating. Penetrating cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol) cause membrane lipid and protein rearrangement resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and increased survival to cryopreservation. [31] Penetrating cryoprotectants are solvents that dissolve sugars and salts in the cryopreservation medium. [65] Precipitation of sugars and salts cause a decrease in osmolarity. [5,89] Spermatozoa are sensitive to osmotic changes causing a decrease in motility. [79] Egg yolk along with non-fat skimmed milk, trehalose, amino acids, dextrans and sucrose are non-penetrating cryoprotectants. A non-penetrating cryoprotectant does not cross the plasma membrane and acts only extracellularly. Non-penetrating cryoprotectant may alter the plasma membrane, or act as a solute, lowering the freezing temperature of the medium and decreasing the extracellular ice formation. [4,41] Reports using egg yolk to cryopreserve sperm date back to 1952. Pace and Graham [57] purified egg yolk and observed that the low-density lipoproteins (LDL) fraction displayed cryoprotective properties. The protective mechanism of the LDL fraction of egg yolk has yet to be fully explained.

Egg yolk undergoes gelation when it is subjected to a freeze-thaw process and LDLs are responsible for this gelation. The other constituents of egg yolk do not directly participate in gelation. [39,82,92] Medeiros [48] and Purdy [65] determined the protective components in egg yolk are the phospholipids (lecithin) and LDL. Low-density lipoproteins will adhere to the sperm cell membrane and form an interfacial film during the freezing–thawing process. [12] The LDL promotes incorporation of phospholipids and cholesterol into the sperm plasma membrane [12], thereby building a complex with seminal plasma proteins and making them unavailable to function in the cell membrane. [12,45] In recent years, numerous studies have confirmed that the LDL extracted from egg yolk is successful for cryopreservation of semen from boars [26,27,29], bulls [5,6,28,37] and dogs [9,44].

The majority of the research on LDL and its use as a cryoprotectant has been done in bulls.[2,6,12,32,50,93] Low-density lipoproteins make up approximately two-thirds of the total solids of egg yolk and are localized in the soluble fraction of egg yolk. This soluble fraction, plasma, is composed of 83-89% lipids and 11-17% proteins. [18,64,67] The LDL structure was described in 1969 as a spherical molecule, 17-60 nm in diameter, with a triglyceride core surrounded by a film of proteins and phospholipids with the polar residues in contact with the aqueous phase.[18] Quinn et al. reported that phospholipids could form a protective film at the surface of spermatozoa membranes after disruption of LDL. The fatty acid composition of the phospholipids in this protective film is important because a low saturated fatty acid (SFA)/polyunsaturated fatty acid (PUFA) ratio tends to render a membrane less susceptible to cold shock. Saturated fatty acids crystallize in a more regular and ordered form in comparison to PUFA as temperatures decrease. [66]

Graham and Foote suggested that phospholipids from egg yolk could merge with spermatozoa membranes and replace some phospholipids thereby decreasing their phase transition temperatures. [26] Phosphatidylserine alone or in combination with phosphatidycholine is the most effective phospholipid to protect spermatozoa. [26] Questions exist about the respective role of lipids and apoproteins on the cryoprotective action of LDL. [50] Removing the LDL fraction from egg yolk also removes detrimental components of the egg yolk, such as HDLs (high-density lipoproteins) and progesterone, to the spermatozoa while improving the cryopreservation of the sperm. [32] In 1992 Vishwanath et al. [90] suggested that egg yolk lipoproteins compete with detrimental seminal plasma (SP) cationic peptides (<5 kDa) by binding to the sperm membrane and thus protecting the sperm from lipid efflux. Manjunath et al. [45] in 2002 showed for the first time that the low-density fraction (LDF) once isolated from the egg yolk interacts with the bovine seminal plasma (BSP) proteins and concluded that this binding is rapid, specific and saturable. The LDF was shown also to have a high capacity for BSP proteins and the complex formed is stable even after freeze-thaw. Bovine seminal plasma proteins are both beneficial and detrimental during fertilization and cryopreservation. One beneficial effect is that BSP proteins bind to capacitation factors such as high-density lipoproteins (HDL) and heparin [16], and potentiate sperm capacitation induced by HDL and heparin[84,86]. Detrimental effects occur by inducing changes in the sperm plasma membrane by stimulating cholesterol and phospholipid efflux. This lipid efflux by BSP proteins is time and concentration dependent. Continuous exposure of sperm to SP containing BSP proteins is detrimental to the sperm membrane by causing an efflux of lipids, rendering the membrane sensitive to cryopreservation. Bergeron et al. [12]proposed that the principal mechanism by which LDL

protected the sperm was by sequestration of the major proteins of BSP, BSP-A1/A2, BSP-A3 and BSP-30-kDa. In addition, LDL was shown to interact specifically with BSP proteins and decreased the binding of the major proteins of BSP to the sperm, preventing lipid efflux from the sperm membrane. Low-density lipoproteins may offer protection to sperm by reducing the deleterious effect of seminal plasma proteins on sperm membranes. [32] Manjunath et al. [45] stated that BSP proteins destabilize the sperm membrane by removing cholesterol and phospholipids. It may be possible that this effect is abolished or minimized by the association of BSP proteins with egg yolk lipoproteins, the major component of extenders used in sperm storage. Manjunath et al. [45] suggested that the scavenging of the BSP proteins by egg yolk (EY) lipoproteins represents the major mechanism of sperm protection by egg yolk.

Whole egg yolk is reported to cause detrimental effects on sperm during cooling and freezing. The detrimental effects of whole egg yolk on sperm viability have been attributed to the action of high-density lipoproteins (HDL). [57,85] Egg yolk is reported to contain substances that inhibit sperm respiration. [32] In addition, it has been demonstrated that the composition of egg yolk is variable based on the breeds and nutrition of the chickens. [93] It has been proposed that egg yolk contains progesterone [15]. It has been shown in several species, cattle, horses and dogs, [1,98] that the progesterone found in egg yolk plays a role in the capacitation of spermatozoa. Capacitation of sperm involves the destabilization of the acrosomal sperm head and membrane facilitated by the removal of steroids and glycoproteins resulting in a fluid membrane with increased permeability to Ca2+. An influx of Ca2+ produces increased intracellular cyclic adenosine monophosphate (cAMP) levels and causes hyper-motility and coincides with the onset of capacitation. [9] During fertilization it is the fixation of the spermatozoon to the zona pellucida that triggers the acrosome reaction. Spermatozoa that spontaneously under go an acrosome reaction after ejaculation or following freezing are incapable of fixing to the zona pellucida and are therefore unable to fertilize the oocyte. [15]

Utilization of certain components of egg yolk in extender creates optimal conditions for spermatozoa undergoing cryopreservation. Evaluating the type of egg, the LDL fraction, and the concentration of both whole egg yolk and LDL's alone is necessary to determine optimal ingredients in the extender. Removal of detrimental fractions of egg yolk may potentially benefit the spermatozoa during the freezing process.

1.6 Types of egg yolk used in cryopreservation

Semen freezing extenders used in many species, boars, goats, stallions, bulls, dog, include chicken egg yolk. [19,25,29,46,55,56,68,69,75,76,78] Chicken eggs are a preferred source of egg yolk due to the ease of acquisition. Studies reporting the use of different types of eggs are emerging. Quail egg yolk extender versus chicken egg yolk extender was compared in Poitou Jackass sperm at concentrations of 0, 2, 5, 10, 15 and 20%. The study found that a 10% quail egg yolk concentration improved the percentages of motile and progressively undulating spermatozoa in comparison to the 10% chicken egg extender. After comparison of the two types of yolk the researchers found that they were similar in composition except the quail egg contained significantly more phosphatidylcholine, less phosphatidylethanolamine, and a smaller ratio of polyunsaturated to saturated fatty acids. [88] Another study compared pigeon egg yolk to chicken egg yolk in Sahiwal bulls. Extenders with pigeon yolk contained concentrations of 5, 10, 15 and 20% compared to a 20% chicken egg yolk concentration. This study found that extender with 20% pigeon or chicken egg yolk had similar post-thaw results. As the concentration of pigeon egg yolk decreased so did the percentage of motility, livability, and intact plasma membranes. [35] An identical study was done with duck egg yolk on Nili-Ravi buffalo bull semen. This study found there was a significant difference between the 20% duck egg yolk extender to the 20% chicken egg yolk extender. The duck extender significantly improved post-thaw motility and livability and reduced tail abnormalities. Reduced percentages of duck yolk in the extender adversely affected the post-thaw parameters. [91]

The most comprehensive study looking at different types of egg yolks compared turkey, duck, omega-3 chicken, chicken and chukar eggs. [97] Not only did the study compare the post-thaw results but also examined the composition of all of the yolks for cholesterol, saturated fats, monounsaturated fat, polyunsaturated fat, trans fat, omega-3, omega-6 and omega-9. Though this study found differences between the compositions of all egg types, only substitution of turkey egg yolk showed a significant increase in motility compared to the chicken egg yolk. The turkey composition was higher in cholesterol, saturated fat, Omega-6 and lower in monounsaturated fat, trans fat and omega-9 compared to the other type of egg yolks. [97] In contrast to the above studies, there are several other reports that show no difference when using duck or other avian species egg yolk. [17] [81] Possible explanations for the different results in these studies could be diet of the animals producing the egg or preparation of the egg yolk, thus producing more or less lipids within the extender.

1.7 Use of LDL alone in extender

The use of egg yolk in semen extenders poses a risk by promoting bacterial growth or a

potential risk of microbial contamination. [3] Bousseau demonstrated that the eggs were often contaminated to some degree depending on the source by bacteria such as *Salmonella* or *Staphlococci*. [14] Due to these risks many investigators have begun looking at the use of LDL's solely as a replacement to whole egg yolk in freezing extenders. In 2004 Amirat [5] demonstrated that the extraction process used to obtain LDL from egg yolk reduces bacterial contamination by 10^7 colony forming units/ml.

Low-density lipoproteins have been reported to provide a protective mechanism either through the stabilization of membrane or by the replacement of membrane phospholipids that are lost during the cryopreservation process. [2] In 2002 Moussa et al. [50] developed the most widely used procedure on the extraction of LDL's from egg yolk for use in extenders. In this study they determined that an 8% LDL extender was superior to a 20% egg yolk commercial extender. Increasing the LDL concentration in the extender above 10% led to decrease in spermatozoa motility post-thaw. It was hypothesized that this effect is due to the precipitation of fructose and salts contained in the extender supplemented with LDL or an increase of LDL concentration leading to LDL aggregation and an inactivation of the effect of aggregrated LDL. Bencharif [10] compared 20% egg yolk extender to 4, 5, 6, 7, 8, and 10% LDL extender in dogs. It was determined that in 20% egg yolk the LDL concentration was 6.6%. A 6% LDL improved the acrosomal, DNA, and flagellar plasma membrane integrity during the freezing process. In addition, they achieved a 100% conception rate with frozen sperm in 6% LDL extender on six beagle bitches. There was no breeding trials performed with the 20% egg yolk or other LDL concentrations.

There have been studies looking at LDL's substitution in multiple species, bovine [2,5,32,50], boar [36], equine[63], caprine [3,95], trout [62] and dog [10,11]. Many of these studies show a positive correlation to use of LDL and post-thaw results. Hu et al. [32] assessed LDL's effect on seminal parameters and anti-oxidant activities of frozen-thawed bull sperm. They found that an 8% concentration of LDL gave the greatest percentages of post-thaw sperm motility, acrosome integrity and membrane integrity in comparison to 20% whole egg yolk. The 8% LDL concentration also favored the highest anti-oxidant activities of CAT, GSH-Px and GSH in comparison with all other extender concentrations. Akhter et al. [2] looked at different concentration of LDL compared to standard 20% egg yolk extender with buffalo bull semen. It was reported that a 10% LDL concentration significantly improved progressive motility, plasma membrane integrity and viability compared to the control (20% egg yolk) and other concentrations of LDL post-thaw. In the same study after post-dilution and pre-freezing, these three parameters were the same between the 10% LDL and control 20% egg yolk. It was noted that other components in egg yolk present cause detrimental effects during the freeze thaw process.

In summary the use of LDL in place of egg yolk appears to have a positive effect on spermatozoa through the freezing process. The ideal concentration range of LDL required varies between species.

1.8 Canine conceptions rates using fresh versus frozen semen

Two retrospective studies looked at fertility rates of frozen semen in the dog. The first analyzed 527 semen inseminations and found bitches inseminated with frozen semen had a 30% lower pregnancy rate and 31% smaller litter size compared to bitches inseminated with fresh semen. [43] The second study showed a conception rate of 60% with frozen semen compared to a 100% conception rate with fresh semen. [77] Both of these retrospective studies demonstrate that the freezing of canine semen is not optimal and additional research is still needed.

1.9 Study Purpose and Hypotheses

Cryopreservation of canine semen continues to grow in popularity. Studies have been completed evaluating different aspects of freezing from packaging, freezing rate, thawing rate and ingredients of the extender. The optimal extender has yet to be produced that allows canine spermatozoa to complete the freezing process capable of fertilization post-freeze due to individual dog variability. In a previous experiment evaluating use of glycerol as a permeating cryoprotectant, it was noted that extender void of glycerol after freezing had motile spermatozoa. The goal of this study was to test the hypothesis that spermatozoa frozen in egg yolk extenders void of glycerol would survive cryopreservation. To test this hypothesis semen from six dogs with normal semen parameters was collected and pooled to test five extenders with varying concentrations of egg yolk 0, 10, 20, 30, and 40%. The semen was evaluated during initial collection, post-cool, and post-thaw for pH, osmolarity, viability, motility, morphology and longevity. The results of the study proved that spermatozoa cryopreserved in extender void of glycerol can successfully complete the cryopreservation process as demonstrated by post-thaw evaluation. The extenders that gave optimal results contained either 20 or 30% egg yolk. The 20% egg yolk extender slightly improved the post-thaw parameters of motility, morphology and longevity of the spermatozoa compared to the 30% egg yolk concentration.

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Chapter 2: Manuscript

Evaluation of Different Concentrations of Egg Yolk in Canine Frozen Semen Extender

Stephanie W. Trout, BS, Beverly J. Purswell, DVM, PhD; Willard H. Eyestone, PhD; Sherrie Clark, DVM, MS; Stephen R. Werre, PhD

Abstract

This study tested different concentrations of egg yolk in canine freezing extender void of glycerol, a commonly used cryoprotectant, by examining the motility and morphology throughout the freezing process: initial (baseline after extender added), post-cool (after three hours at 5°C) and post-thaw (after freezing.) Initial values of pH, osmolarity, motility and morphology were obtained for comparison of the samples. Spermatozoa from six normal dogs as determined by progressive linear motility > 70% and normal morphology > 60% was used. Semen was collected and pooled for five freezing trials. The concentrations of egg yolk used in the extender were: 0%, 10%, 20%, 30% and 40%. Assessment of each sample was blinded to the treatments until all results were obtained and statistics had been analyzed. Based on this study a 20% egg yolk concentration is slightly superior to a 30% egg yolk concentration when assessing post-thaw motility, morphology and longevity and significantly superior to a 0%, 10% or 40% egg yolk concentration. The study also

showed motility and normal post-cool and post-thaw sperm morphology did not always correlate. Utilization of 0% and 10% concentrations of egg yolk has negative effects on semen quality as measured by the motility and/or morphology. Results confirm freezing does not affect secondary sperm abnormalities, abnormalities of the tail and distal section of the middle piece, during cooling or freezing. Primary abnormalities, abnormalities of the head and midpiece, increased in the 0% extender during cooling and all extenders during freezing. The pH of the extenders before the addition of sperm was significantly different. Once sperm was added to the extenders, there was no longer a significant difference in pH. There was a positive correlation for both motility and normal morphology percentages post-cool and post-thaw for the extenders with similar osmolarity to the semen.

Introduction

1. Introduction

The cryopreservation of canine sperm has been under investigation since 1969. As the canine breeding industry continues to grow, the need for better extenders to improve the fertility of the semen frozen increases. Since there are individual variations, not every dog's semen is able to survive freezing in the extenders currently available. [8,15] Evaluation of egg yolk in current semen extenders was under taken to further elucidate the cryoprotection provided by various concentrations of egg yolk.

Various cryoprotectants are used for the process of freezing semen with glycerol being the most frequently used cryoprotectant in canine freezing extender. Previous research indicates that between 3-6 percent glycerol concentration is optimal for sperm viability. [3], [11] A second common ingredient found in canine semen freezing extenders is egg yolk. The phospholipid, cholesterol, and low-density lipoprotein content of chicken egg yolk have been identified as the protective components in chicken egg yolk. [14], [4], [10,13] In cattle, it has been shown that the low-density lipoproteins (LDL) found in egg yolk help to improve the cryopreservation of sperm. [7],[1] It is believed that these low-density lipoproteins aid in cryopreservation by binding with seminal plasma proteins that would otherwise disrupt the phospholipid layer of the plasma membrane on the sperm cells. When sperm cells are not in the presence of low-density lipoproteins they are subjected to seminal plasma proteins that coat the sperm membrane and induce cholesterol and phospholipid efflux. [6] Through this efflux the plasma membrane is compromised and is

subjected to cold shock and eventually death during cryopreservation. Bergerson et.al, 2004 went one step further to show that the LDL's were binding to bovine seminal plasma (BSP) proteins, which kept the BSP's from binding with the sperm so that the phospholipids on the head of the sperm were free to bind with the cryoprotectant in the extender.

This study evaluated the role egg yolk plays as a cryoprotectant in canine semen by comparing five different concentrations of egg yolk in a freezing extender that is void of any other cryoprotectant. The initial baseline parameters evaluated were pH, osmolality, motility and morphology. After the prefreeze cooling, motility and morphology were again evaluated. After freezing, post-thaw motility, morphology and longevity were evaluated. By evaluating the above parameters, the optimal concentration of egg yolk as a cryoprotectant in canine semen was determined.

Material and Methods

2. Materials and Methods

The Virginia Polytechnic Institute and State University IACUC committee approved the procedures performed during this research.

2.1. Animals

Six reproductively mature male dogs ranging in age from approximately 2.5 – 4yrs old were used during the experiment with normal parameters for motility and morphology. In order to establish normal parameters for the dogs, semen was collected twice, two days apart and evaluated for sperm motility and morphology under a phase/contrast scope. Sperm morphology was evaluated using the vital stain, eosin/nigrosin. Sperm motility of greater than 70% and normal sperm morphology greater than 60% were minimal requirements for the dog to be considered normal. Once accepted, the dogs underwent a two-week acclimation period. The dogs were handled and manually ejaculated every other day. The dogs were all single housed in 4'x8' indoor kennels and fed Hill's Science Diet Maintenance®/™ . Amount of food fed daily to each dog was determined by a boarded veterinary nutritionist. They were given water ad libitum.

2.2. Trials

The dogs were collected every three to four days over a 24-day period allowing for a total of 8 trials. Three trials were excluded due to incomplete data collection. Five different concentrations of egg yolk, 0%, 10%, 20%, 30% and 40%, were used in an extender void of any other cryoprotectant. During the trials, extenders were randomly selected. The individual processing the semen during all stages of the study remained blinded to the treatment until all trials were completed and analyzed.

2.3. Freezing and Thawing Extender

All extenders with different egg yolk concentrations were prepared a week prior to the trials with fresh non-fertilized chicken eggs. (Appendix 1) All extenders were held at -20°C freezer in 3 ml aliquots until use. Thawing media[i] was made per manufacturer instructions, placed in 1.8 ml micro centrifuge tubes in 0.75 ml aliquots, and frozen at -20°C until use.

2.4. Semen collection

The dogs were minimally restrained with a leash. Semen was manually collected directly in a sterile baby bottle liner.[ii] All six dogs were collected and semen was immediately sent to the lab for evaluation and processing.

2.5. Semen processing and evaluation

After collection, the semen samples from all six dogs were pooled into one sterile 50 ml conical centrifuge tube[iii] in the lab. Total volume of the pooled sampled was measured then divided into five equal aliquots in 15 ml centrifuge tubes.[iv] Before each evaluation, samples were mixed slowly by inverting the tube five times.

A motility slide for each aliquot was prepared, read immediately and recorded as initial motility. Slides, 25x75x1 mm,[v] and cover slips, 22x22 mm,[vi] were pre-warmed to 37°C. A 10μL drop of semen was placed on the pre-warmed slide and covered with a pre-warmed cover slip. The slide was then read using a phase contrast microscope[vii] at 400x magnification. Five randomly

selected areas on the slide were viewed before recording the average motility for each slide by visual assessment.

Simultaneously a morphology slide for each aliquot was made using approximately 10 μL of eosin/nigrosin stain[viii] and 10 μL of semen. The stain and semen were placed on one end of a microscope slide[ix] mixed together gently with a second slide, and pushed across the slide to obtain a uniform distribution for air-drying. Once dry the morphology slide was labeled with the treatment group, stage of freezing process (initial, post-cool or post-thaw), trial number and date. After drying the slide was placed into a slide carrier and read at a later date using bright field microscopy at 1000x magnification under oil immersion. A total of 200 sperm were counted per slide. The percent of normal spermatozoa, primary abnormalities and secondary abnormalities for each slide were recorded. Primary abnormalities counted included detached acrosomes, folded acrosomes, abnormal shaped heads, vacuoles, proximal droplets and tightly coiled tails. Secondary abnormalities counted included distal droplets and detached heads.

Lastly, 10 μl of each aliquot was placed into a 1.8 ml micro centrifuge tube[x] containing 990 μl of a 10% formalin solution.[xi] Each sample was then inverted slowly five times before 4μl was distributed on each side of a Neubauer haemacytometer. The haemacytometer was then place on a phase contrast microscope at 400x and five of the twenty-five squares were read from each side. The counts from each side had to be within 10% of each other for quality control. If not within 10% the haemacytometer was cleaned and new samples were placed in the wells. Once both sides were within 10% an average was taken. The average was then multiplied by five giving the total sperm concentration in millions per ml.

The pH readings were taken of the extender, semen, and extended semen. A Mettler Toledo pH meter[xii] was used and directions per the manufacturer were followed to analyze the samples.

Osmolarity readings were taken of the extender, semen and extended semen. An Advanced Instruments, INC, 3300 osmometer (Norwood, MA) was used and directions per the manufacturer were followed to analyze the samples.

2.6. Extenders and freezing method

The five 15 ml aliquots of semen were centrifuged at 500g for 10 minutes. Centrifuge tubes were immediately removed after centrifugation and supernatant was aspirated off leaving the semen pellet. The pellet volume was recorded for each sample. To determine the amount of extender to be added, the pellet volume was then multiplied by two, this volume of extender was added initially and again one hour later. The extenders were allowed to come to room temperature prior to use. Each

extender was inverted five times to mix prior to adding to the semen pellet. Once the extender was added, the sperm and extender were mixed until the pellet was no longer visible. The 15 ml centrifuge tube containing the treatment and semen was placed in a room temperature water bath along with the extender to be added at one hour. The waterbaths were then placed into a 5°C refrigerator. After an hour of incubation, an additional aliquot of extender was added making the final extender: semen ratio 4:1. The semen was replaced into the waterbath and refrigerated for another two hours. Glass pipettes were placed in the refrigerator to equilibrate to 5°C. At the end of the two-hour incubation, semen was deposited on dry ice, in which wells were formed by using ½" screw heads attached to a board. Using the glass pipette the semen sample was mixed by aspirating and releasing it within the centrifuge tube three times. After mixing the sample, two drops of semen were added to each well to form pellets of frozen semen. The pellets were allowed to sit on the dry ice for 3-5 minutes before plunging into LNO2. After equilibration in the LNO2, the pellets were placed into 2 ml cryovials.[xiii] The vials were pre-labeled with treatment group, trial and date. Each cryovial had been punctured three times with a 16gauge needle ahead of time to allow venting of the vials when removing them from the LNO2. Vials were stored in LNO2 until the post-thaw evaluations were made.

2.7. Post-cool semen evaluation

The extended cooled semen sample was evaluated for motility and morphology as previously described immediately after the freezing process was completed.

2.8. Thawing method

Post-thaw evaluation of each treatment group was performed. Thaw media was incubated at 37°C for one hour prior to thawing the semen pellets. One pellet from each treatment group was placed in the thaw media and gently mixed until the pellet was no longer visible, approximately 15-30 seconds.

2.9. Post-thaw semen evaluation

After thawing, each sample was assessed for motility and morphology as previously described. The motility slide was immediately read and time was recorded as time zero. The motility assessment was repeated for each sample at the following times: 30mins, 1 hour, 2 hour, 4 hour, 8 hour, 24 hour, 36 hour, 48 hour and 72 hour. In between each time the samples were stored in a

Styrofoam box at room temperature. After the samples reached ≤10% motility for two consecutive time sessions, they were discarded. Post-thaw morphology slides were made immediately following the initial motility assessment. These slides were assessed and recorded.

3.0 Statistical Analysis

Data were entered into a computer spreadsheet and analyzed by Dr. Stephen Werre, statistical analysis lab supervisor at the VA/MD Regional College of Veterinary Medicine, utilizing SAS® software[xiv]. A mixed-model ANOVA utilizing a Tukey-Kramer adjustment was used to assess the effects of treatment within and between treatment groups for pH, osmolarity, viability and initial, post-cool and time zero post-thaw motility and morphology. A mixed-model ANOVA utilizing the Glimmix Procedure was used to assess the effects of treatment and time within and between treatment groups for post-thaw motility over time post-thaw. Differences were considered significant when the p-value was < 0.05.

Results

3.1 pH and Osmolarity

The pH and osmolarity of the extenders, semen and extended semen were evaluated for initial differences. The pH of the extenders showed significant differences between 20% egg yolk and 30% egg yolk, 7.124±0.5 and 7.022±0.04, respectively (p= 0.0026), 10% and 30%, 7.120±0.04 and 7.022±0.04, respectively (p= 0.0038) and 20% and 40% 7.124±0.5 and 7.054±0.06, respectively (p=0.0449). The pH of the semen (7.020±0.5) and extended semen (range 6.972±0.06 to 7.03±0.05) showed no significant differences. (table 1)

The osmolarity of the semen throughout the trials was 319.6±12.98 mOsm (p=1). Osmolarity for the extenders at 0%, 10%, 20%, 30% and 40% egg yolk concentrations were 234.1±1.82mOsm, 260±1.29mOsm, 292.6±2.63mOsm, 325.7±1.99mOsm and 372±2.46 mOsm, respectively, which were significantly different when compared to one another (p <0.0001.) The 20 and 30% egg yolk concentration extenders had optimal post-thaw results and were the closest in osmolarity to the semen (319.6±12.98mOsm) at 292.6±2.63 and 325.7±1.99, respectively. Osmolarity for the extender semen at 0%, 10%, 20%, 30% and 40% egg yolk concentrations were 248.9±1.08mOsm, 272.8±2.01mOsm, 298.1±3.76mOsm, 328±2.62mOsm and 361.5±4.39 mOsm, respectively, which were significantly different when compared to one another (p <0.0001.) (table 2)

3.2 Motility
3.2.a Initial Motility

The initial motility of the extended semen was evaluated immediately after the addition of extender. Initial motility for the 0%, 10%, 20%, 30%, and 40% egg yolk extenders added to semen were 91±5.48%, 90±3.95%, 90±5%, 89.5±2.7%, and 92±2.74%, respectively, showing no significant differences in motility between extenders (1< p >0.5.) (table 3 and graph 1)

3.2.b Post-cool Motility

The post-cool motility was evaluated after the extended semen equilibrated for 3hrs in a waterbath at 5°C. Post-cool motility for the 0%, 10%, 20%, 30%, and 40% egg yolk extenders were 92.5±2.5%, 90.5±2.74%, 90.5±2.09%, 91.5±3.35%, and 93±2.74%, respectively, showing no significant differences in motility between extenders (1< p >0.541.) Comparison of initial and post-cool motility of the extended semen indicates similar values within egg yolk concentrations over these two time points. After cooling for three hours the extenders have no affect on motility. (table 3 and graph 1)

3.2.c Post-thaw Initial Motility between Treatments

The initial or time zero post-thaw motility was evaluated and compared between extenders. Significant differences were noted between 20% egg yolk extender compared to 40% egg yolk extender and 0% egg yolk extender (62.5%, 41% and 22% motile respectively, p= 0.0378 and p<0.0001, respectively), 30% egg yolk extender to 0% egg yolk extender (49% and 22% motile respectively, p= 0.0046) and 0% egg yolk extender to 10% egg yolk extender (22% and 61% motile respectively, p= 0.0001.) No significant differences were noted between the remaining egg yolk concentrations. The two egg yolk concentration extremes of 0% and 40% produced the lowest initial post-thaw motilities at 22% and 41% respectively. (table 3)

3.2.d Post-thaw Motility Total Time Motile

The total length of time that each post-thaw sample remained motile until motility was less then 10% for two consecutive readings was evaluated. The 20% egg yolk extender was optimal in comparison to the remaining extenders by remaining motile out to 72hrs. The remaining concentrations of 0%, 10%, 30%, and 40% remained motile out to 8hrs, 36hrs, 48hrs, and 36hrs, respectively. (table 4 and graph 2)

3.2.e Post-thaw Motility significant drop in motility within treatment

The motility of semen in the various extenders was evaluated to determine the first significant drop in motility compared to time zero post-thaw motility result. In the 0% egg yolk extender the drop occurred at eight hours post-thaw with an initial time zero post-thaw motility of 22±5.19% decreasing to 4.3±3.12% (p= 0.0515). In the 10% the drop occurred at thirty minutes

post-thaw with an initial time zero post-thaw motility of 61±5.19% decreasing to 47±5.55% (p= 0.0437). In the 20% the drop occurred at two hours post-thaw with an initial time zero post-thaw motility of 62.5±5.19% to 42.5±5.39% (p = 0.0070). In the 30% the drop occurred at two hours post-thaw with an initial time zero post-thaw motility of 49±5.19% to 26.5±5.39% (p= 0.0016). In the 40% extender the drop occurred at four hours post-thaw with an initial time zero post-thaw motility of 41±5.19% to 19±3.9% (p= 0.0039). (table 4)

3.3 Morphology
3.3.a Initial Morphology

Normal morphology of extended semen samples with 0%, 10%, 20%, 30%, and 40% egg yolk concentration were 80.6±5.59%, 85.6±2.3%, 84.4±3.78%, 80.08±4.21, and 82.6±5.13%, respectively, showing no significant differences between samples ($1 < p > 0.2363$.) Primary abnormalities of extended semen samples with 0%, 10%, 20%, 30%, and 40% egg yolk concentration were 16.8±5.54%, 11.8±2.77%, 12.8±5.45%, 14.8±6.61%, and 14.8±4.82%, respectively, showing no significant differences between samples ($1 < p > 0.4041$.) Secondary abnormalities of extended semen samples with 0%, 10%, 20%, 30%, and 40% egg yolk concentration were 2.6±1.34%, 2.6±1.14%, 2.8±2.49%, 4.4±2.79%, 2.6±1.52%, respectively, showing no significant differences between samples ($1 < p > 0.6063$.) (table 5)

3.3.b Post-cool Morphology
3.3.b.1 Post-cool Normal Morphology

Normal morphology after cooling at 5°C for three hours was evaluated. Significant differences of normal sperm post-cool between the 0% egg yolk extender and the remaining extenders with 10%, 20%, 30%, and 40% egg yolk were noted (69.6±7.37% compared to 82.2±4.08%, 83.8±4.09%, 85.2±3.63% and 85±5.34% respectively, p =0.0039, p= 0.0013, p= 0.0005 and p = 0.0006, respectively.) No differences were seen between extenders with 10, 20, 30 or 40% egg yolk concentrations normal morphology ($1 = p > 0.8357$). (table 5)

3.3.b.2 Post-cool Morphology Primary Abnormalities

After cooling at 5°C for three hours the primary abnormalities were evaluated. Significant differences of primary abnormalities post-cool between the 0% egg yolk extender and the remaining

extenders with 10%, 20%, 30%, and 40% egg yolk were noted (27.6±7.8% compared to 17.2±4.0%, 15±4.06%, 13.2±3.56% and 13.6±5.55% respectively, p =0.0089, p= 0.0017, p= 0.0004, and p = 0.0006, respectively.) No differences were noted of the primary abnormalities between the extenders with 10, 20, 30 or 40% egg yolk concentrations ($0.9829 < p > 0.5682$.) (table 5)

3.3.b.3 Post-cool Morphology Secondary Abnormalities

The secondary abnormalities of all extenders were evaluated post-cool and no significant differences were noted between extenders. Secondary abnormality results for the 0%, 10%, 20%, 30% and 40% egg yolk extenders were 2.8±1.3%, 1.0±0%, 1.2±0.45%, 4±4.24%, and 1.75±0.96%, respectively ($0.9950 < p > 0.1022$.) (table 5)

3.3.c Post-thaw Morphology
3.3.c.1 Post-thaw Normal Morphology

Significant differences of normal sperm post-thaw were noted between 20% egg yolk concentration extender compared to 0% and 10% egg yolk concentration extender (36.4±8.56% compared to 20.6±6.11% and 19.2±4.76% respectively, p = 0.0051 and p= 0.0024 respectively.) No significant differences in post-thaw normal morphology were seen between the remaining egg yolk concentrations. The 20% egg yolk overall had the highest post-thaw normal morphology at 36.4±8.56%. (table 5)

3.3.c.2 Post-thaw Morphology Primary Abnormalities

Significant differences of primary abnormalities were noted during post-thaw evaluation of the extenders. The 20% egg yolk concentration had the least amount of primary abnormalities with 61.2±9.12%, which was not significantly different then the 30% egg yolk concentration with 68.8±11%. This percent of primary abnormalities in 20% egg yolk concentration was significantly less compared to extenders with 0%, 10% and 40% egg yolk concentrations (61.2±9.12% compared to 76.4±7.44%, 77.6±4.83%, and 72±2.83% respectively, p=0.0041, p=0.0041, p=0.0495, respectively.) No differences in primary abnormality percentages were seen between extenders containing egg yolk concentrations of 0, 10 and 40%. Additional these results show that extreme concentrations of egg yolk in the extenders whether low or high (0%, 10% and 40%) had increased number of primary abnormalities in comparison to 20% egg yolk concentration. (table 5)

3.3.c.3 Post-thaw Morphology Secondary Abnormalities

The secondary abnormalities of all extenders were evaluated post-thaw and no significant differences were noted between extenders. Secondary abnormality results for the 0%, 10%, 20%, 30% and 40% egg yolk extenders were 3.75±2.63%, 3.5±1.91%, 2.4±1.14%, 2.5±2.38% and 2.5±1.29%, respectively ($0.9992 < p > 0.6742$.) (table 5)

Discussion

The results of this study indicate the 20% egg yolk concentration extender was preferred during cryopreservation over the 0%, 10%, 30%, and 40% egg yolk concentrations. Parameters pH, osmolarity and initial, post-cool and post-thaw motility and morphology were evaluated to derive this conclusion. Parameter results varied in their correlation with the final result and will be discussed below.

Results of this study indicate pH was not a reliable measurement in predicting the protective qualities of the extender for the sperm during cryopreservation. The 20% and 30% egg yolk concentration extenders were optimal in comparison to the remaining extenders in their final post-thaw motility and morphology though their initial pH represented the highest and lowest measurements, 7.12±0.04 and 7.022±0.04 respectively.

Cryopreservation results of the spermatozoa positively correlated when the osmolarity of the semen and extender were similar. During this study, the 20% and 30% egg yolk concentration extenders gave optimal results during both freezing and cooling compared to the remaining extenders. Both extenders' mean osmolarities were closest to the semen osmolarity with differences of 27 mOsm and 6.1 mOsm respectively. In contrast the remaining egg yolk concentrations, 0, 10 and 40%, had a difference of 85.5, 59.6 and 52.4 mOsm respectively to the osmolarity of semen. Osmolarity is a parameter that has been discussed but is often over looked when evaluating extenders. Two previous studies showed that once osmolarity was ≥500 mOsm spermatozoa decreased in motility. [5,12] Eilts in 2005 [2] stated extenders help stabilize the cell membrane and that the osmotic sensitivity of the cells is important during the cryopreservation process.

Initial morphology and motility evaluation verified that no significant differences were noted between the 0%, 10%, 20%, 30%, and 40% concentrations of egg yolk extender prior to cryopreservation.

During post-cool evaluation of the various extenders no differences were found in the motility between extenders. This observation was anticipated, as there are canine semen extenders commercially available void of egg yolk. An unexpected result was the significant 11% decrease of normal morphology in the 0% egg yolk concentration extender after 3 hours of cooling at 5°C with no significant change in the remaining extenders. This drop in normal morphology is notable as many extenders used for shipment of cooled semen lack egg yolk as a component. A conclusion can be made that egg yolk may be indicated in cooling extenders as well as in freezing extenders. Additionally the 0% extender had the largest drop in post-thaw motility, 70% compared to 52-28% in the other extenders. Thus a drop in post-cool morphology has a negative correlation with post-thaw motility and as discussed below post-thaw morphology.

Evaluation of secondary abnormalities within all extenders through the initial, post-cool and post-thaw results demonstrated a similarity in results between time points indicating the stability of secondary abnormalities through the cryopreservation process.

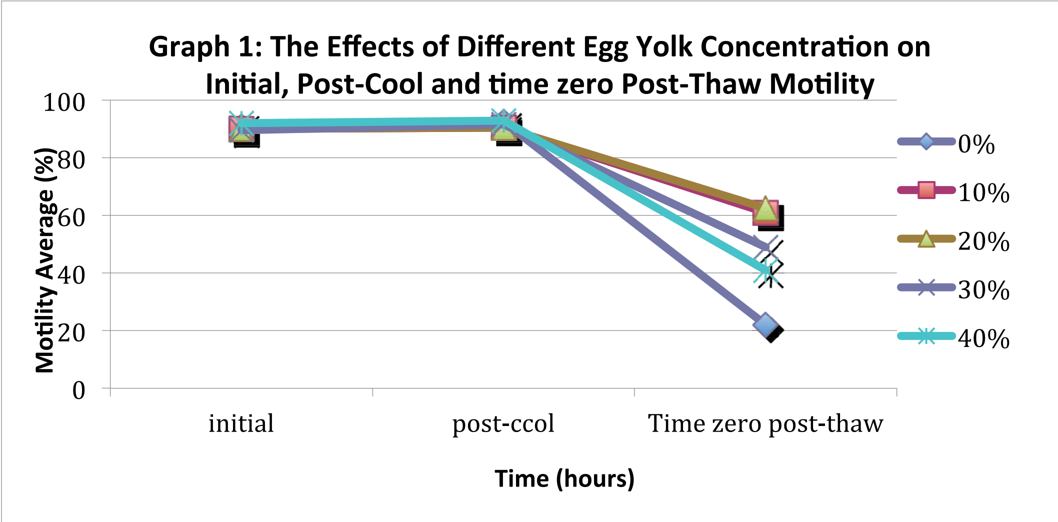
Post-thaw sperm motility and morphology results demonstrate that a concentration of 20% egg yolk produced slightly better results than a 30% concentration and was optimal in comparison 0%, 10%, and 40% egg yolk concentrations. Extreme egg yolk concentrations of 0% and 40% resulted in the lowest initial post-thaw motilities at 22±5.19% and 41±5.19% respectively. The 10% extender had the lowest normal post-thaw morphology at 19.2±4.76% but the second highest post-thaw motility, 61±5.19%. The implication is that motility and morphology do not necessarily correspond to each other. Dogs could have semen that has excellent motility but poor morphology. Oettle [9] showed in 1993 that a 40% normal morphology with fresh semen only gave a 13%

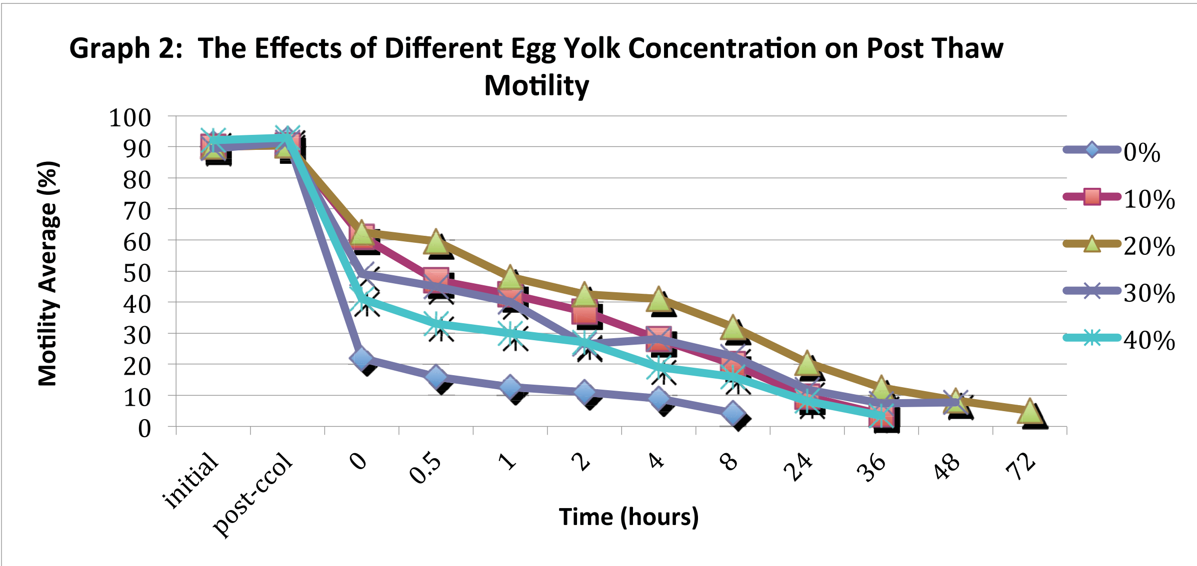
conception rate and that a 60% normal morphology was needed to for a greater then 61% conception rate. These results emphasize that if only initial morphology is evaluated the results may not correlate with the final post-thaw morphology results and motility alone is inadequate when assessing semen quality.

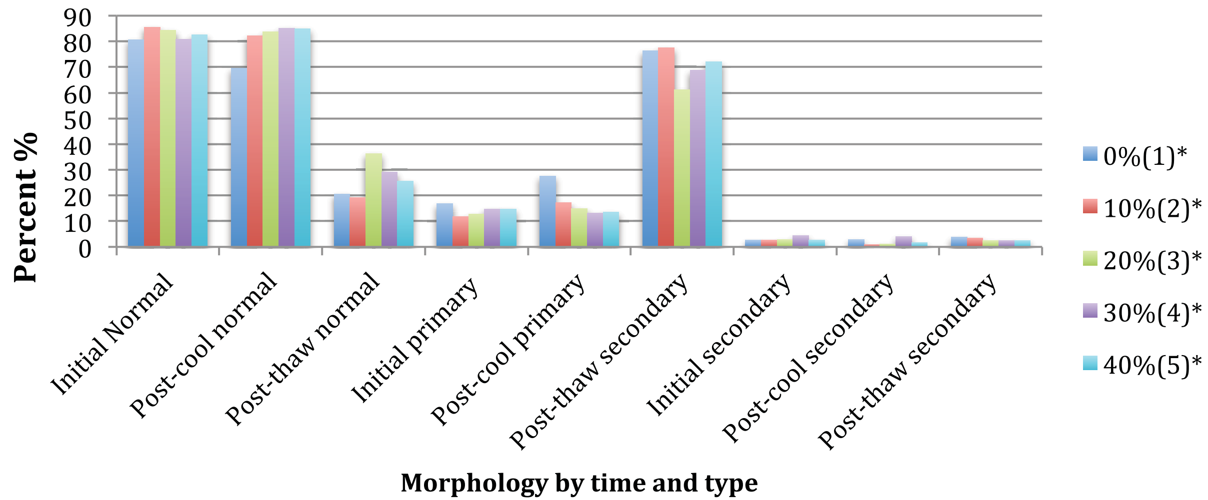
Post-thaw morphology results show that extreme concentrations of egg yolk in the extenders whether low or high (0%, 10% and 40%) had increased number of primary abnormalities in comparison to 20% egg yolk concentration. These results indicate though primary abnormalities occur naturally during spermatogenesis the abnormalities also occur during cryopreservation.

Though void of both a penetrating and non-penetrating cryoprotectant the 0% egg yolk concentration completed the cryopreservation process with 20.6±6.11% normal spermatozoa and 22±5.19%. Indicating additional cryoprotectant in the remaining extender ingredients and/or natural cryoprotective properties of spermatozoa.

From these findings morphology, motility, and osmolarity minimally should be evaluated through out the freezing process. Motility did not always correlate with morphology noted during the post-cool evaluation, emphasizing the importance of evaluating morphology after each step of cryopreservation. Significant differences in osmolarity between semen and extender can negatively affect the spermatozoa during cryopreservation. Lastly, in this study a 20% egg yolk concentration was preferred over a 0%, 10%, 30% or 40% egg yolk concentration.


Graph 1: The Effects of Different Egg Yolk Concentration on Initial, Post-Cool and time zero Post-Thaw Motility
Motility Average (%)
100
80
60
40
20
0
initial
post-ccol
Time zero post-thaw
Time (hours)
0%
10%
20%
30%
40%


Graph 2: The Effects of Different Egg Yolk Concentration on Post Thaw Motility
Motility Average (%)
100
90
80
70
60
50
40
30
20
10
0
initial
post-ccol
0
0.5
1
2
4
8
24
36
48
72
Time (hours)
0%
10%
20%
30%
40%



*Number represents the order in the graph the data appears for each result.

Table 1

pH of extender only, semen only and extender with semen given in mean % with standard deviation

% of egg yolk	Extender only	Extender with semen	Semen only
	pH	pH	pH
0	7.066±0.02	6.972±0.06	7.020±50
10	7.120±0.04[b]	7.03±0.05	
20	7.124±0.5[ac]	7.024±0.03	
30	7.022±0.04[ab]	6.912±0.03	
40	7.054±0.06 [ac]	6.976±0.16	

Within a column, different superscript letters denote values that differ significantly ($P < 0.05$).

Table 2

Osmolarity of extender only, semen only and extender with semen given in mean value with standard deviation

% of egg yolk	Extender only	Extender with semen	Semen only
	Osmolarity (Osm/L)	Osmolarity (Osm/L)	Osmolarity (Osm/L)
0	234.1±1.82[a]	248.9±4.08[b]	319.6±12.98
10	260±1.29[a]	272.8±2.01[b]	
20	292.6±2.63[a]	298.1±3.76[b]	
30	325.7±1.99[a]	328±2.62[b]	
40	372±2.46[a]	361.5±4.39[b]	

Within a column, different superscript letters denote values that diffe significantly ($P < 0.0001$).

Table 3

Motility mean values with standard deviations for initial, post-cool and mean value with standard error for time zero of post-thaw

Extender % of egg yolk	Motility (%)		
	Initial	Post-cool	Post-thaw (time zero)
0	91±5.48	92.5±2.5	22±5.19^{abc}
10	90±3.95	90.5±2.74	61±5.19^{a}
20	90±5	90.5±2.09	62.5±5.19^{bd}
30	89.5±2.74	91.5±3.35	49±5.19^{c}
40	92±2.74	93±2.74	41±5.19^{d}

Within a column, different superscript letters denote values that differ significantly (P < 0.05).
No significant differences were noted between any egg yolk concentrations for initial motility. (1< p >0.5.)
No significant differences were noted between any egg yolk concentrations for post-cool motility. (1< p >0.541.)

Table 4

Post-thaw motility over time for each concentration of extender shown are mean values and standard error

Extender % of egg yolk	Time in hours with % of motility defined below									
	0	0.5	1	2	4	8	24	36	48	72
0	22±5.19^{acd}	16±5.55	12.6±6.28	11±5.39	9±3.9	4.3±3.12*	0	0	0	0
10	61±5.19^{d}	47±5.55*	42.5±6.28	37±5.39	28±3.9	20±3.12	9.6±3.43	3.8	0	0
20	62.5±5.19^{ab}	59.5±5.55	48±6.28	42.5±5.39*	41±3.9	32±3.12	20.4±3.43	12.4	8.33	5
30	49±5.19^{c}	45±5.55	40±6.28	26.5±5.39*	28±3.9	22.5±3.12	11.9±3.43	7.4	7.67	0
40	41±519^{b}	33±5.55	30±6.28	27±5.39	19±3.9*	16±3.12	8.2±3.43	3.4	0	0

Within a row, superscript asterick denotes first signficant drop in motility (P < 0.05).

Within a column, different superscript letters denote values that differ significantly (P < 0.05).

Table 5

Normal, Primary Abnormalities and Secondary Abnormalities of Morphology for initial, post-cool and post-thaw given with mean values and standard deviations

Extender % of egg yolk	Normal morphology (%)			Primary abnormalities (%)			Secondary Abnormalities (%)		
	Initial	Post-cool	Post-thaw	Initial	Post-cool	Post-thaw	Initial	Post-cool	Post-th
0	80.6±5.59	69.6±7.37^{abcd}	20.6±6.11^{a}	16.8±5.54	27.6±7.8 ^{abcd}	76.4±7.44^{a}	2.6±1.34	2.8±1.3	3.75±2
10	85.6±2.3	82.2±4.08^{a}	19.2±4.76^{b}	11.8±2.77	17.2±4.0^{a}	77.6±4.83^{b}	2.6±1.14	1.0±0	3.5±1.
20	84.4±3.78	83.8±4.09^{b}	36.4±8.56^{ab}	12.8±5.45	15±4.06^{b}	61.2±9.12^{abc}	2.8±2.49	1.2±0.45	2.4±1.
30	80.8±4.21	85.2±3.63^{c}	29.2±10.13	14.8±6.61	13.2±3.56^{c}	68.8±11.0	4.4±2.79	4±4.24	2.5±2.
40	82.6±5.13	85±5.34^{d}	25.6±3.36	14.8±4.82	13.6±5.55^{d}	72±2.83^{c}	2.6±1.52	1.75±0.96	2.5±1.

No significant differences were seen between treatment groups during initial evaluation of normal morphology, primary abnormalities or secondary abnormalities, 1<p>0.2363, 1<p>0.4041, and 1<p>0.6063 respectively.

No significant differences were noted between 0%, 10%, 20%, 30%, and 40% egg yolk concentrations after any of the time point evaluations secondary abnormalities, 0.9992<p>0.1022

Within a column, different superscript letters denote values that differ significantly ($P < 0.05$).

References Chapter 2

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Chapter 3: Discussion and Conclusion

The use of cryopreserved dog semen was first reported in 1969. In the past two decades, the demand for this process is increasing in a pet industry that is upwards of 40 billion dollars annually. The cryopreservation of semen is no longer limited to the elite show dog but is utilized in a variety of situations from genetic preservation of rare breeds to family pets. There is an increasing need to optimize the cryopreservation of canine semen to ensure the needs of the industry are met. Sperm are prone to damage during cryopreservation thus it is important to ensure that the optimal cryoprotectants are being utilized in the extender. Although glycerol is a widely used cryoprotectant, it was observed that glycerol was not needed in extenders that utilized egg yolk (unpublished data). It has been shown that egg yolk acts as a cryoprotectant of sperm.[18] The phospholipid, cholesterol, and low-density lipoprotein content of chicken egg yolk have been identified as the protective components in chicken egg yolk. [17], [3], [13,16] The present study was undertaken to determine the optimal concentration of egg yolk needed when freezing canine semen in an extender composed of tris-citrate-glucose. This study evaluated five concentrations of egg yolk in an extender void of glycerol ranging from 0-40%.

Semen parameters in individual dogs are known to affect successful cryopreservation. [11,19] To minimize this variability, pooled semen from six individual dogs were utilized. The dogs chosen met minimum requirements of greater than 70% motility and 60% normal morphology in two consecutive collections.

The pH of the extender, semen, and extended semen were evaluated on the day of freeze. Significant differences in pH were noted between extenders containing 10% and 30% egg yolk, 20% and 30% egg yolk and 20% and 40% egg yolk. Once the extenders were added to the semen the pH between all samples was no longer significantly different. In the 0%, 30%, and 40% egg yolk extenders, once added to the semen, fell in pH below the pH of both the extender and semen. The 10% and 20% egg yolk concentrations represented the highest initial pH at 7.12±0.04 and 7.124±0.5 respectively whereas the semen had a pH of 7.020±0.5. Though the 20 and 30% egg yolk concentration extenders were optimal in their final post-thaw motility and morphology their initial pH represented the highest and lowest measurements, 7.12±0.04 and 7.022±0.04 respectively. The findings of this study show pH would not be a reliable measurement in determining the protective qualities of the extender for the sperm through the freezing process.

The osmolarity for extender, semen, and extended semen were evaluated on the day of freeze. Osmolarity was found to be significantly different for all egg yolk concentrations and extended semen. The osmolality for the semen remained a constant of 319.6±12.98 mOsm. Osmolarity is a parameter that has been discussed but is often over looked when evaluating extenders. Two previous studies showed that once osmolarity was ≥500 mOsm spermatozoa

decreased in motility. [6,15] In the present study, the two extenders that gave optimal results during both freezing and cooling were the 20 and 30% egg yolk concentrations. Both extenders' mean osmolarities were closest to the semen at 292.6±2.63 and 325.7±1.99 mOsm, a difference of 27 mOsm and 6.1 mOsm respectively to the osmolarity of semen. In contrast the remaining egg yolk concentrations, 0, 10 and 40%, had a difference of 85.5, 59.6 and 52.4 mOsm respectively to the osmolarity of semen. Osmolarity is a parameter that should be considered when evaluating extenders for use in cryopreservation of canine semen due to its affect on post-thaw viability. Eilts in 2005 [2] stated extenders help stabilize the cell membrane and that the osmotic sensitivity of the cells is important during the cryopreservation process. Osmotic stress causes stress in the cell water volume and thus the cooling rate of the sample affects the membrane permeability and cell survivability.

During post-cool evaluation of the various extenders, both sperm motility and morphology were examined. No differences were found in the motility between extenders. This observation was anticipated, as there are canine semen extenders commercially available void of egg yolk. What was unexpected was the significant 11% decrease of normal morphology in the 0% egg yolk concentration extender after 3 hours of cooling at 5°C. The remaining extenders, 10%, 20%, 30% and 40% had no significant changes. This drop in normal morphology is notable as many extenders used for shipment of cooled semen lack egg yolk as a component. A conclusion can be made that egg yolk may be indicated in cooling extenders as well as in freezing extenders. The 0% extender was the only extender to show a drop in morphology during post-cool and had the largest drop in post-thaw motility, 70% compared to 52-28% in the other extenders.

The post-thaw sperm motility and morphology results show that a concentration of 20% egg yolk produces slightly better results than a 30% concentration and is significantly optimal over the remaining concentrations utilized in this study. After the thawing process, the 0% and 10% egg yolk concentrations had significantly lower percentages of normal morphology compared to the remaining extenders. The extender containing 0% egg yolk concentration had lower motility and morphology compared to all extenders containing egg yolk. The 10% extender had a slightly lower normal morphology but the second highest post-thaw motility. The implication is that motility and morphology do not necessarily correspond to each other. Dogs could have semen that has excellent motility but poor morphology. Oettle [12] showed in 1993 that a 40% normal morphology with fresh semen only gave a 13% conception rate and that a 60% normal morphology was needed to for a greater then 61% conception rate. However, if only the initial morphology is taken into account when determining how well a dog freezes this may not actually represent the final post-thaw results.

The 10% extenders' post-thaw motility was 61%, which is normally considered acceptable motility. These results indicate that motility alone is inadequate when assessing semen quality.

Future experiments to help improve canine freezing extenders are still required to find the optimal ingredients. To better elucidate the role of egg yolk as a cryoprotectant in canine sperm an evaluation of a wider variety egg yolk types compared to chicken egg yolk is required. Some of the current types of egg yolk being used in other species are chucker, goose, duck and quail. [8], [5] It is important to utilize eggs that are fresh and easily accessible in preparing freezing extenders. In addition to evaluating different types of eggs, their low-density lipoprotein concentrations should be determined. Several studies in bulls and a few studies in dogs are available to help to determine the best way to extract the LDL's from the egg yolk allowing a concentrated volume of LDL's void of other egg yolk components. [13], [1] Utilizing this technique and repeating the above experiments with the concentrated form of LDL's may further help to protect spermatozoa during cryopreservation as seen in previous studies with other species. [7], [10], [14] Another study comparing glycerol and egg yolk or LDL may further elucidate which cryoprotectant is optimal for canine semen. Morphology, motility, pH and osmolarity should minimally be evaluated through out the freezing process.

Finding the right combination of ingredients for a freezing extender is a challenge. Due to individual animal variability, it would be desirable to find an extender that minimizes this variable response to semen freezing between individuals. Preservation of canine semen will continue to be in demand, not only for the domestic canid but for the endangered species as well. For example, the Red Wolf population is seeing a decline in their number of animals over recent years [4,9]. The implication of cryopreservation of semen continues to be expanded for the greater good of species preservation.

References Chapter 3

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Appendix A: Simplified Canine Freezing Extender Recipe

Ingredients:	Tris	2.42g
0%	Citric Acid Mono	1.26g
	Glucose	0.90g
	Egg Yolk	0mls
	Distilled Water	100mls
	Penicillin, K+ (500 IU/ml)	0.1ml
	Streptomycin Sulfate	0.1g
	Total	100mls

Ingredients:		
10%	Tris	2.42g
	Citric Acid Mono	1.26g
	Glucose	0.90g
	Egg Yolk	10mls
	Distilled Water	90mls
	Penicillin, K+ (500 IU/ml)	0.1ml
	Streptomycin Sulfate	0.1g
	Total	100mls

Ingredients:		
20%	Tris	2.42g
	Citric Acid Mono	1.26g
	Glucose	0.90g
	Egg Yolk	20mls
	Distilled Water	80mls
	Penicillin, K+ (500 IU/ml)	0.1ml
	Streptomycin Sulfate	0.1g
	Total	100mls

Ingredients:		
30%	Tris	2.42g
	Citric Acid Mono	1.26g
	Glucose	0.90g
	Egg Yolk	30mls
	Distilled Water	70mls
	Penicillin, K+ (500 IU/ml)	0.1ml
	Streptomycin Sulfate	0.1g
	Total	100mls

Ingredients:		
40%	Tris	2.42g
	Citric Acid Mono	1.26g
	Glucose	0.90g
	Egg Yolk	40mls
	Distilled Water	60mls
	Penicillin, K+ (500 IU/ml)	0.1ml
	Streptomycin Sulfate	0.1g
	Total	100mls

1. Gather all the above ingredients plus the following:
    a. 50 ml conical tubes

b. Parafilm
c. Graduated cylinder
d. Filter paper
e. Egg separator
f. Scale (supplies needed for weighing)
g. 12 ml syringe
h. 3 ml syringe
i. Tubes for extender storage
j. Ziploc freezer bag
k. .45 μl filter
l. Mix the following in the graduated cylinder sealed with parafilm thoroughly:
m. Distilled water
n. Tris
o. Citric Acid Mono
p. Glucose
q. Egg yolk – obtain by cracking the egg, separating out the egg yolk from the white with use of an egg separator. Place the egg yolk onto the filter paper and carefully either roll the yolk on the paper so that the egg white is no longer on the yolk or dab the yolk with the filter paper to obtain the same result. If you try rolling a few times and the yolk keeps breaking then dab, your yolk membrane is too fragile to roll. Once the egg white is off use the 12 ml syringe to poke through the membrane then aspirate the yolk out of the egg into the syringe. It may take several eggs to obtain the desired amount of yolk. **egg white is toxic to sperm so it is necessary to remove the white before obtaining the yolk

2. After mixing pour the extender into 50 ml conical tubes then centrifuge at 1000G for 10 minutes.
3. After centrifugation if it appears that the supernatant is clear of debris poor it directly into a new 50 ml conical tube being careful not to disturb the pellet.
4. If it appears there is still a lot of debris run the supernatant through a .45 μl filter.
5. Once you have the final supernatant add the last two ingredients: Kpen and Streptomycin
6. Make sure to mix thoroughly
7. Now take a 3 ml syringe and draw 3 mls at a time of the extender and put in the storage tubes.
8. After all extender is gone label a Ziploc bag with the treatment letter, freezing extender and the date, then store in a -20 freezer.

Foot Notes

[i] BF extender (Animal Reproduction Systems, Chino, CA)
[ii] Gerber Products Co., Fremont, MI
[iii] Corning Inc., Corning, NY
[iv] Corning Inc., Corning, NY
[v] Fisher Scientific, Pittsburgh, PA
[vi] Corning Inc., Corning, NY

[vii] Nikon Instruments Inc., Melville, NY
[viii] Society of Theriogenology, Nashville, TN
[ix] Corning Inc., Corning, NY
[x] Corning Inc., Corning, NY
[xi] Animal Reproduction Systems, Chino, CA
[xii] Mettler-Toledo, LLC Columbus, OH
[xiii] NUNC/Thermo Scientific, Rochester, NY
[xiv] The SAS system version 9.2, Cary, NC