Effects of cooling time and thaw rate on membrane integrity and motility of frozen-thawed canine spermatozoa using commercial semen extenders.

A research project for 5th year veterinary medicine students at the University of Utrecht, the Netherlands.

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Contents

Abstract

Cryopreservation of bovine spermatozoa has been successful for many years. More recently, cryopreservation of canine spermatozoa has been requested. Protocols using different extenders, different freeze rates and different thaw rates have been empirically derived over the years. The objective of this study was to compare the current laboratory protocol with two other protocols, one using different cooling times and a different extender and one using different thaw temperatures and a different extender [1].

A single ejaculate was collected from 11 mature dogs of different breeds. Each ejaculate was prepared for cryopreservation with two different commercial semen extenders, a human extender (Irvine Scientific, Santa Ana, CA, 92705-5588)(HEYE) or a canine extender (3560 Pine Grove, Unit 227, Port Huron, MI 48060)(CEYE). For each extender, two different cooling times were used, 30 (30m) and 60 (60m) minutes, before adding the corresponding extender containing 12% glycol (resulting in a final glycerol concentration of 6%). Each of the four aliquots were loaded into 0.5 mL straws, placed on a boat 4 cm over liquid nitrogen for 10 minutes and then plunged. For each aliquot, two different thawing protocols were used, one 50°C for 10 sec (50°) and one 37°C for 30 sec (37°). Five minutes after thawing, each sample was assessed for 13 different motility parameters using a computer assisted sperm analyzer (SpermVision, Minitüb, 419 Venture Court, Verona, WI 53593, USA) and for membrane integrity using SYBR-14/PI (LIVE/DEAD® Sperm Viability Kit, by Invitrogen™, 5791 Van Allen Way Carlsbad, CA 92008 USA). Only membrane integrity, progressive and total motility data are presented. Results showed that changing the extender, the cool down protocol or the thawing protocol offered no advantage over the current employed laboratory protocol.

Introduction

Since the introduction of glycerol as a permeable cryoprotective agent (pCP) and the subsequent discovery and use of the cryoprotective agent dimethylsulfoxide (ME_2SO), many cells and tissues have been cryopreserved, often by empirically derived methods (Woods, Benson et al. 2004). Semen cryopreservation has had a great influence on animal breeding, particularly the dairy industry (Holt 2000). Semen cryopreservation, enabling the wide-spread dissemination of superior genetics, has been used to improve domestic breeds and to preserve rare breeds by creating semen banks [2]. Canine semen is cryopreserved when it has to be stored for a later use, such as artificial insemination or research.

Most research on semen cryopreservation has been performed on human and farm animal semen, however recently canine semen cryopreservation has been studied more extensively. Protocols for cryopreserving canine semen using different extenders, different freezing rates and different thawing rates have been empirically derived by different laboratories over the years.

In order to maximize the number of viable and fertile spermatozoa that survive the cryopreservation process, the goal of any protocol is to prevent the adverse stresses that occur during cryopreservation. Cryopreservation of spermatozoa consists of initial cooling of the cells to 4°C, freezing and subsequent thawing. During cryopreservation, the cell is stressed by the decreasing temperature during cooling, the movement of water out of the cell and the formation or intra- and extra-cellular ice during freezing, as well by the movement of water out of the cell and the possible

re-crystallization of intracellular ice during thawing. Procedures have been developed to protect the cells during each of these processes.

Cooling

Mammalian spermatozoa are very sensitive to cooling from body temperature to near the freezing point (≈ 4°C). Damage to spermatozoa during cooling, known as cold shock, is observed as an irreversible loss of motility upon re-warming. Cold shock leads to the loss of selectivity in membrane permeability, which can be observed by staining with dyes that do not penetrate the intact plasma membrane [3].

Normally, cellular membrane lipids are in a crystalline-liquid phase. Cooling alters the cellular membrane, changing it from a crystalline-liquid phase to a gel phase (lipid phase transition) and inducing lipid packing faults. The lipids in a gel phase are, unlike the liquid phase, are locked in place and are not able to move within the bilayer. With lipid packing faults, when small holes occur during the gel phase, they can't be spontaneously resealed; which normally happens when the membrane is in a liquid phase. The changed packing of the lipids also can lead to altered function of membrane ATPases, whose activity depend on the physical state of certain lipids. The damage caused by cooling depends on the combination of the different membrane constituents, which include the cholesterol/phospholipid ratio, the content of non-bilayer-preferring lipids, the degree of hydrocarbon chain saturation, and the protein/phospholipid ratio in the membrane [3, 4]. Because different species have different cellular membrane constituents, the effect of cooling differs among them. When looking at the sensitivity of different species' spermatozoa to cooling, most farm animals have spermatozoa that are very sensitive, dogs and cats have spermatozoa that are somewhat sensitive and rabbit, rooster and humans have spermatozoa that are less sensitive to cold shock [3, 5].

Cold shock can be prevented by controlling the cooling rate and by adding protective compounds to the semen. Empirically, it has been determined that using non-permeating cryoprotectants, such as egg yolk or milk, and cooling semen from body temperature to 5°C at a rate of ≤ 10°C/hr diminished the damaging effects of the cold shock. The exact mechanisms by which egg yolk and milk protect the cell are not yet fully known. The phospholipids and low density lipoprotein (LDL) from the egg yolk seem to stabilize the cell membrane [3]. It has been suggested that the LDL adhere to cell membranes during the freeze–thaw process, thus preserving the spermatozoal membranes (Figure 1B) [6-8]. Recently, there have been studies on the use of 'pure' LDL in canine (freezing) extenders that have shown promising results, although small sample sizes were used [9, 10]. The benefit of using specific phospholipids and/or LDL rather than egg yolk or milk would be to create an extender void of animal protein. The presence of egg yolk represents a potential risk of microbial contamination, which can be a particular problem with the international exchange of cryopreserved semen [11].

The standard procedure in this lab is to initially cool the semen in a refrigerator at 5°C for one hour, which seems to be quite standard [9, 10, 12-15]. Different cooling times have been used by other groups; 2 hours at 4°C or 5°C [16-19] and 3 hours at 4°C for [1], as well as others. Rota et al noted that the samples cooled 1 hour at 4°C reached 4°C in 45 minutes [14]. Yu et al found epididymal derived spermatozoa stored at 4°C attained 4°C after only 10 minutes [20]. Shortening the cool-down equilibration time during the semen cryopreservation process would be more efficient in a busy clinical situation.

Freezing and thawing

After cooling, spermatozoa need to be frozen to effectively preserve them for long time periods. Reducing the temperature to 4^{o} C reduces the cellular metabolic activity and increases the life-span of the spermatozoa. Continued metabolic activity would lead to cell death, due their very limited metabolic activity (depending mostly on catabolic processes to function). To totally halt this process, the spermatozoa need to be frozen below -130^{o}C, at which the thermally driven chemical reactions do not proceed, effectively stopping time [21].

During freezing however, the spermatozoa can be damaged by various stressors. At temperatures around -5°C the intra- and extra-cellular water remains unfrozen in a super cooled metastable state. Between approximately -5°C and -10°C, ice forms in the extracellular medium, while intracellular water remains super-cooled. The maintenance of intra- and extra-cellular liquid water in chemical equilibrium ensures cellular dehydration. At this point, the rate of cooling must be slow enough to permit cellular dehydration to occur and avoid the freezing of the intracellular water, yet fast enough to avoid exposing the cell to a hyperosmotic condition subsequent to dehydration. Severe dehydration leads to solution-effects, for which several hypotheses exist, which are too detailed to mention in this report. Intracellular freezing of water leads to the formation of intracellular ice crystals. Another damaging effect is the mechanical stress caused by ice formation around the cell, which constrains the cells to a very limited space with unfrozen solutes (Figure 1) [21]. The crystallization of ice and the solution-effects lead to cellular injury and often to cell death [21].

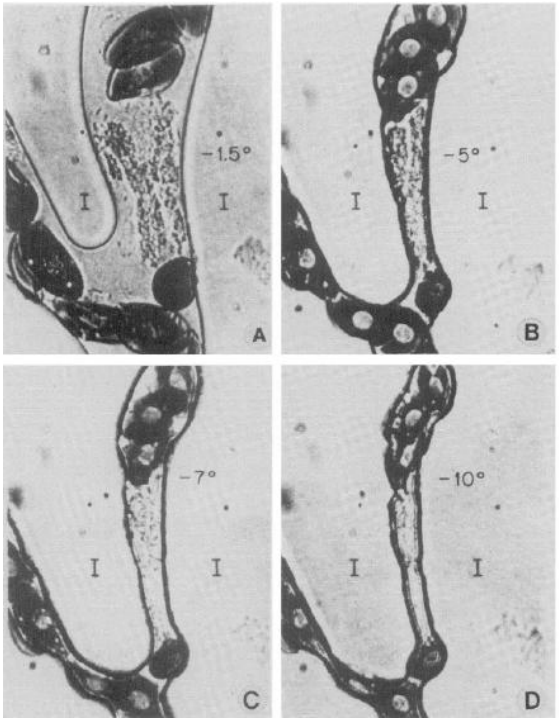

Figure 1. *Photomicrographs of frog erythrocytes in serum during course of slow freezing from -1.5 to -10°C. Note that cells are confined to channels of unfrozen solution between ice crystals (I), that channels decrease in diameter with decreasing temperature, and that cells shrink* [21].

To protect against the damaging effects of freezing, most semen freezing protocols incorporate a permeating cryoprotectant (pCP), such as glycerol. Glycerol can penetrate the sperm cell (unlike LDL from egg yolk) and has positive intracellular and extracellular effects. Intracellular; because the pCPs pass through the cell membrane, replace the water necessary for the maintenance of cellular volume, and depresses the freezing point of water (Figure 2). Extracellular; the pCPs increase the osmolarity and cause cellular dehydration, thus decreasing the amount of water that can be frozen within the cell. This coupled in- and out-flow causes the water to be displaced from within the cell faster than it would be displaced without the glycerol, even at lower freezing rates [3].

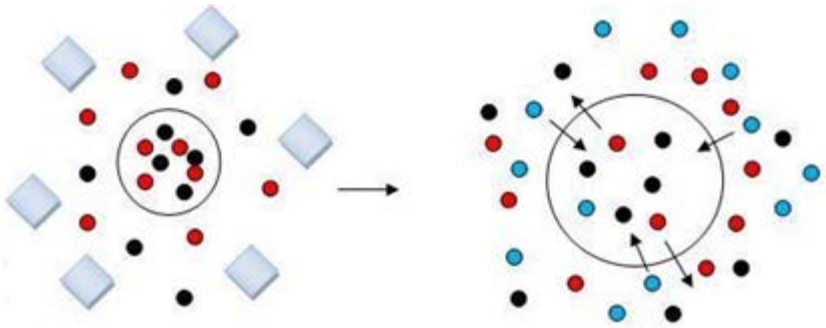

Figure 2. *During thawing the extracellular ice melts and the water can flow back into the cell, while the glycerol moves out. Black dots represent the solutes, blue dots water, red dots glycerol, the blue squares ice, and the blue stars re-crystallized ice.*

Glycerol also can insert into the membrane bi-layer and may have a negative (toxic) effect on the structure and function of the cell membrane. There can be large differences in glycerol tolerances between species [4]. Songsasen et al found that canine spermatozoa were rather resistant to high concentrations of glycerol [22]. Other pCPs such as ethylene glycol (EG) and dimethylsulfoxide (Me_2SO) have occasionally been used, but the beneficial use of glycerol has rarely been challenged [23]. Songsasen et al found that canine spermatozoa were rather resistant to high concentrations EG, but that Me_2SO was toxic. Me_2SO decreased the motility, but did not affect the membrane integrity of the canine cells [22]. It has to be noted that they only used three dogs for their experiment, however. Martins-Bessa et al compared glycerol with ethylene glycol at different concentrations in egg-yolk TRIS extenders [19]. They found no advantages to ethylene glycol over glycerol. They noted that other studies on ethylene glycol found the same conflicting results.
Different research has led to the development of different commercial canine extenders such as: Uppsala Equex II[1], CLONE[2], CanFreeze™[3], Caniplus Freeze and Triladyl Canine[4], Next Generation® Universal™ and Next Generation® Dr. Kenny semen extenders[5].
In most studies on semen extenders, non-commercial extenders are being used. These extenders vary in buffer content, glycerol concentration, using egg yolk or LDL and in the use of Equex STM paste.

[1] CaniRep, Fjallbo 110, SE 75597 Uppsala, Sweden
[2] CLONE RI, Greenwich Valley Veterinary Clinics, 725 Quaker Ln, West Warwick, RI 02893
[3] Partnar, 3560 Pine Grove, Unit 227, Port Huron, MI 48060
[4] Minitube of America, Inc. P.O. Box 930187 419 Venture Court Verona, WI 53593
[5] Exodus Breeders Corporation, 5470 Mount Pisgah road, York, PA 17406

The extenders used for this research were a human extender[6](HEYE) and the canine extender CanFreeze™ (CEYE), The components for HEYE are listed as TES 176 mM, Tris 80 mM, Dextrose 9 mM, Gentamicin Sulfate 10 µg/mL, and heat-inactivated egg yolk 20 % (v/v). The CEYE components are only listed as a dual sugar proprietary formulation containing ticarcillin and penicillin.
After spermatozoa are frozen, they need to be thawed. Rapid freezing leads to the formation of small, intracellular, thermodynamically unstable, ice crystals. If rapidly frozen cells are warmed at a slow rate, these thermodynamically unstable ice crystals have enough time to recrystallize and form much larger crystals within cell organelles (Figure 3). Rapid warming will melt the ice crystals before they can re-crystallize. In theory, slow cooled cells also should benefit from fast warming, thereby avoiding exposure of the dehydrated hyperosmotic condition for a long period to the hyperosmotic solutions. Long term exposure of dehydrated cells to hyperosmotic conditions, may cause the cells to swell [21].

Figure 3. *The intra- and extra-cellular ice melts during the thawing process. Due to a low thaw rate, the intracellular water can re-crystallize. Black dots represent the solutes, blue dots water, red dots glycerol, the blue squares ice, and the blue stars re-crystallized ice.*

There has to be an optimal freezing rate, where these negative stresses are minimized. However, even at the optimal freezing rate these cells are subjected to these stresses. The optimal freezing rate varies greatly between different cell types [21]. Yu et al. studied the effects of different cooling (freezing) and warming rates on canine spermatozoa collected from the cauda epididymis[20]. Their results showed a relationship between freezing and thawing rates when looking at motility and plasma membrane integrity. Sperm motility was low at low cooling rates, increased to a maximum at a cooling rate of 11°C/min and then decreased with higher freeze rates (Figure 4). The membrane integrity followed the same pattern, however with slow freezing rates; slow warming resulted in a higher motility compared to the rapid warming. This did not seem to correspond with the hypothesis that slow warming exposes the spermatozoa for a longer period to the solution-effects and therefore should result in more damage to the spermatozoa. They concluded that for epididymal spermatozoa the maximum survival was obtained when they used a cooling rate of -11°C/min and a high warming rate.

[6] Irvine Scientific, Santa Ana, CA, 92705-5588

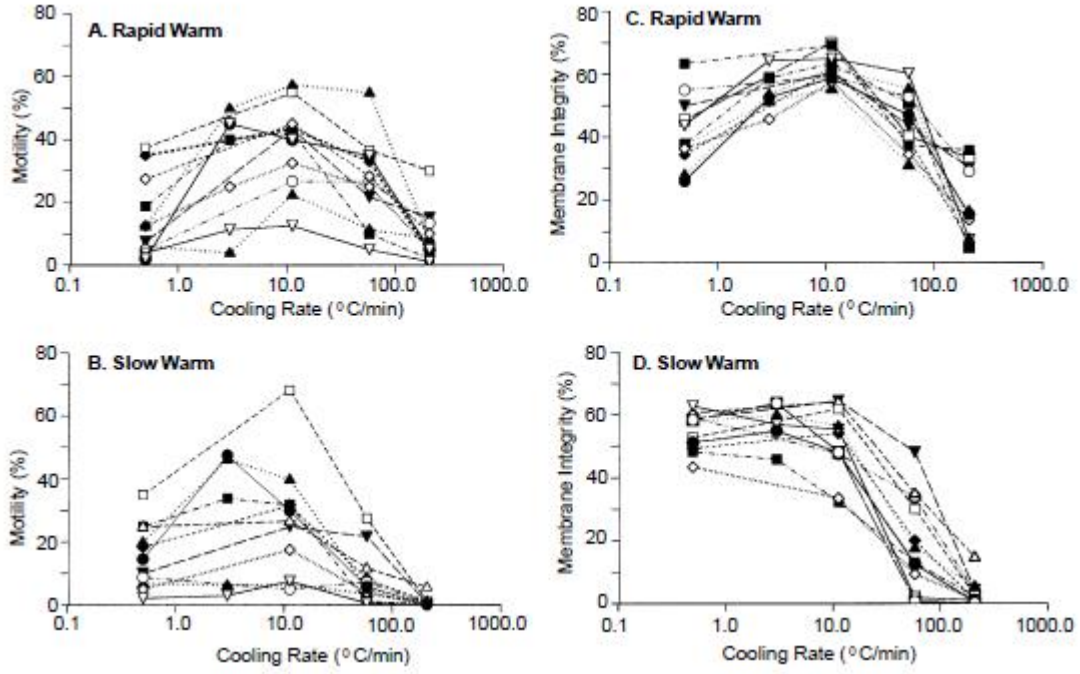

Figure 4. *Survival of epididymal spermatozoa of 11 dogs after their spermatozoa were cooled at each of five rates (0.5, 3, 11, 58 or 209°C/min), stored briefly in LN2, and then warmed either at 830°C/min or at 33°C/min. The results plotted are the means of duplicate samples for each dog assayed either for motility or for membrane integrity. (A) Motility after rapid warming. (B) Motility after slow warming. (C) Membrane integrity after rapid warming. (D) Membrane integrity after slow warming* [20].

This seems to be in agreement with Thirumala et al, who calculated the theoretical optimal freezing rate for dog spermatozoa and found it to be between -10 and -30°C/min [24]. They concluded that this was close to the empirically derived freezing rates.

In most laboratories freezing is done by means of liquid nitrogen (LN_2) in either a tank or a Styrofoam™ box or with a programmable freezer. Freezing with the tank involves 3 steps in which the semen samples are frozen on LN_2 vapors in a tank filled with LN_2 and are held at 3 different heights above the liquid, before being immersed in the LN_2 [25-29]. Freezing with a Styrofoam box uses the same principal, only involves less steps. The box is first filled with LN_2 to a certain height (the exact height differs between studies) and the samples are then placed a on floating 'boat' and frozen for 10 minutes in the LN_2 vapors, before being immersed in the LN_2. The height above the liquid determines the freezing rate. Some studies have been done to determine the appropriate height for their protocols [17, 20]. Most studies used a height in the range of 4 to 8cm [9, 10, 12, 13, 16-19, 30-32]. Infrequently a programmable freezer is used, which allows the freezing rate at any given moment of time to be set and adjusted. With a programmable freezer, specific freezing rates can be compared [14, 18, 33]. These freezers tend to be quite expensive and therefore not used often.

As is the case with cooling times, different thaw times and temperatures have been used by different laboratories. Several studies compared low thawing temperatures (37-38°C) to higher thawing temperatures (55 or 70°C), with variations in time exposure to the different temperatures. A higher thawing temperature resulted in a higher thawing rate. It was generally found that higher thaw rates gave better overall better results when testing for motility. Nothling et al. acknowledged that the high thaw rate at 70°C was better and tested it against thawing in just boiled water (98°C)

[16]. They found that there was hardly a difference; they however felt that the 70°C thaw was more practical. Comparisons of other rates have been done, but resulted in the same conclusion; a higher rate is better. In this laboratory, the semen is usually thawed at 50°C for 10 seconds, which has yielded good results. Many researchers used the lower thaw rates [9-11, 14, 26, 32, 34, 35]. The CEYE producer advises to thaw at 37°C for 30 sec. They also recommend that after thawing, the semen should be diluted with their thaw media, but do not state the composition of the thaw media. The thaw media cited by others primarily contains at least a buffer (mainly TRIS), a sugar (mainly glucose, sometimes fructose) and an antibiotic (mainly streptomycin, sometimes penicillin) and the sample:media ratio is often 1:2 or 1:3 [12, 13, 19, 25-28, 36, 37]. Some other thaw extenders, with different compositions, have occasionally been used [32, 33].
It should be clear that developing a good cryopreservation protocol is a difficult task. Besides the different processes mentioned here, there are a lot more factors that should and can be considered, but that lays beyond the reach of this study.
In this study a cool time of 30 minutes was compared to a cool time of 60 minutes and a thaw time of 30s at 37°C was compared against a thaw time of 10 seconds at 50°C. The 60 minute cool time and the 10 second cool time are used in the standard protocol in this laboratory, which is described by Eilts et al. in: Small Animal Theriogenology [38]. The thaw time of 30 sec at 37°C was advised by the CEYE manufacturer.
Statistical analysis included a repeated measures analysis in an ANOVA of a 2^3 factorial arrangement of treatments with dog as a random effect in a mixed effects model using the SAS mixed procedure. Pair-wise t-tests of least squares means were performed.

Semen analysis

In order to determine the quality of a semen cryopreservation protocol, the fertilizing potential should be analyzed. The ideal method would be to inseminate a group of bitches of normal fertility, however this would require a large number of animals and add many extra variables such as the fertility of the female, the variability of each female's estrous cycle, and the insemination method [39]. Most researches have therefore chosen to quantitatively measure sperm functions. These sperm functions have however never been properly related to fertility. The most widely used functions are: motility, morphology, membrane integrity, acrosomal status and zona binding assays [39, 40].
The motility can be analyzed by bright field microscopy or by the use of computer-assisted sperm analysis (CASA). The visual assessment by microscopy is subject to evaluator bias and can only estimate the percentage of motile spermatozoa. The CASA gives an objective measurement, and can give a wide range of motility parameters such as total and progressive motility, slow, medium and rapid moving spermatozoa, linearity of sperm movement. Several studies found high correlations between the computer-calculated motility, progressive motility and concentration, and the conventional light microscopic evaluation [40].
Morphology can, as for motility, be analyzed visually by means of a bright field microscope or CASA [40]. Visually, the larger abnormalities can be detected and scored: unusual size and shape of the head, bending of the midpiece, presence of proximal or distal cytoplasmic droplets, bent or coiled tails and detached heads. The CASA can give more detailed information on sperm morphology, but has so far hardly been used primarily in research situations.

The cell membrane integrity can be assessed by different means. Routinely, this has been done by means of light microscope stains, such as eosin/nigrosin. With this stain, the dead cells stain red and

the live cells are not stained, due to the stains being unable to penetrate an intact membrane [39, 40]. More recently, several fluorescent dyes have been developed and validated for the assessment of sperm membrane integrity in dogs. These stains include CFDA/Pi and SYBR 14/Pi. Both CFDA and SYBR 14 are membrane-permeant and are rapidly converted by intracellular esterases into highly fluorescent, membrane impermeant green fluorophores, which then remain intracellular and bind to the nucleic acids. The membrane-impermeant PI labels the nucleic acids of membrane-compromised sperm with red fluorescence and displaces or quenches the CFDA or SYBR 14[41, 42]. These changes can then be detected by flow cytometry or by visual observation using a fluorescence microscope. The acrosomal status can be determined with non-fluorescent stains or fluorescent stains that stain the intact membranes [40]. Cryopreservation can trigger the acrosomal reaction and therefore render the spermatozoa infertile [39].
The zona binding capacity of canine spermatozoa can be evaluated by two types of assays: one using intact homologous oocytes (ZBA), the other using bisected hemizonae (HZA). In the ZBA the spermatozoa are co-incubated with oocytes and the number of spermatozoa bound to the ZP is counted a using phase-contrast or a fluorescence microscopy. In the HZA the zona is dissected from the oocyt and then incubated with the spermatozoa and again counted [40].

Research objective

The objective of this study was to compare the current laboratory protocol with two other protocols, one using different cooling times and a different extender and one using a different thaw protocol and a different extender.

Hypothesis 1: cryopreservation of canine spermatozoa using a new, commercially available canine extender using a shorter cooling time would yield increased post-thaw motility and more intact membranes than the currently employed laboratory protocol.
Hypothesis 2: cryopreservation of canine spermatozoa using a new, commercially available canine extender using a lower thawing temperature in combination with a longer thaw time would yield a lowered post-thaw motility and intact membranes than the currently employed laboratory protocol.

Materials and Methods

A total of 11 animals were used for semen collection. Two Walker-type hounds and four Beagles dogs from the Division of Laboratory Animal Medicine at the school of Veterinary Medicine and five client-owned dogs (one Pit-bull, two Labrador Retrievers, one Golden Retriever and one Siberian Husky) had semen collected for cryopreservation. The two Walker-type hounds from the teaching colony were housed at the Louisiana State University School of Veterinary Medicine and routinely used for semen collection. The four Beagles, also housed at the School of Veterinary Medicine, never had semen collected before the study and had to be trained for the semen collection procedure. Two of the Beagles were housed individually and two were housed together. These six dogs were maintained according to the Guide for the Care and Use of Laboratory Animals (2010, National Institutes of Health). All procedures for handling and treatment of the animals were reviewed and approved in advance by the Louisiana State University Institutional Animal Care and Use Committee. The clients provided written consent to have semen collected for research purposes.
None of the dogs had any known disease or received any treatment that influenced their

reproductive function for the 60 days immediately prior to the semen collection used for cryopreservation. They were however not tested for Brucella canis. Each dog had at least five days of sexual rest, but no more than 10 days, before the semen. The dog's ages ranged from 13.7 months to 116.6 months (median age 25.4 months).
Semen samples with semen parameters of < 50% motility and/or <50% normal cells were excluded from the study. Experience from the laboratory has shown that samples with these low parameters have very low survivability during cryopreservation and would adversely impact the results. Samples containing blood were not used, since blood has a negative effect on the motility and membrane integrity of canine spermatozoa after cryopreservation [42].

Semen analysis

The raw semen was evaluated for volume, concentration, motility, morphology and membrane integrity.

Volume and concentration

The volume as measured in a 15 mL centrifuge tube. To determine concentration, a 1:10 and a 1:100 dilution of the raw semen was made with formal buffered saline (FBS). The concentration was determined by using the 1:100 dilution in a hemocytometer at 400x magnification under a phase-contrast microscope.

Motility

The raw semen sample was first assessed under bright field microscopy at 400x magnification to estimate the motility. This was done by placing a drop of semen on a 37^{o} slide using two small wooden sticks, which are thermo-neutral and therefore do not cold-shock the sperm cells. Motility was assessed by computer assisted semen analysis (CASA)[7]. Two drops of semen were then placed in a 3ml Eppendorf cup, which was in a heated stage (37^{o}C), and then diluted to a concentration of about 50x10^{6} cells/ml with the Inra 96 extender. This dilution was incubated for 5 minutes, after which the motility was analyzed. A 3µl drop was placed in a chamber slide type of a heated (37^{o}C) 20 micron Standard Count 4 chamber slide[8] and placed under the microscope. The sample was viewed at 200x using a phase contrast microscope[9]. Four fields were recorded, containing a total of at least of 100 cells. After recording the images, each field was manually edited for mis-identified cells. The fields were recorded in the middle of the chamber and on four different points, along the longitudinal axis. Afterwards recording the images, the each field was evaluated manually and edited for mis-identified cells.

The following motility parameters were assessed by the CASA system:

1. *Curvilinear velocity (VCL, mm/s),* the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time.
2. *Linear velocity (VSL, mm/s)*, the straight trajectory of the spermatozoa per unit of time (= straight line distance from beginning to end of track divided by time taken).
3. *Mean velocity (VAP, mm/s)*, the mean trajectory of the spermatozoa per unit of time.

[7] SpermVision, Minitüb, 419 Venture Court, Verona, WI 53593, USA
[8] Leja, Luzernestraat 10, 2153 GN Nieuw-Vennep, The Netherlands
[9] Olympus BX41; Olympus America, Inc., Center Valley, PA, USA

4. *Mean coefficient (STR, %),* which indicates the linearity of the mean trajectory and is defined as (VSL/VAP) x 100.

5. *Linear coefficient (LIN, %),* the ratio of the straight displacement in the sum of elementary displacements during the time of the measurement and it is defined as (VSL/VCL) x 100.

6. *Wobble coefficient (WOB, %),* which indicates the oscillation of the curvilinear trajectory upon the mean trajectory and is defined as VAP/VCL) x 100.

7. *Frequency of head displacement = beat cross frequency (BCF, Hz),* the number of lateral oscillatory movements of the sperm head around the mean trajectory.

8. *Amplitude of lateral head displacement (ALH, mm),* which is the mean width of sperm head oscillation.

9. *Distance curved line (DCL, mm),* the actual distance that the sperm cell moved during the analysis period.

10. *Distance straight line (DSL, mm),* the distance from the point in which the cell was first found in the analysis to the location of the cell at the last frame of the analysis in a straight line.

11. *Distance average path (DAP, mm),* the measured distance using a smoothed line as a reference.

12. *Average orientation change (AOC, degrees),* the average number of degrees that the head of the sperm moved from left to right during the analysis.

Sperm cells were marked as immotile, if AOC < 4.5mm, they were marked local if DSL <6mm. Progressive sperm cells were divided in 4 different categories; hyperactive cells were: VCL >80mm, LIN < 80 and ALH > 6.5mm, linear cells were: STR > 0.9 and LIN > 0.5, non-linear: STR < 0.9 and LIN < .5, Curvalinear: DAP/Radius >= 3 and LIN < 0.5. These parameters were the laboratory standards. Total motility was calculated as the sum of the motile and progressive sperm. Other settings were as followed: Cell identification Area: 22 to 80 μm^2, Field-of-view depth: 20 µm, Pixel to µm ratio: 150 to 100, Points to use in cell path smoothing: 11. Assessment requirements: 5000 cells or 4 fields.

Morphology

Sperm morphology was assessed by the researcher under phase contrast microscopy lens at 1000x magnification. Sperm cells were classified as normal, proximal droplet, distal droplets, abnormal mid-piece, coiled tail, kinked/bent tail, abnormal head shape and detached head.

Membrane integrity

The sperm membrane integrity was determined using SYBR14/PI[10]. The SYBR 14 and PI were each diluted to a stock 0.02M concentration before the start of the study and were frozen in small aliquots and stored in a freezer. The small aliquots were frozen inside light-proof 3mL Eppendorf cups.

Before staining, one aliquot of each was thawed for 10 minutes at 37°C. A 10 ml tube with phosphate buffered saline (PBS), which was stored in a refrigerator at 5°C, was warmed for the same amount of time. After 10 minutes, 50 µl of semen was diluted in an appropriate amount of PBS to

[10] LIVE/DEAD® Sperm Viability Kit, by Invitrogen™, 5791 Van Allen Way Carlsbad, CA 92008 USA

make a solution of $50x10^6$ cells/ml. This concentration proved after preliminary testing to be optimal for manual counting. A volume of 3 µl of SYBR 14 was then added and each sample was stored for 10 minutes in the warmer at 37°C, to let the stain permeate the cells. After this, 2 µl of PI was added to each sample and incubated at 37°C for 5 minutes to let the PI stain the membrane compromised cells. The supplier states that in this order of adding the stains, they both need 5-10 minutes before the next step can take place. After experimenting with different times, it showed that the SYBR14 worked best with a 10 minute incubation period and that the PI only needed a 5 minute incubation period. This ensured that the sperm was properly stained, but exposed to the negative influence of the left-over glycerol for the shortest period of time.
A 3 µl drop was placed under a cover slip on a heated slide, viewed at using a fluorescent microscope[11]. Images were digitally recorded[12] using a camera[13]. At least 100 cells were counted manually. Spermatozoa having green fluorescence were considered membrane-intact and those having red or orange fluorescence were considered to have damaged membranes. The percentage of membrane-intact spermatozoa was calculated as a fraction of the total. Blurry recordings, due to movement of the fluid on the slide, were discarded and not counted. The percentage of membrane-intact spermatozoa was calculated as a fraction of the total. A flow sheet, depicting the process can be seen in Figure 5.

Cryopreservation process

After semen collection the volume, concentration and motility were analyzed in a small sample. After preparing the motility sample, the sample for the SYBR 14 and propidium iodide (PI) stains were made and recorded. Each sample was split into two separate aliquots. One aliquot was prepared for cryopreservation with a two-part human egg-yolk-based extender (HEYE)[14] and the other aliquot was prepared with a two-part canine egg-yolk-based extender (CEYE)[15]. Each aliquot had the appropriate refrigeration extender added and was centrifuged for 10 minutes at 900x g after which the supernatant was removed, leaving a pellet. The pellet was re-suspended with the appropriate refrigeration extender (HEYE or CEYE) to a concentration of $100x10^6$ cells/ml in 15 mL plastic tubes. The motility was again assessed by light microscope, as a quality control.
The two semen samples were then split into two subsequent aliquots again and were cooled for either 30 or 60 minutes in a refrigerator at 5°C. During the cooling process eight 0.5 ml French straws were labeled, attached to a syringe and cooled alongside the semen samples.
After the appropriate cooling period (30 or 60 minutes), the samples were taken out of the refrigerator and put into a cold-box and extended with the appropriate freezing extender to a final concentration of 50×10^6 cells/ml. The cold box was a Styrofoam box containing frozen canisters, which ensured that the straws stayed at 5°C while being processed. The box was prepared a half hour before freezing to ensure it was at the right temperature when used. After addition of the freezing extender, the motility was again checked under the light microscope for quality control. The

[11] BX51, Olympus America Inc., 3500 Corporate Parkway, P.O. Box 610, Center Valley, PA 18034-0610
[12] DP2 BSW, Olympus America Inc., 3500 Corporate Parkway, P.O. Box 610, Center Valley, PA 18034-0610
[13] DP72, Olympus America Inc., 3500 Corporate Parkway, P.O. Box 610, Center Valley, PA 18034-0610
[14] Refrigeration extender: Refrigeration Media - TEST Yolk Buffer (TYB) with Gentamicin
Freezing extender: Freezing Medium - TEST Yolk Buffer (TYB) with 12% Glycerol and Gentamicin
Irvine Scientific, Santa Ana, CA, 92705-5588
[15] Refrigeration extender (Step One, CB-027)
Freezing extender (Step Two, CB-028)
Partnar, 3560 Pine Grove, Unit 227, Port Huron, MI 48060

straws were filled in the cold-box and sealed with a microwave sealer. The straws were then placed on a screen that was attached to a 3 cm thick Styrofoam frame which floated on the LN_2 vapors. After 10 minutes the screen was flipped and the straws were plunged into the LN_2. After being immersed, the straws were put in a labeled storage cane. The canes were stored vertically in a storage tank with filled with liquid LN_2 at a temperature of -196^{o}C. The straws were stored for at least 7 days before they were thawed and analyzed.

The straws were thawed one at a time at either 37^{o}C for 30 seconds or at 50^{o}C for 10 seconds in a warm water canister, depending on their allocated protocol. The straws were removed from the canister, wiped dry, cut at the sealed end and then held over an Eppendorf cup, after which the plug end was cut. The 3 ml Eppendorf cup was placed in a 37^{o}C heated block. The CEYE samples were extended to a concentration of 25 x 10^6 cells/ml with the CEYE thaw medium and the HEYE samples were extended to the same concentration with the a commercial non-egg-yolk stallion semen extender[16]. Five minutes after thawing, the samples were analyzed as described.

Statistical analysis included a repeated measure analysis in an ANOVA of a 2^3 factorial arrangement of treatments with dog as a random effect in a mixed effects model using the SAS mixed procedure[17]. Pair-wise t-tests of least squares means were performed as follows, for the cooling down: HEYE 60m and CEYE 60m, HEYE 30m and CEYE 30m, HEYE 60m and HEYE 30m, and CEYE 60m and CEYE 30m and for the thawing rate: HEYE 50° and CEYE 50°, HEYE 37° and CEYE 37°, HEYE 50° and HEYE 37°, and CEYE 50° and CEYE 37°.

[16] Inra 96, INRA, IFR135, 37380 Nouzilly, France

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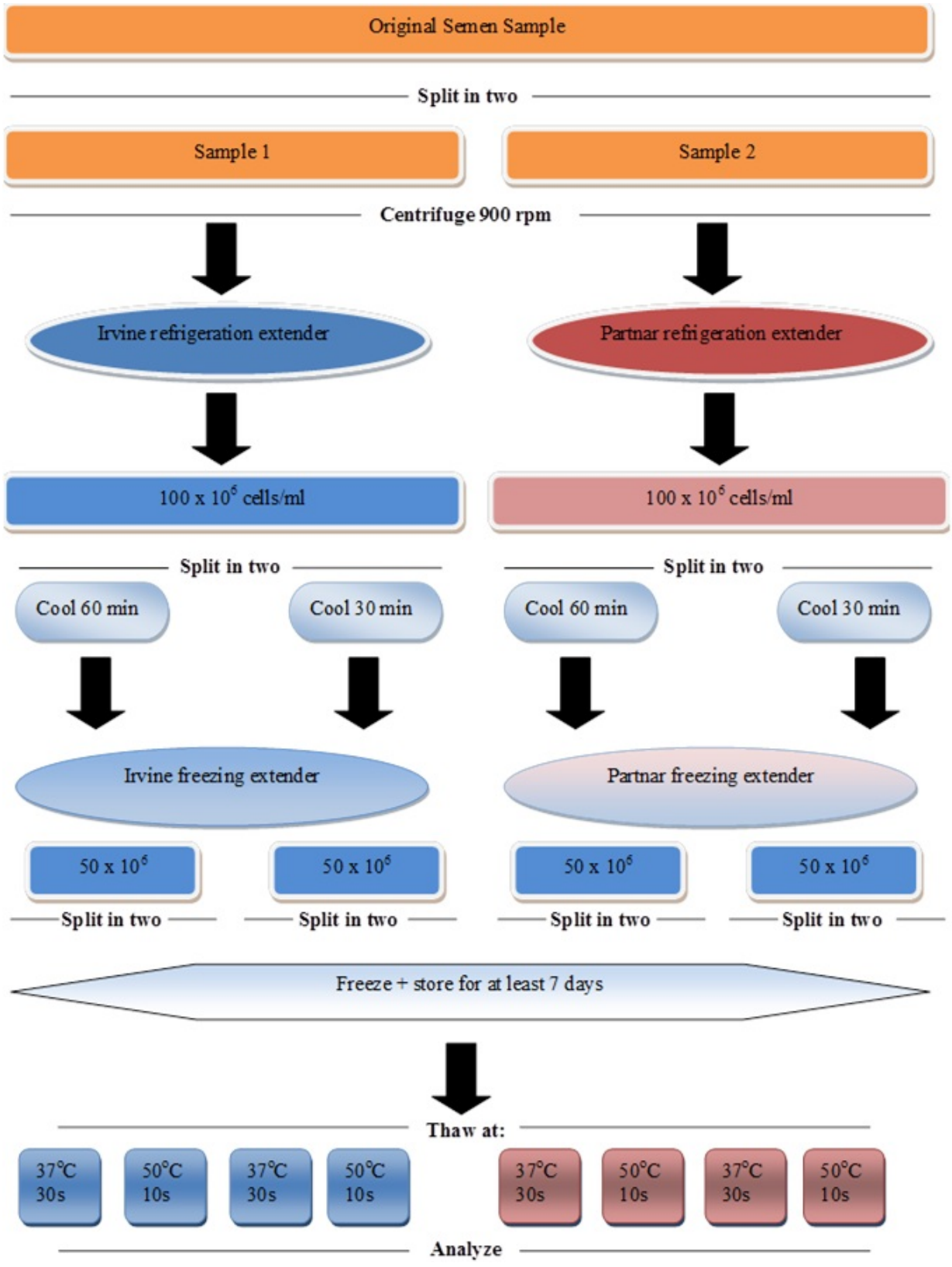

Figure 5. *The cryopreservation protocol used.*

Results

The results for the total motility (%), the progressive motility (%) and the intact membranes (%) are shown below in Tables 1 to 6. The remaining data parameters can be found in the supplement, with all the raw data.

Effect of different cooling time and extenders

The results for post-thaw percent total motility (%), percent progressive motility (%), and percent intact membrane integrity (%) are presented in Table 1, Table 2, and Table 3, respectively. All the samples for this part of the research had a thaw protocol of 50°C for 10 seconds.
Using the HEYE extender resulted in significantly greater total motility compared to using the CEYE extender when a 60 minute cool down was used, but not when a 30 minute cool down was used. Using the HEYE extender resulted in significantly greater total motility when samples were cooled for 60 minutes than when cooled for 30 minutes in HEYE, however when using the CEYE extender total motility was not different for the two cooling times.
Using the HEYE extender resulted in significantly greater progressive motility compared to using the CEYE extender when a 60 minute cool down was used, but not when a 30 minute cool down was used. Using the HEYE extender resulted in significantly greater progressive motility when cooled for 60 minutes than when cooled for 30 minutes in HEYE, however when using the CEYE extender the progressive motility was not different at the two cooling times.
There was no difference in the membrane integrity between the HEYE and CEYE extender comparing either a 30 or 60 minute cool down between extenders. There was a difference in the membrane integrity comparing 30 and 60 minute cool down times within the HEYE, but not for the CEYE extender.

Extender 1	Post Thaw Total Motility (mean ± SE)	Extender 2	Post Thaw Total Motility (mean ± SE)	
HEYE 60m	$55.9^{a,1} \pm 3.5$	CEYE 60m	$43.5^{b,3} \pm 4.1$	(^{a,b} $P = 0.007$)
HEYE 30m	$44.7^{c,2} \pm 4.3$	CEYE 30m	$46.0^{c,3} \pm 3.8$	(^{c} $P = 0.763$)
	(^{1,2} $P = 0.0150$)		(^{3} $P = 0.5676$)	

Table 1. *Post thaw percent total motility (mean ± SE) from single ejaculates of 11 dogs using two different commercial egg yolk based extenders at two different cool-down equilibration times. Values within rows with different letter superscripts differ; values within columns with different number superscripts differ.*

Extender 1	Post Thaw Percent Progressive Motility (mean ± SE)	Extender 2	Post Thaw Percent Progressive Motility (mean ± SE)	
HEYE 60m	$49.6^{a,1} \pm 4.1$	CEYE 60m	$36.7^{b,3} \pm 4.6$	(^{a,b} $P = 0.0037$)
HEYE 30m	$39.4^{c,2} \pm 4.8$	CEYE 30m	$40.3^{c,3} \pm 4.6$	(^{c} $P = 0.8448$)
	(^{1,2} $P = 0.0205$)		(^{3} $P = 0.4125$)	

Table 2. *Post thaw percent progressive motility (mean ± SE) from single ejaculates of 11 dogs using two different commercial egg yolk based extenders at two different cool-down equilibration times. Values within rows with different letter superscripts differ; values within columns with different number superscripts differ.*

Extender 1	Post Thaw Percent Intact Membrane Integrity (mean ± SE)	Extender 2	Post Thaw Percent Intact Membrane Integrity (mean ± SE)	
HEYE 60m	$51.8^{a,1} \pm 3.3$	CEYE 60m	$44^{a,3} \pm 3.3$	(^{a,b} $P = 0.0611$)
HEYE 30m	$41.9^{c,2} \pm 5.3$	CEYE 30m	$49.4^{c,3} \pm 4.1$	(^{c,d} $P = 0.0683$)
	(^{1,2} $P = 0.0175$		(^{3} $P = 0.1905$)	

Table 3. *Post thaw percent intact membrane integrity (mean ± SE) from single ejaculates of 11 dogs using two different commercial egg yolk based extenders at two different cool-down equilibration times. Values within rows with different letter superscripts differ; values within columns with different number superscripts differ.*

Effect of different thaw temperatures

The results for post thaw percent total motility (%), percent progressive motility (%), and percent intact membrane integrity (%) are presented in Table 4, Table 5, and Table 6, respectively. All the samples for this part of the research had a cooling time of 60 minutes.

Using the HEYE extender resulted in a significantly greater total motility compared to using the CEYE extender, when a 50°C as well as a 37°C thaw protocol was used. There was no significant difference for the total motility comparing the 50°C and 37°C protocols within the HEYE of the CEYE extenders.

Using the HEYE extender resulted in a significantly greater progressive motility compared to using the CEYE extender, when a 50°C as well as a 37°C thaw protocol was used. There was no significant difference for the progressive motility comparing the 50°C and 37°C protocols within the HEYE of the CEYE extenders.

Using the HEYE extender resulted in a significantly larger number of intact membranes compared to using the CEYE extender when using the 37°C thaw protocol, but not for the 50°C. There was no significant difference for the number of intact membranes comparing the 50°C and 37°C protocols within the HEYE of the CEYE extenders.

Extender 1	Post Thaw Total Motility (mean ± SE)	Extender 2	Post Thaw Total Motility (mean ± SE)	
HEYE 50°	$55.89^{a,1} \pm 3.5$	CEYE 50°	$43.5^{b,3} \pm 4.1$	(^{a,b} $P = 0.007$)
HEYE 37°	$54.49^{c,1} \pm 4.5$	CEYE 37°	$36.87^{d,4} \pm 3.9$	(^{c,d} $P = 0.017$)
	(^{1} $P = 0.078$)		(^{3,4} $P = 0.1452$)	

Table 4. *Post thaw percent total motility (mean ± SE) from single ejaculates of 11 dogs using two different commercial egg yolk based extenders at two different thaw temperatures. Values within rows with different letter superscripts differ; values within columns with different number superscripts differ.*

Extender 1	Post Thaw Percent Progressive Motility (mean ± SE)	Extender 2	Post Thaw Percent Progressive Motility (mean ± SE)	
HEYE 50°	$49{,}63^{a,1} \pm 4.1$	CEYE 50°	$36.72^{b,2} \pm 4.6$	(^{a,b} $P = 0.0037$)
HEYE 37°	$45.23^{c,1} \pm 5.1$	CEYE 37°	$31.78^{d,2} \pm 4.5$	(^{c,d} $P = 0.0026$)
	(^{1} $P = 0.3112$)		(^{3} $P = 0.2546$)	

Table 5. *Post thaw percent progressive motility (mean ± SE) from single ejaculates of 11 dogs using two different commercial egg yolk based extenders at two different thaw temperatures. Values within rows with different letter superscripts differ; values within columns with different number superscripts differ.*

Extender 1	Post Thaw Percent Intact Membrane Integrity (mean ± SE)	Extender 2	Post Thaw Percent Intact Membrane Integrity (mean ± SE)	
HEYE 50°	$51.80^{a,1} \pm 3.3$	CEYE 50°	$44.03^{a,2} \pm 3.3$	(^{a} $P = 0.0611$)
HEYE 37°	$56.32^{c,1} + 3{,}7$	CEYE 37°	$40.61^{d,2} \pm 2.8$	(^{c,d} $P = 0.0003$)
	(^{1} $P = 0.2704$)		(^{2} $P = 0.4034$)	

Table 6. *Post thaw percent intact membrane integrity (mean ± SE) from single ejaculates of 11 dogs using two different commercial egg yolk based extenders at two different thaw temperatures. Values within rows with different letter superscripts differ; values within columns with different number superscripts differ.*

Conclusion

Both the hypotheses were rejected. Results showed that changing the extender, the cool down protocol or the thawing protocol offered no advantage over the current employed laboratory protocol.

Discussion

Shortening the cooling time from 60 to 30 minutes using the HEYE extender resulted in a significant decrease in motility and membrane integrity. It could be that 30 minutes is not long enough to cool down the spermatozoa to 4^0C. This would be in concurrence with Rota et al, who found that their samples cooled for 1 hour at 4^oC reached 4^oC in 45 minutes [14]. Yu et al found epididymal derived spermatozoa cooled at 4^oC attained 4^oC after only 10 minutes, which makes a cooling time too short to reach the appropriate temperature unlikely[20]. There was no such difference for the CEYE. The total and progressive motility of the HEYE were significantly higher when cooled for 60 min, but were not significantly different when cooled for 30 minutes. It seems that when cooling for 60 minutes it benefits to use the HEYE extender, but this beneficial effect is not present when cooling for 30 minutes. It could be that the egg yolk in the HEYE extender needs longer to stabilize the cell membrane. It is possible that the CEYE lacked egg yolk or another non-permeating cryoprotectant. This could explain the significant lower total and progressive motility at 60 minutes and the lack of difference at 30 minutes, when the egg yolk in the CEYE might not yet exert its beneficial effect. The exact content of the CEYE has not been given, so a systematic evaluation of the individual components would be required.

Changing the thaw protocol from 50°C for 10 seconds to 37°C for 30 seconds, did not result in a significant decrease in either the total or progressive motility or the number of intact membranes for either extender. The lack of significant difference between the two thaw rates could be explained by the freezing rate used. The freezing rate in the current protocol is likely near 58°C/s. Yu et al measured a freezing rate of -58°C/min when freezing the straws 3.4 cm above the liquid nitrogen; a height of 3 cm was used in this study [20]. The freezing rate in this study therefore was greater than the theoretical optimal freezing rate of -10° to -30°C/min, which would require a relative high thaw rate. It seems that thawing at 37°C for 30 seconds is fast enough for the freezing rate used in our protocol, however some researchers have found different results. Ivanova et al compared a rate of 55°C for 5 seconds to a thawing rate at 37°C for 1 minute and found a significant higher motility at 55°C when looking at the total motility [1]. Several of these studies compared 70°C with 37°C and thus compared a higher thawing rate (vs. 50) with than this study. Peña and Linde-Forsberg found that for total motility, progressive motility, and membrane integrity thawing for 8 seconds at 70°C was significantly better than thawing for 15 seconds at 37°C[12]. Nothling et al however found that at 5 min post-thaw the progressive motility was not significantly different when comparing thawing for 5 seconds at 70°C and 2 minutes at 37°C[17]. Rota et al reported the same for total motility and membrane integrity, but used a 1 minute thaw time at 37°C[14]. Their conclusions seem unlikely, since most of their data points fall within a single standard deviation from each other.

The problem with comparing the present data with data from other studies is that every researcher uses a different cryopreservation protocol. Besides the aforementioned extender components and freezing rate, there are many other variables that differ between each protocol and each variable will have its own influence on the spermatozoa during the cryopreservation process. Besides the protocol itself, the way data is collected differs. The membrane integrity and the progressive and total motility are often assessed subjectively, instead of using a more objective method such as CASA or a flowcytometer. This subjective assessment and counting can lead to observer bias and less accurate data.

In this study, the most commonly used parameter tests (and available at the time) were used; motility and membrane integrity. The motility was assessed by the use of CASA to ensure as little observer bias as possible. The CASA recorded many different motility parameters, however most of these parameters are rarely reported and therefore were not used in comparing the different protocols in this study. Only the total and progressive motility from CASA were used in this study. The membrane integrity was assessed manually. This manual assessment of the membrane integrity could explain the lack of significant difference between the different protocols. Besides the live and dead staining spermatozoa, there is a third group, the moribund group. They become non-motile and have their stain changed from live to dead within one minute [41]. This led, during the assessment, to spermatozoa sometimes being counted as alive, while they were actually dying. Besides this problem, the stains were light sensitive, losing their color after exposure to (fluorescent) light. Motile spermatozoa, when photographed in one area, could have already lost their color while being exposed to fluorescent light in a previous recorded area. This could lead to motile/alive spermatozoa not being counted. To limit these problems, recordings were made far apart on a slide. These problems could have led to less accurate data and therefore a lack of significant difference. A more accurate estimate of the membrane integrity could have been obtained by using a flowcytometer or recording with the use of CASA. Unfortunately it was not possible to use either one for this purpose. The motility parameters however were analyzed objectively by CASA. There were some limitations of the software program, however. As described before, the recorded fields had to be edited after recording. Occasionally editing could not be done properly, due to close interactions between spermatozoa. It would occasionally record the path of a motile sperm cell over another sperm cell. Two problems could arise: the path of the motile sperm was split in two or the underlying sperm cell could not be edited. The first problem lead to a count of two motile sperm cells, which had to be corrected by deleting one of the paths, but gave a wrongful recording of some of the motility parameters. The second problem could lead to an immotile sperm cell being recorded as motile. It was also sometimes unclear if a particle was a sperm cell, because it lacked a tail. Detecting tails was particularly hard in the HEYE samples, due to many random particles in the samples. The sperm cell-like particles were deleted for all the samples. These particles closely resembled detached sperm heads, which made it impossible to discriminate between the two. In order to not create an artificial difference in the number of detached sperm heads between the HEYE and CEYE samples they were never counted. This led to a consistent slight overestimate of the motility parameters. These close interactions were concentration related, at high concentrations the individual spermatozoa would overlap each other more and had more interactions. This problem was minimized when the samples had a concentration of $25x10^6$ cells/ml, which post-thaw was reached when the original sample was re-suspended with the appropriate refrigeration extender to 100×10^6 cells/ml.

The CEYE extender protocol included a cooling, freezing and thaw extender, which would lead to the $25x10^6$ cells/ml. The HEYE protocol, as employed in the laboratory, only contained a cooling and freezing extender and the end-concentration would then be $50x10^6$ cells/ml. There was not enough CEYE thaw media to be used in the protocols with the HEYE extender, so a different diluent had to be used. During a preliminary to study, the effect on the post-thaw total and progressive motility was assessed. The CEYE thaw medium, the INRA cooling extender, the HEYE cooling, freezing extender or a 0.9% saline solution were compared. The INRA extender gave the least difference when comparing it to the CEYE thaw medium. The use of the INRA extender could have influenced the results, when comparing the HEYE with the CEYE extender. The HEYE extender yielded

significantly greater total and progressive motility then the CEYE extender when using the standard protocol (60 min and 50°C). When using the CEYE advised protocol (60 min and 37°C), the HEYE extender yielded significantly greater membrane integrity, total and progressive motility. Thus there may be components in the HEYE and/or the equine extender used for semen thawing that favor the sperm motility function over that seen with the CEYE.
In conclusion, the freezing protocol currently used by the laboratory yielded the greatest total and progressive motility, however membrane integrity did not differ. The difference in fertility among the treatments, as with most semen studies, was not examined.

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