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Effect of different subzero ice formation cooling rates during cryopreservation of Canine sperm on Zona binding ability

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Abstract:
The objective of the study was to determine the effect of different subzero ice formation cooling rates during cryopreservation of canine sperm on zona binding ability. Semen (n=18 ejaculates) was collected from 6 healthy, fertile dogs by digital stimulation and divided into two aliquots. After initial screening, one aliquot of semen was extended in Tris-egg yolk medium. Diluted semen samples were cooled to 5 ºC and then a Tris-egg yolk containing 8% glycerol was added. Sperm were packaged in 0.25 mL straws and cooled by programmable and conventional freezer. Cumulus oocyte complexes (COCs) (n=1616) retrieved from canine ovaries (n=52) after routine ovario-hysterectomy were graded and only grade I and II COCs were subjected to in vitro maturation in TCM 199 medium. Both fresh and frozen semen were processed by swim up method and subjected to in vitro maturation and fertilization. After 16-18 h of co-incubation, sperm-oocyte complex were stained with 1% aceto-orcein to determine oocyte penetration rate and the number of sperms attached to the zona pellucida of each oocytes were recorded. A mean (± SE) of 31 (± 4) oocytes were recovered per ovary. The percentage of grade I, II and III oocytes per ovary were 25.9, 43.1 and 31.0, respectively. The post-thaw sperm structural and functional parameters were greater for sperm cryopreserved following slow cooing compared to medium and fast cooling rates (P<0.05). The mean (± SE) number of fresh sperms bound to zona pellucida was 6.63 ± 0.09 which was significantly higher (P< 0.01) than that of frozen-thawed spermatozoa (1.68± 0.05).Canine sperm fertility was greater following cryopreservation of canine semen using slow cooling compared to medium or fast cooling. Zona pellucida binding assay could be used as a valuable in vitro tool for assessing the fertilizing capacity of both fresh and frozen-thawed canine spermatozoa.

***Key words**: Canine ovary, Cumulus oocyte complexes, Sperm, cooling rate, Zona pellucida binding assay*

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I. Introduction

Improving genetic diversity is a major concern both for conservationists and reproductive physiologists. One among the techniques available for the preservation of valuable genetic resources is gamete cryopreservation. Cryopreservation of canine semen is a process that provokes changes in the sperm membrane structure and function, particularly during the extension, cooling and thawing process. As most of the damage to the spermatozoa occurred during the critical temperatures at which water was converted to solid state, optimizing the cooling rate in this critical zone (5°C to –20°C) would minimize cryoinjury and may result in maximum recovery of morphologically normal and active spermatozoa. With the advent of programmable freezers, it has now been possible to have control over the cooling rates leading to higher post-thaw survivability of spermatozoa. However, even the best cryopreservation techniques were found to cause lethal and sub-lethal cryoinjury and hence, standard diagnostic evaluation based on physical parameters such as sperm motility pattern, sperm morphology, number of spermatozoa in an ejaculate were not sufficient to account for the fertility status of an ejaculate at any time.[1]

Further, assays such as hypo-osmotic swelling test and Spermac staining technique provide information regarding the integrity of plasma membrane and acrosome of the sperm and the predictive value of these

parameters could be further improved when used in conjunction with zona pellucida binding assay that evaluates the fertilizing ability of sperm. Such assays have been established for evaluating the fertilizing ability of human[2], bull[3] , boar [4],cat [5] and dog sperm[6]. In addition, studies have also shown a positive correlation between zona penetration rates and in vitro fertilization rates in humans[2] and within vitro fertility in boars[4]. Thus, the need for development of an ideal freezing technique for dog semen that would result in high fertilizing ability would open up new prospects for dog breeding.

Canine sperm was cryopreserved using different freezing media[7], different storage period at 4-5°C before freezing [8], different cooling, thawing rates, and freezing methods[9], and collection and preservation of epididymal sperm. Some protocols for sperm cryopreservation employing freezing machines have incorporated cooling to -5 to -10°C in pigs and dogs.[10,11] However, the effect of cooling to different subzero temperatures on sperm damage particularly zona binding, has not been tested in dog spermatozoa. The objective of the study was to determine the effect of different subzero ice formation cooling rate during cryopreservation of canine sperm on zona binding ability.

II. Materials and Methods

Animals: Eighteen healthy and sexually matured, mediums-sized dogs (age: 2 to 6 yrs.), owned by private dog breeders were used in this study.

Semen collection: Semen (n=18 ejaculates) samples were collected by digital manipulation described by Linde-Forsberg . The pre-sperm, sperm-rich and post-sperm fractions were collected separately in a clear, graduated, sterile semen collection cups. Semen samples with a minimum of 70 % normal, vigorous and forward linear motion were considered for further processing. Each sample was divided in to tow aliquots, assigned for use as fresh and frozen.

Semen preparation

Tris-Fructose-Citric acid (20% egg yolk; 8% glycerol) extender was used to dilute the semen samples for freezing. This extender was prepared following the method described by Silva *et* al. [12] with some modifications. The sperm rich fraction of semen was diluted with the extender to 1:2 or 1:3 ratio, to ensure an average sperm concentration of diluted semen was 200×10^6 sperm / ml. The extended semen samples were processed in a cold cabinet at 5 ^oC. French mini straws (0.25 ml; 50 10^6 sperm/straw) of different colors for different cooling rates were used to load the diluted semen samples. After loading, the semen straws were equilibrated in a water bath at 5 ^oC for 2 h. At the end of the equilibration period the straws were collected from the cold-water bath and dried by a precooled paper towel.

Programmable freezing: The dried straws were arranged on racking platform inside the cold handling cabinet at 5 ^oC. The straws were subjected to programmable freezing (Programmable freezer: Kryo 10 Series III, Planer, U.K) at different cooling rates, slow, medium and fast. The cooling rates are described in table 1. The computer graph showing different cooling rates under programmable freezing are given in Figure 1. After freezing, the frozen straws were immediately immersed in liquid nitrogen and transferred into goblets and stored at -196 ^oC.

Conventional freezing: Conventional freezing was performed using a thermocol box. Liquid nitrogen was poured into the thermocol box to a level of 5 to 6 cm from the bottom. The straws were arranged on a freezing rack at 7 to 8 cm above the liquid nitrogen level. Freezing was done by holding the straws horizontally and exposing the straws in the liquid nitrogen vapor for 30 min until frozen. After freezing, the frozen straws were immediately immersed in liquid nitrogen and transferred into goblets and stored at -196 ^oC.

Zona binding assay

Oocyte recovery: Ovaries were collected from routinely spayed bitches and transported to the laboratory in physiological saline at 35 °C to 38 °C within 2 to 3 h of collection. Following removal of ovarian bursa, the exposed ovary was rinsed with saline to remove blood and other contaminants. Cumulus oocyte complexes (COCs) were retrieved from canine ovaries by slicing method described by Nickson *et.* al. [25] Immature oocytes were screened under stereo zoom microscope at 10 × magnification, rinsed in modified HEPES-buffered Tyrodes medium in 35 mm petridish and graded as described by Hewitt *et* al. [26] Only grade I and grade II COCs were selected for further processing.

In vitro maturation (IVM) of oocytes: Cumulus oocyte complexes (grade I and grade II) were transferred into individual 100 µl droplets (15 COCs per droplet) of pre-equilibrated TCM-199 medium supplemented with 10 per cent heat inactivated fetal calf serum, Folltropin (1 µg/ml), LH (0.02 units/ml), estradiol (1 µg/ml), penicillin (100 IU/ml) and streptomycin (50 µg/ml) and cultured for 72 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

Sperm preparation: Both the fresh and frozen (4 straws were thawed at 37 °C for 40 sec) semen samples were processed by swim up method outlined by Parrish *et* al[14]. The sperm pellets were collected, and sperm

concentration was determined with a hemocytometer. The concentration of sperm was adjusted to 40 x 10^6 sperm / ml using fertilization medium for dilution.

Oocyte preparation*:* After 72 h of maturation, COCs were washed thrice in pre-equilibrated fertilization medium in a 35 mm petridish. The expanded cumulus cells were removed mechanically by repeated gentle pipetting. The matured oocytes were then transferred into pre-equilibrated 75 µL droplets (10 to 15 oocytes / droplet) of IVF-TALP medium overlaid with sterile mineral oil.

Sperm-oocyte co-incubation: The IVF droplets with oocytes were inseminated with 5 µl of sperm suspension to achieve a final concentration of 2 x 10^6 sperm / mL and co-incubated for 16 to 18 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After co-incubation, the sperm-oocyte complexes were washed in PBS and transferred to 1 per cent (w/v) aqueous sodium citrate for 10 min to remove loosely attached spermatozoa.

Determination of zona binding

Sperm-oocyte complexes (Figure 2) were rinsed in PBS and transferred to a clean, grease free glass slide overlaid with a coverslip supported by 4 drops of vaseline and paraffin mixture. The wax sealed oocytes were first fixed in acetic acid: ethanol: chloroform mixture (3:6:2 v/v) for 2 to 3 min to remove lipids in the ooplasm and then fixed with acetic acid: ethanol mixture (1:3 v/v) for 24 h. Later the slides were air dried and stained in 1 per cent aceto-orcein (1% orcein in 4.5% acetic acid) and examined under phase contrast microscope (400× magnifications). The number of sperm remaining on or in the zona pellucida of each oocyte was recorded and the results were expressed as the mean number of sperm per oocyte (Number of sperms penetrating the zona / Total number of oocytes inseminated) and the percent penetration ([Number of oocytes with sperm penetrating the zona / Total number of oocytes inseminated] × 100).

Statistical analysis: The differences in sperm parameter including zona binding between different cooling rates were analyzed by ANOVA method (SAS version 9.4, Cary, NC, USA).

III. Results

Post thaw semen characteristics for different cooling rates are given in table 2. The post-thaw semen characteristics were superior for slow freezing compared to medium and fast freezing ($P<0.01$).

A total of 1616 oocytes were recovered from 52 ovaries with a mean (± SE) yield of 31.07 ± 3.47 oocytes per ovary. The percentage and mean recovery rate of grade I, II and III oocytes per ovary were 25.9, 43.1 and 31.0 and 8.05 ± 1.00, 13.38 ± 1.79 and 9.63 ± 1.39, respectively.

The mean (± SE) number of fresh sperms bound to zona pellucida (Figure 2) was 6.63 ± 0.09 giving a penetration rate of 92.1 % which was significantly higher (< 0.01) than the mean (± SE) number of 1.68 ± 0.05 frozen thawed spermatozoa bound to zona pellucida with a penetration rate of 78.0%. The number of spermatozoa bound to zona pellucida and rate of penetration were significantly higher ($P < 0.01$) in fresh than frozen thawed spermatozoa.

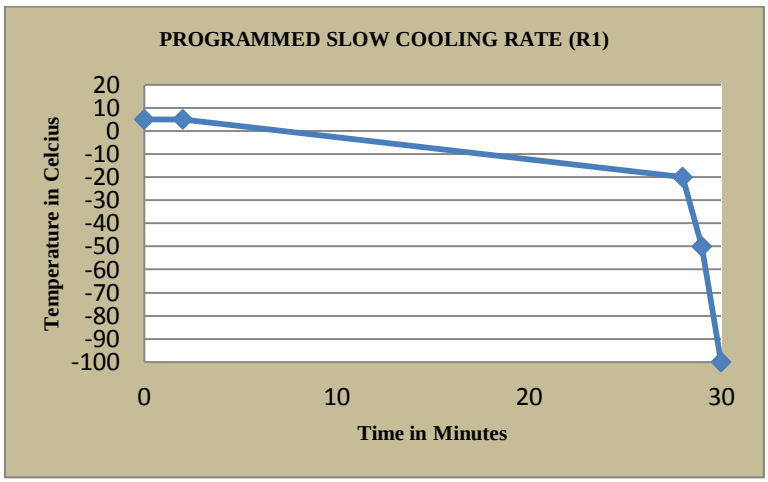
The penetration rate for slow medium and fast cooling were 79.9, 56.1 and 42.0 %, respectively. The penetration rate for slow freezing was greater compared to medium and fast freezing rates ($P<0.05$). The penetration rate for medium freezing was greater compared to slow freezing.

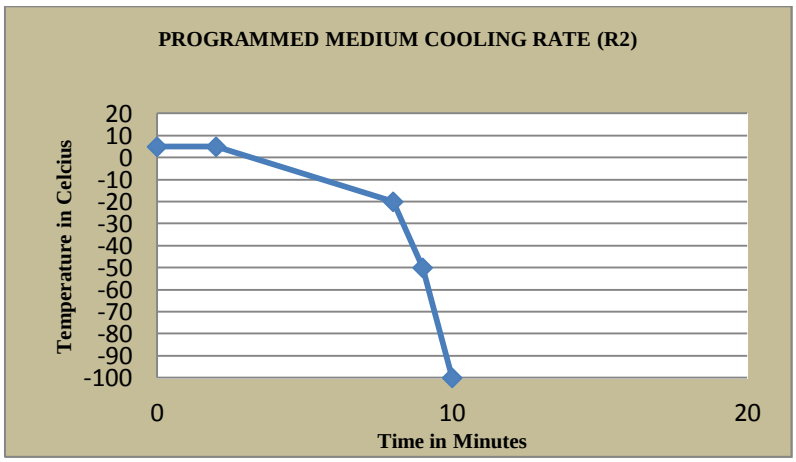
Table 1. Cooling rates for slow, medium and fast freezing methods

Method	Start temp	Hold	Rate 5 to -20 ° C	Rate -20 to -50 ° C	Rate -50 to -100 ° C
Slow	5 ºC	2 min	-1 ºC/min	-20 ºC/min	-30 ºC/min
Medium	5 ºC	2 min	-5 ºC/min	-20 ºC/min	-30 ºC/min
Fast	5 ºC	2 min	-10 ºC/min	-20 ºC/min	-30 ºC/min

Table 2. Post thaw semen characteristics for different cooling rates

Freezing method	Cooling rate	Post-thaw motility (%)	Live spermatozoa (%)	Abnormal spermatozoa (%)	Acrosomal integrity (%)	Plasma membrane integrity (%)	Penetration rate (%)
Programmable Freezing	Slow (n=18)	51.66^{a} ± 1.22	60.55^{a} ± 1.79	36.83^{a} ± 1.11	46.38^{a} ± 1.46	53.00^{a} ± 1.01	79.9
	Medium (n=18)	35.00^{b} ± 1.66	40.38^{b} ± 1.68	42.16^{b} ± 0.91	31.50^{b} ± 1.98	37.77^{b} ± 1.73	56.1
	Fast (n=18)	30.26^{b} ± 1.29	32.77^{b} ± 1.57	48.33^{c} ± 0.94	28.38^{b} ± 1.20	34.27^{b} ± 1.56	42.2
Conventional freezing	(n=18)	49.44^{a} ± 1.70	57.19^{a} ± 1.43	35.83^{a} ± 1.16	42.30^{a} ± 1.49	50.33^{a} ± 1.47	76.1





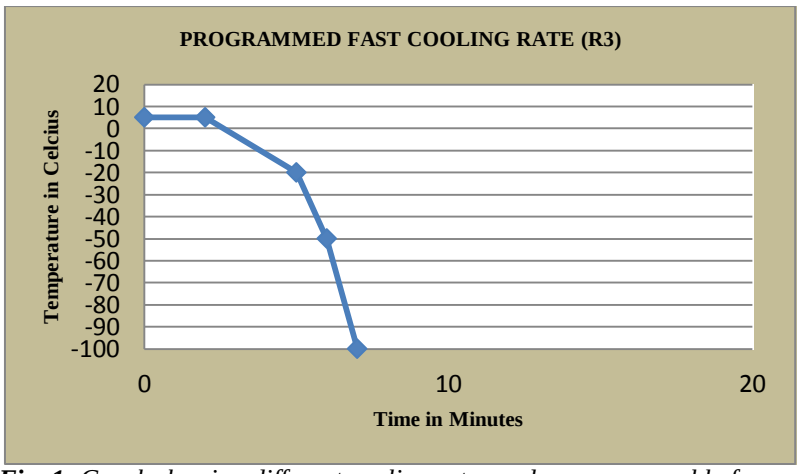


Fig. 1: *Graph showing different cooling rates under programmable freezer*
Plate 1a, Programmed slow cooling rate (R1); Plate 1b, Programmed medium cooling rate (R_2);
Plate 1c, Programmed medium cooling rate (R3)

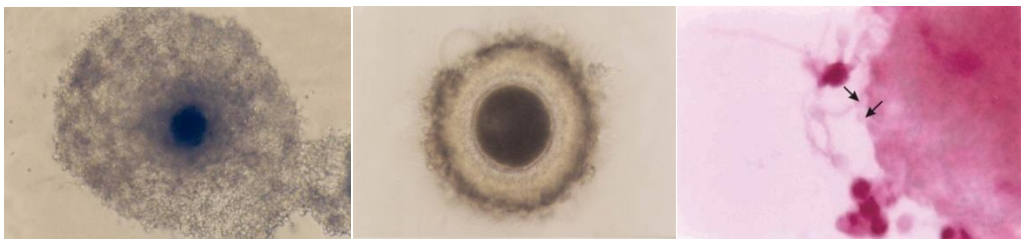


Fig. 2: *Binding of sperm with oocyte*
Plate 2a, matured canine oocyte with expanded cumulus cells; Plate 2b, Sperm-oocyte co-incubation; Plate 2c, binding of sperms with matured canine oocyte (Aceto-orcein staining).

IV. Discussion

Optimal cryopreservation can vary depending on the cryoprotective agent choice, cooling and freezing methods, and thawing rate.[15] The present work indicates a positive interaction of sub-zero slow cooling, whereas the inverse was true for medium or fast rates. It is possible medium to fast sub-zero cooling rates resulted in decreased sperm quality likely as a result of limited CPA exposure, insufficient dehydration and thus intracellular ice formation.

Motility is critical for sperm transport in the female reproductive tract to the site of fertilization and also is required for fertilization .[16] The motility following slow sub-zero cooling is greater than medium and fast cooling. Plasma membrane structure and integrity appear to be an important component associated with reduced motility of frozen-thawed sperm .[17] It is possible that during thermotropic phase transition, the medium and fast cooling induced changes in plasma membrane structure and integrity contributed to reduced motility. Further, cold shock-induced modifications are characterized by metabolic damage, which may negatively influence motility[18], as well as non-specific bilayer faults in membranes [19] that permit for wrong loss and gain of vital intracellular constituents such as calcium ions, nucleotides, antioxidants, and enzymes. Loss of adenine nucleotides depletes ATP stores and without ATP, motility ceases.

In this study, a mixed population of oocytes was retrieved from bitches at various stages of the estrous cycle, when follicles with different characteristics at different stage of development were present which may have accounted for the variation observed. Hewitt et al. (1998) reported that oocytes released directly from punctured follicles would provide a smaller and more uniform population, but it was impossible to puncture individual follicles, as they remain below the surface of the ovary and do not become apparent until a few days before ovulation[20].Further, the number of spermatozoa bound to zona pellucida and rate of binding were significantly higher for fresh than frozen-thawed sperm. Similar differences in the zona binding capacity of fresh and frozen thawed samples have been reported.[21] It should be noted that prolonged exposure to extenders with glycerol may result in toxic effects to spermatozoa and cause osmotic changes that impair the post-thawing sperm quality.[22]

The binding rate of spermatozoa to zona pellucida represents a critical event in gamete interaction and was predictive of sperm fertilizing potential. It should be noted that a positive correlation existed between zona penetration rate and in vitro fertility.[23] Calcium is a major mediator of sperm function; in which Ca2+ ATPase pumps maintain low ion concentration to allow correct signaling by calcium influxes, which are needed to initiate motility, hyperactivation, capacitation, acrosome reaction, and therefore fertilization *in vivo*.[24] An intracellular calcium accumulation has been reported during low temperature storage and this influx can be attributed to modified plasma, mitochondrial, and nuclear membrane interactions that introduce leakage and ion pump dysfunction. Further, membrane proteins that are associated with the lipid bilayer and their function may be expected to be altered, especially those that perform the function of transport channels for calcium absorption. The permeability of these channels is increased on cooling, affecting calcium regulation.[25] These facts have serious consequences for cell functioning and many changes may be incompatible with sperm viability. Therefore, it is critical to preserve the viability and motility of sperm using appropriate sub-zero cooling. It should be noted that cooling-induced calcium release triggered by compromised membranes may cause premature motility activation and subsequent depletion of ATP stores necessary to maintain motility post thaw. Intracellular calcium increase is also linked to loss of membrane integrity.[24] Based on our results; it is probable that slow transit through lipid phase transition temperatures achieved through slow cooling rates resulted in the least amount of membrane damage and thus leakage of cellular components essential to sperm function.

V. Conclusion

In conclusion, canine sperm fertility was greater following cryopreservation of canine semen using slow subzero cooling compared to medium or fast subzero cooling. Zona pellucida binding assay could be used as a valuable in vitro tool for assessing the fertilizing capacity of both fresh and frozen-thawed canine spermatozoa.

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