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Different Cooling Rates for Cryopreservation of Semen in Various Livestock Species: A Review

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A B S T R A C T

Artificial insemination is the most valuable tool for the dissemination of superior germplasm and control of venereal diseases. To accomplish this task, successful and effective cryopreservation of semen is imperative. Semen cryopreservation facilitates its transportation from long distances and use of semen even after the death of sire. However effective cryopreservation of semen of many domestic animals is still not achieved because large proportion of sperms does not survive after freezing and thawing process. During cryopreservation of semen, sperms are damaged if semen is not cooled with optimum cooling rates. The objective of this review is to summarize and compare the work on effects of cooling rates and critical temperature range on post thaw semen quality of different animals. It could be concluded from the present review that optimum freezing protocol for buffalo bull, sheep, pig and stallion is not standardized, so far. However, till to some extent success has been achieved in cattle bulls.

Introduction

It has long been known that cooling bovine, ovine, caprine, swine stallion semen too rapidly between 30°C and 0°C induces a lethal stress in sperms proportional to cooling rate and temperature range (Watson, 1981). Eight times more cryopreserved sperms are required as compared to fresh sperms to achieve fertilization *in vivo* (Shannon and Vishwanath, 1995). It is generally accepted that at least 50% of sperm die during the freezing and thawing procedures (Watson, 2000) which is known as cold shock. Fast cooling of semen from 30 to 4^0C leads to injuries known as “cold shock” (Gilmore *et al.,* 1998; Watson, 2000). There are large interspecies differences with respect to the sensitivity of spermatozoa to cold shock; for example, ram and boar spermatozoa are very susceptible, whereas human spermatozoa are relatively resistant (Watson, 1981; Holt and North, 1984; De Leeuw *et al.,* 1990; Drobnis *et al.,* 1993). For successful semen

cryopreservation, the composition of an extender and optimal freeze rate play important roles to minimize extra- and intra-cellular stresses (Hammerstedt *et al.,* 1990; Curry *et al.,* 1994). During cooling, temperature change induces stress on sperm membranes which causes phase changes in lipids and altered functional state of sperm membranes. Such stresses on the membranes may be continued below 0°C since phase changes are not complete at 0°C. It is well known that a major phase change occurs near 5° to–15°C (Drobnis *et al.,* 1993) and this may be the prime temperature range for temperature dependent injury. An optimum freeze rate must be slow enough to allow water to leave the cells to prevent intracellular ice crystal formation, and fast enough to avoid severe cell dehydration and the solution effect (Mazur, 1970). Polge (1957) for the first time reported that a critical temperature zone between -15 and -30°C is responsible for exerting most of the damage to spermatozoa and if the cooling rates were not optimal, all the cells might be damaged by -80°C.Noiles*et al.,* (1995) observed a temperature-dependent discontinuity of the water permeability of mouse sperm plasma membranes between 4 and 0^0C. These data suggest that a membrane-phase transition occurs between 4 and 0^0C in mouse sperm during the freezing process. It is generally recognized that the duration of exposure to such events should be minimized for optimal cell survival implying that the cooling rate should be rapid. However, the cooling rate must be slow enough to allow water to leave the cells by osmosis preventing intracellular ice formation which is lethal. Sperm cells are generally frozen at quite rapid rates in the range 15–60°C/min, which have been empirically determined as giving the best survival rates, Watson (2000).

There are two main temperature ranges wherein damage to sperm take place during freezing: the period of supercooling (0°C to -5°C) and the formation of ice crystals (-6°C to -15°C) (Woelders *et al.,* 1997). Further, Mazur (1965) has opined that damage to sperm membranes occurs during the temperature range of -15°C to -60°C during freezing and thawing, which is called as critical temperature range. Various scientists have worked on critical range to improve post thaw motility and viability with contradictory results. Kumar *et al.,* (2003) has defined the critical temperature range between 5 to -50 °C, when ice crystal forms and consequently cell dehydration resulting into sperm damage under extreme situations (Table 1 and 2).

Cooling rates for buffalo bull semen

Buffalo spermatozoa are more susceptible to damages during freezing than cattle spermatozoa (Raizada *et al.,* 1990). These damages can be minimized by optimizing the cooling and freezing rates and using appropriate diluting media (Kumar *et al.,* 1992). Dhami*et al.,* (1996) compared four cooling protocols using Tris-citric acid-fructose-yolk-glycerol extender in Murrah buffalo bull semen (10 to 5°C in 1-2hours; 30 to 5°C in 1-2 hours) and evaluated post thaw motility at thawing rate of 40°C/min. It was observed that post thaw motility was similar (35, 36, 41 and 41%) under both the conditions. Talevi*et al.,* (1994) studied the slow (28°C to 5°C in 1 hour) and rapid cooling (28°C to 5°C in 15 minutes) protocols for buffalo bull semen and no significant differences in post-thaw motility of spermatozoa was observed. Dhami and Sahni (1994) compared different cooling protocols for Murrah buffalo bull semen (30°C to 5°C in 60 and 120 min) and found the later protocol more advantageous and yielded highest fertility rate (68.1%) upon insemination. On the other hand, Ramakrishnan and Ariff (1994) did not found any significant difference in post thaw motility by using different cooling rates (room temp to 5^0C in 45, 65 and 90 min). Del Sorbo*et al.,* (1995) examined two freezing

protocols for buffalo bull semen; a) In step wise cooling in which the straws were exposed to temperatures of 5°C for 4 min, -15°C for 7 min, -80°C for 15 min and -130°C for 15 min and then plunged into liquid nitrogen; b) In continuous cooling, temperature decreased from 5°C to -30°C @ 20°C/min, from -30°C to -100°C @15°C/min, then standing for 5 min at -100°C, before immersion into liquid nitrogen. The post-thaw recovery rates were better for ''step-wise'' than for continuous cooling method. Sukhato *et al.,* (2001) studied the effects of three cooling rates (10, 20 or 30°C ⁄ min) from 4°C to each of either -40°C, - 80°C or -120°C before plunging into liquid nitrogen. on post thaw motility and fertility of buffalo bull spermatozoa. It was found that cooling rates of 20 or 30°C ⁄ min yielded better progressive post thaw motility and fertility. Bhosrekar *et al.,* (1994) compared the conventional freezing method (over liquid nitrogen in static vapours for 10 min) and programmable freezing methods for buffalo bull semen. It was found that freezing @ 17.32°C⁄ min between 4°C and -40°C with programmable freezer produced better quality frozen semen than the conventional method of freezing. Rasul (2000) studied the effects of freezing rates on post-thaw viability of buffalo spermatozoa extended in Tris–citric acid-based extender. The freezing rates examined between 4 and -15°C were 3 or 10°C⁄ min, whereas the freezing rates investigated between -15 and -80°C were 10, 20 or 30°C⁄ min. It was concluded that the different freezing rates tested gave comparable results in terms of post-thaw spermatozoa viability. Dhami *et al.,* (1992) and Ali (2001) reported that a slow cooling rate (30 to 5ºC in 2hrs) of diluted bovine semen was necessary for successful freezing with good fertility. Contrary to this, Weidler and Zaugg (1975) and Dhami and Sahni (1994) have suggested that fast pre-freeze cooling (30 ºC to 5ºC in less than 2 h) to freeze buffalo semen without affecting its freezability. Andrabi (2009) outlined the cryogenic procedures for buffalo semen as; cooling from 37 or 39 to 4^0C at the rate of 0.2–0.4^0C⁄ min, equilibration, at least 2 h at 4^0C, freezing of straws approximately 4 cm above liquid nitrogen for 10–20 min, or by the fast-freezing rates (programmable freezing), and thawing at 45–60^0C for at least 15 seconds. Previously, Dalal *et al.,* (2016) studied three different programmable namely protocol A (4 to -10°C @ -05 °C/min, -10 to -100°C @ -40 °C/min and -100 to -140°C @ -20 C/min), protocol B (4 to -10°C @ -05, -10 to -40°C @ -60 °C/min -40 to -100 °C @ -40°C/min and -100 to -140°C @ -20°C/min and protocol C (4 to -12°C @ -04 -12 to -60°C @ -40°C/min and -60 to -140°C @ -50°C/min). In this study, we used twenty-one ejaculates from three Murrah buffalo bulls (7 ejaculates per bull) and extended semen was frozen according to three different freezing protocols as described above using a bio-freezer. Post thaw semen samples were evaluated and the average percentage of individual motility, progressive motility, viability, sperm membrane integrity and total sperm abnormalities was similar (p>0.05) in three protocols freezing protocols. In all three protocols, freezing rates were similar in the critical range. It might be the reason for similar outcome of post-thaw parameters. Recently, in an extensive study conducted by Dalal *et al.,* (2018) with different cooling rates applied in critical temperature zone revealed that best post thaw semen quality was obtained with cooling rate at -30 ^{0}C /min, -40^0C/50 ^{0}C /min and -50 ^{0}C /min in the between 4 ^{0}C to-15 ^{0}C, -15 ^{0}C to -60 ^{0}C and -60 ^{0}C to -140^0C respectively.

Cooling rates for cattle bull semen

Januskauskas *et al.,* (1999) studied the effects of two different cooling rates on post thaw motility of Swedish dairy bull semen. Semen was extended in Triladyl and was cooled

within 20 min to 19°C to 21°C and further cooled to 4°C at 4.2°C/min (fast) and at 0.1°C/min (slow) cooling rates and equilibrated at 4°C for 4 hours. Further, cooling was done to -10°C at a rate of 4°C/min and from -10°C to -150°C at a rate of 40°C/min. No difference was observed in post thaw motility using fast and slow cooling rates.

Woelders (1997) studied the effect of cooling rates between -10 and -70^{0}C in the four freezing protocols -40, -76, -140, and -300^{0}C/min on viability of bull sperm and found that cooling rate was found to be between 76 and 140^{0}C/min as optimum. Anzar*et al.,* (2011) studied the effects of three cooling rates between -10 and -80°C (-10°C, -25°C, and -40°C/min) on post thaw motility of bull spermatozoa and found similar post thaw motility. Rodriguez *et al.,* (1975) studied the effects of cooling rates on survival of beef bull spermatozoa. Straws were cooled from 5^{0}C to -130^{0}C in 3.5min (fast cooling), in 20 min (moderate cooling) and in 40 min (slow cooling). It was found that fast cooling resulted into better post thaw motility as compared to others. Kumar *et al.,* (2003) studied the effects of cooling rate on cryo-survival of bull and ram spermatozoa using biofreezer. Semen was extended at 22°C and cooled to 5°C in 90 min (0.2°C/min) and from 5°C to -5°C @ 5°C/min. From -5°C to -50°C, semen was cooled using three cooling rates (1, 30, and 50 °C/min) and then held for 5 min before plunging into liquid nitrogen. Post thaw motility in bull semen was observed as 27.5, 38.3 and 38.3% and viability as 49.6, 50.2 and 50.3% at 1°C/min, 30°C/min and 50°c/min, respectively. Post-thaw motility in ram semen was observed as 15.8, 15.8 and 25.8% and viability as 31.2, 45.7 and 34.8% at 1°C/min, 30°C/min and 50°c/min, respectively. It indicates post thaw motility is highest at 50°C/min and viability is highest at 30°C/min. It is worth mentioning that for cattle bull semen currently a freezing rate of ≥40°C is practiced in general for cryopreservation during the critical temperature zone (Anzar *et al.,* 2002).

Cooling rates for ram semen

Salamon and Maxwell (1995) reported that in the ram spermatozoa, maximum damage occurs between -10°C and -25°C which is the region of ice crystallization. Post-thaw motility in boar semen was observed as 14.2, 21.3 and 10% and viability as 36.7, 43.2 and 38% at 1°C/min, 30°C/min and 50°C/min, respectively. It indicates that post thaw motility and viability is highest at the cooling rate of 30°C/min. Byrne *et al.,* (2000) compared the effects of two freezing rates @ -5°C/min (fast) and @ -0.5°C/min. (slow) through critical temperature zone (0°C to -25°C) on the fertility of ram spermatozoa *in vivo* and *in vitro*.

Semen frozen with fast cooling rate yielded higher cleavage rate (57% vs. 26%) and more blastocyst per oocyte (28% vs. 13%) than the semen with slow cooling rate. Insemination with semen frozen with fast cooling rate resulted in a significantly higher pregnancy rate. So, these observations indicate that the post thaw recovery of sperm depends on species and cooling rates. The optimum sperm freezing rate has been reported amongst distinct species, −50^{0}C to −100^{0}C/min for bulls (Woelders *et al.,* 1997), −15 ^{0}C/min to −60 ^{0}C/min for ram (Byrne *et al.,* 2000; Anel *et al.,* 2003) and −30^{0}C/min to −60^{0}C/min for boars (Fiser and Fairfull,1990).Cooling rate within (5^{0}C to –100°C) results in less intracellular dehydration, less intracellular solute concentrations and less shrinkage of the cells (Mazur, 1984; Woelders, 1997). The best survival of human spermatozoa is obtained when the cooling rate from room temperature to 4/5°C is of 0.5 to 1.0°C/minute (Henry *et al.,* 1993).

Table.1 Summary of critical temperature range reported by various authors

S.No	Authors	Critical temperature range
1	Drobnis*et al.,* (1993)	5°C to -15°C
2	Woelders*et al.,* (1997)	0°C to -15°C
3	Mazur (1965)	-15°C to -60°C
4	Kumar *et al.*(2003)	-5°C to -50°C
5	Mazur(1970)	-10°C to -50°C
6	Marshall (1984)	-5°C to -50°C
7	Polge(1957)	-15°C to -30°C

Table.2 Summary of optimum cooling rates reported by various authors

S.No	Author	Species	Optimum cooling rates
1	Dalal *et al.*(2018)	Buffalo bull	-30 °C/min (4 °C to -15 °C), -50 °C/min (-15 °C to -140 °C)
1	Woelders*et al.*(1997)	Bull	50^0C/min to −100^0C/min
2	Anel*et al.,* (2003); Byrne *et al.*(2000)	Ram	−15 ◦C/min to −60 ◦C/min
3	Fiser and Fairfull, (1990)	Boars	−30^0C/min to −60^0C/min

In experiments with the spermatozoa of the bull, ram, stallion and boar it has been found that during slow cooling to -79^0C, there is a critical temperature range between -15^0C and -25^0C at which the greatest amount of damage occurs (Polge, 1957).

Cooling rates for boar semen

Medrano *et al.,* (2002) conducted series of experiments using were set up using 20 ejaculates from 13 boars to investigate the effect of different cooling rates on boar sperm cryosurvival using cryomicroscopy. The cooling protocols were split into two stages: (i) from +5 °C to -5 °C and (ii) from -5 °C to -50 °C. Cooling rates in the range 3 °C/min to 12 °C/min did not cause significant damage to the sperm plasma membrane between +5 °C and -5 °C; however, spermatozoa cooled at 24 °C/min to -5 °C were slightly damaged. Cooling rates in the range 15 °C/min to 60 °C/min did not produce differences in sperm cryosurvival during freezing between -5 °C and -50 °C, or after thawing. In addition, cooling rates in the range 3 °C/min to 80 degrees C/min did not produce significant differences in sperm cryosurvival. However, slow freezing i.e. 3°C/min induced a slight increase in the percentage of plasma membrane-damaged spermatozoa (propidium iodide-positive) at -50 °C. Fiser (1990) recommended optimal rate for boar semen is 30^0 C/min this was specifically tested as one

of the protocols. All samples were rewarmed at 60°C/min.

Cooling rates for stallion semen

Devireddy *et al.,* (2002) reported that optimization of equine sperm cryopreservation protocols requires an understanding of the water permeability characteristics and volumetric shrinkage response during freezing. They employed cell shape-independent differential scanning calorimeter (DSC) technique was used to measure the volumetric shrinkage during freezing of equine sperm suspensions at cooling rates of 5^0 C/min and 20^0 C/min in the presence and absence of cryoprotective agents (CPAs). By fitting a model of water transport to the experimentally obtained DSC volumetric shrinkage data, the best-fit membrane the reference membrane permeability at a reference temperature T_R= 273.15 K; and apparent activation energy (kJ/mol) or the temperature dependence of the cell membrane permeability were determined. On the basis of these parameters and mathematical simulation they concluded that the optimal cooling rate for equine sperm in the absence of any CPA is 29^0 C/min and is 60^0 C/min in the Kenney extender with CPAs. In another study, Oldenhof *et al.,* (2017) investigated the interplay between cooling rate and protectant concentration for cryopreservation of stallion sperm. Glycerol (GLY), ethylene glycol (EG), dimethyl formamide (DMF), propylene glycol (PG), and dimethyl sulfoxide (DMSO) were tested as cryoprotective agents (CPAs), using concentrations up to 1500mM and cooling rates ranging from 5^0 C to 55^0 C/min through modeling and experimental approach. They found that maximal survival of stallion sperm was obtained using intermediate concentrations (250-500 mM) combined with higher cooling rates ($26\text{-}53^0$ C/min), with GLY being more effective compared with EG. PG and DMSO appear to be less effective as CPAs for stallion sperm.

Cooling rates for rabbit semen

Maeda *et al.,* (2012) studied the effect of primary cooling rates on the motility and fertility of frozen-thawed rabbit spermatozoa. Rabbit semen diluted with an egg-yolk acetamide extender was cooled from room temperature to 5°C at 4 different rates (–0.1, –0.2, –0.4, –0.8°C/min) and then semen was frozen in liquid nitrogen vapour. After thawing, sperm cooled at –0.1°C/min showed the highest motility (40.7±7.3%); there were no significant differences between the motilities of the –0.1, –0.2, and –0.4°C/min groups. Niasari-Naslaji *et al.,* (2007) studied effects of cooling rates on semen cryopreservation in Bactrian camel (*Camelus bactrianus*) using Shotordiluents (Tris based extender, with the osmolality of 330 mOsm/kg and pH of 6.9, favors the short-term preservation of the Bactrian camel spermatozoa under chilled condition). Extended semen was kept in conical flasks at 37°C in water bath and gradually cooled to 4°C in 4 hours (0.14 °C/min: average slow cooling rate). The other cooling rate was 4°C in 1 hour (0.55°C/min: average fast cooling rate). It was observed that fast cooling protocol was better than slow cooling.

In conclusion, each species sperm has different membrane composition in terms of cholesterol, phospholipids and various protein domains which interact differently with environment in which they are suspended therefore have different phase transition temperature. Infect, there are animal to animal variation in sperm membrane composition that depends on nutrition and hormonal milieu in the spermatogenesis environment. So, each species sperm has their own optimum freezing protocol. However, over the globe in routine, liquid nitrogen freezing is being used

for cryopreservation of various species sperm. We discourage it and recommend the programmable freezing with optimal cooling rates.

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