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Effect of cooling rate and equilibration time on pre-freeze and post-thaw survival of buck sperm ☆

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ABSTRACT

Survival of buck sperm is affected due to duration and temperature of stages of refrigerated or frozen storage. This study investigated interactive effect of cooling rates (moderate; MC and rapid cooling; RC); and equilibration times (0, 2, 4 and 8 h) on survival before freezing at 4 °C and post-thaw quality of buck sperm. Semen was collected (three Beetal bucks; replicates = 6), pooled and diluted with Tris-citrate extender. Pooled semen samples were subjected to either RC (−2.2 °C/min) or MC (−0.3 °C/min) from 37 °C to 4 °C in separate aliquots and further equilibrated at 4 °C for 8 h. Semen was frozen using standard procedure after completion of each equilibration period i.e. 0, 2, 4 and 8 h. Semen was evaluated for motility, viability, plasma membrane integrity (PMI) and normal apical ridge (NAR) before freezing and after thawing. The survival time (time for survival above threshold limit i.e. 60%) at 4 °C, of motility and PMI was observed 5 and 6 h respectively in RC group while >8 h in MC group. Rate of decline (slope) in motility and viability was higher ($P < 0.05$) in RC overtime during equilibration at 4 °C while PMI and NAR declined at equal rate in both cooling groups. Post-thaw motility and NAR were higher ($P < 0.05$) in MC when equilibrated for 2–8 h while viability and PMI of RC was observed equal to MC group. In conclusion, survival of buck sperm is higher when cooled with moderate rate. However, RC can maintain post-thaw sperm viability and PMI equal to MC when equilibrated for 2–8 h. The methods should be explored to maintain motility and NAR during rapid cooling of buck sperm.

Introduction

The preservation of male germplasm through process of cryopreservation potentiates the transfer of improved genetics to wide geographical regions, duration and across the breeds. The success of breeding programs, through artificial insemination (AI), depends upon quality of cryopreserved semen. The storage of sperm through cryopreservation deteriorates the structural and functional capacity of sperm [10,20]. At least, 50% sperm get destructed during process of freezing and thawing [2,37]. It is necessary for sperm to possess at least 60% motility and viability before freezing which are major determinants of success of cryopreservation procedure, to acquire 30% progressive motility [2,5] and 10–20 million viable sperm after thawing for optimum fertility through AI in goats [20]. The well defined processes of extension of semen with suitable diluents [21], proper cooling rate, equilibration time, and freezing rate are key elements to develop suitable and species specific protocol of cryopreservation [35] and ensure optimal post-thaw quality of semen in ram [33], bull [39] and buck [20].

Cooling rate of semen from temperature of dilution (37 °C) to 4–5 °C, determines the success of freezing protocols in terms of sperm survival [17] which depends upon most appropriate cooling rate; balanced homeostasis and metabolism [23]. Mechanism of cooling lowers down the metabolic activity of sperm cells. Improper cooling rate instigates temperature shock and causes membrane disruption due to structural disorganization of proteins, disturbance of ion channels, production of reactive oxygen species (ROS) and loss of mitochondrial membrane potential [17,23,36]. Various cooling regimens have been investigated in different species, mainly considering as "rapid cooling", "moderate cooling" and "slow cooling" processes. Even with slow cooling, sperm cell face thermal stresses and become compromised. The extent of damage depends upon rate of lowering of temperature [36]. Rapid cooling to 0–4 °C creates a lethal state for sperm [9], known as "cold shock" [12] which induced osmotic and oxidative changes

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resulting in disturbance in chemical and physical functions of cell; causing imbalanced homeostasis and up regulation of ROS. The intensity of these deleterious alterations depends upon composition of cell membrane as less ratio of polyunsaturated fatty acids makes the sperm more resistant to cold shock [6] and have been explored in multiple species [8,20]. However, the response of buck sperm to cooling regimes has not been explored yet in detail.

The time during which cryoprotectants, penetrate within sperm cell is known as "equilibration time" and more specifically "glycerolization time" because glycerol is more widely used cryoprotectant. It follows stage of cooling during cryopreservation [21]. Equilibration time affects the sperm survival after freeze–thawing. Multiple equilibration times have been examined in wide variety of species. Generally, it is claimed that 2–4 h is most suitable duration for cryoprotectant to penetrate [40] but it also depends upon type and concentration of cryoprotectant [18]. It has been investigated earlier that equilibration time exceeding 4 h can be harmful to sperm [15]. It has been further manifested that this duration in combination with rate of cooling affects the sperm survival. [27]. This relationship of cooling and equilibration time is important to develop the strategy for steps and timing of stages of cryopreservation. This interactive effect is still needs to be explored during buck semen cryopreservation regimes.

Therefore, the present study investigated the effect of cooling rates (moderate and rapid) and equilibration times (0, 2, 4 and 8 h) on survival and change in sperm parameters before freezing (pre-freeze) and post-thaw quality of Beetal buck sperm.

Materials and methods

Semen collection and initial evaluation

Three mature bucks (Beetal breed, 2–3 years of age), stationed at animal shed of University of Veterinary and Animal Sciences (31°34/N, 74°17/E), were used for semen collection and were provided uniform housing and lighting conditions. They were fed with green fodder and concentrate according to body requirement. Semen was collected (replicates = 6) using artificial vagina (IMV France; temperature 42 °C) twice a week from each buck during breeding season (October–December). Each ejaculate was transferred to water bath maintained at 37 °C and evaluated for sperm motility and concentration. The ejaculates possessing >65% progressive motility and $>2.5 \times 10^9$ sperm/mL, were pooled (three ejaculates per replicate) by bucks to eliminate buck-to-buck variation and to conduct sperm evaluation on time with precision. Each pooled ejaculate was considered as one replicate.

Semen processing

Each pooled semen sample was extended using Tris-based extender (pH 6.8) containing Tris (2.42 g/100 mL; MP Biomedicals LLC, Illkirch, France), citric acid monohydrate (1.4 g/100 mL; Merck, Darmstadt, Germany), egg yolk (20 mL/100 mL), fructose (1 g/100 mL; Sigma–Aldrich Chemicals, St. Louis, MO, USA), glycerol (7 mL/100 mL; BDH Laboratory Supplies, Poole, England), and antibiotics (benzyl penicillin 1000 i.u./mL and streptomycin sulfate 100 μg/mL, Sigma) at 37 °C [31] to a final concentration of 50×10^6 sperm/mL. Diluted semen was split into two aliquots for cooling to 4 °C at two rates i.e. moderate (MC) and rapid cooling (RC) group. Semen in glass tubes of both aliquots were placed in water holding beaker separately which were placed in cooling cabinet. The temperature was lowered from 37 °C to 4 °C in ~90 min in MC group (−0.3 °C/min) while in RC group; the decrease in temperature was accelerated by adding standard size ice cubes at constant rate in water surrounding the semen tube so that temperature lowered to 4 °C in ~15 min (−2.2 °C/min). This constant lowering of temperature was standardized by continuous recording of temperature with thermometer placed in semen tubes. The same procedure was applied to each replicate. After completion of cooling period, both groups were subjected to equilibration at 4 °C for 0, 2, 4 and 8 h in the same cooling cabinet. This equilibration at 4 °C was also considered as chilled storage and its data were used for pre-freeze (before freezing) survival (at 4 °C for 8 h) analysis. At end of each equilibration time, one fourth of semen volume (as there were four freezings for each cooling group subjected to four durations of equilibration) was filled into French semen straws (0.5 mL, IMV, France), subjected to vapor freezing (3 cm above liquid nitrogen surface for 7 min) and plunged into liquid nitrogen. Post-thaw analysis was performed after 1 week of freezing. Frozen semen in two straws (per replicate for each equilibrated group of MC and RC group) was thawed at 37 °C for 30 s and pooled in glass tube to minimize possible straw-to-straw variation. Each semen sample of MC and RC group was evaluated after extension, after completion of cooling, at pre-freezing after completion of 0, 2, 4 and 8 h of equilibration for respective group, and after freeze–thawing (post-thaw) of each equilibrated group for progressive motility, intactness of plasma membrane (viability), plasma membrane integrity (PMI) through hypo-osmotic swelling test (HOST) and intact acrosome (normal apical ridge; NAR) as given below.

Semen assays

Progressive motility

Motility was determined subjectively using a phase-contrast microscope (20× objective lens; BX51, Olympus, Japan) with a warm stage maintained at 37 °C. A wet mount was prepared by placing a 10 μL drop of semen at a glass slide which was covered with a cover slip (22 × 22 mm). Motility was assessed in five different microscopic fields of slide. The average of five fields was recorded as final motility. Rectilinear forward movement of sperm was considered as progressive motility.

Plasma membrane integrity (PMI)

Functional integrity of plasma membrane of sperm was determined using a hypo-osmotic swelling test (HOST) [3]. The HOS solution (190 mOsmol/kg) was prepared by dissolving tri-sodium citrate (0.75 g; Merck, Darmstadt, Germany) and fructose (1.351 g; Sigma–Aldrich Chemicals, St. Louis, MO, USA) in 100 mL de-ionized water. It was performed as; 50 μL of each semen sample was mixed with 500 μL of HOS solution and incubated at 37 °C for 30 min. After incubation, a 5 μL drop of prepared sample was examined under phase-contrast microscope (400×) at 37 °C. A minimum one hundred sperm were counted for their swelling ability in HOS solution. The sperm characterized by coiled or swollen tail of varying degree were considered to have biochemically active plasma membrane.

Acrosomal integrity

Intact acrosome was determined using normal apical ridge (NAR) assay [7]. It was performed using formol citrate solution which was prepared by dissolving tri-sodium citrate (2.9 g; Merck, Darmstadt, Germany) in 1% solution of formaldehyde (stock solution 37%; Merck, Darmstadt, Germany). A 500 μL of each semen sample was fixed in 50 μL of formal citrate solution. Two hundred sperm were counted for normal, loose and damaged acrosome using a phase contrast microscope (1000×, BX 51, Japan). Crescent shaped acrosome attached with head were considered as normal. Data of normal acrosome only was used for analysis.

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