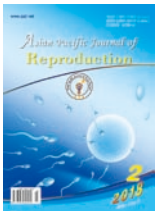

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Improvement in cryosurvival of buffalo bull (*Bubalus bubalis*) sperm by altering freezing rate within critical temperature range

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ABSTRACT

Objective: To optimize the cryopreservation of buffalo bull semen by altering freezing rates within critical temperature range (4 ℃ to -60 ℃). **Methods:** A total of 20 ejaculates each from 5 Murrah buffalo bulls were cryopreserved using programmable biofreezer in 2 phases. In the 1st phase, 9 freezing rates were applied at -2, -5, -10, -20, -30, -40, -50, -60 or -4 ℃/min (control) from 4 ℃ to -15 ℃; at -40 ℃/min from -15 ℃ to -60 ℃. In the 2nd phase, a fixed freezing rate was applied at -30 ℃/min from 4 ℃ to -15 ℃. Six freezing rates were applied at -10, -20, -30, -40 (control), -50 or -60 ℃/min from -15 ℃ to -60 ℃. The freezing from -60 ℃ to -140 ℃ were fixed at -50 ℃/min in both the phases. Post thaw semen quality was assessed in terms of motility, viability, membrane integrity (hypo-osmotic swelling test), sperm abnormalities, and active mitochondria. Data were arc sine transformed and analyzed through one-way analysis of variance using SPSS software. **Results:** In the 1st phase, percent individual motility, progressive motility and viability were similar among various protocols. Percent hypo-osmotic swelling reactive sperm was higher with freezing at -30 ℃/min. In the 2nd phase, percent individual motility, viability and hypo-osmotic swelling reactive sperm was higher with freezing at -50 ℃/min. Sperm head abnormalities were lower at -30 ℃/min in the 1st phase, but were similar among the protocols of the 2nd phase. Percent active mitochondria were higher at -30 ℃/min in the 1st phase and at -50 ℃/min in the 2nd phase. **Conclusions:** The optimum post thaw semen quality of buffalo bull could be obtained by applying freezing rate at -30 ℃/min (4 ℃ to -15 ℃) and at -50 ℃/min (-15 ℃ to -140 ℃), followed by plunging of straws in into liquid nitrogen for storage.

1. Introduction

Semen cryopreservation is a widely used technique across the world for conservation and proliferation of genetically superior germplasm through artificial insemination[1,2]. The process of semen cryopreservation is stressful[3] and around 40%–50% of sperm lose their motility and viability due to loss of structural and functional capabilities[4]. One of the factors which affect post thaw survival of sperm is the freezing rate. Maximum damage to sperm during cryopreservation takes place within the critical temperature range, which is categorized by various authors as: period of super

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cooling (0 ℃ to -5 ℃) and period of ice crystal formation{(-6 ℃ to -15 ℃)[5], (5 ℃ to -60 ℃)[6–12] , (-5 ℃ to -50 ℃)[13]}. During this phase, ice crystals form resulting into cell dehydration lead to sperm damage[14]. On the basis of critical temperature range, maximum damage to bull sperm has been reported from 5 ℃ to -50 ℃[14,15]. The freezing protocol for cattle bull semen is well optimized as: from 4 ℃ to -12 ℃ at -4 ℃/min, from -12 ℃ to - 40 ℃ at - 40 ℃/min, and from -40 ℃to -140 ℃ at -50 ℃/min, before plunging into liquid nitrogen[16]. Similarly, in another protocol, the cooling rate from 4 ℃ to -10 ℃ at - 5 ℃/min, from -10 ℃ to -100 ℃ at - 40 ℃/min, and from -100 ℃ to -140 ℃ at -20 ℃/min, before plunging into liquid nitrogen has been reported[17]. However, no such established freezing protocol is available for buffalo bull semen. Buffalo bull sperm are more prone to damage during cryopreservation as compared to cattle bull. One of the reasons behind excessive cryo-damage to buffalo bull sperm is the difference in the ratio of membrane phospholipid[6,18]. Among total sperm membrane phospholipids, the level of phosphatidylcholine (unsaturated) and phosphatidylethanolamine (saturated) constitutes approximately 66% and 23%[19] against only 50% and 10% in cattle bull, respectively[20]. Further, the glutathione concentration, an antioxidant in buffalo bull semen is less as compared to cattle bull semen. These factors result in more lipid peroxidation induced damage to buffalo bull sperm[21], resulting in poor post thaw survival[22,23]. The present study was to evaluate the various freezing rates in critical temperature range to improve post thaw semen quality of buffalo bulls.

2. Materials and methods

2.1. Animal care

The approval from the Institutional Animal Ethics Committee to carry out this study was not required as no invasive techniques were applied on animals. Semen was being collected, evaluated and frozen by using standard methods under progeny testing program.

2.2. Selection of buffalo bulls

The study was carried out on five Murrah buffalo bulls (Bubalus bubalis) aged around 4 years, being maintained at bull station, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India (30.55 °N, 75.54 °E), under similar conditions of nutrition and management. These bulls were under progeny testing program and were being used for semen collection by artificial vagina method. Bulls were being maintained under loose housing system (covered area 12 ft × 10 ft and uncovered area 25 ft × 10 ft) and standard feeding schedule along with adlib green fodder.

2.3. Preparation of semen extender

Tris egg yolk extender was prepared fresh on the day of semen collection by using following chemicals: Tris (Tri hydroxy methyl amino methane, Cat # BC0195A-2.5KG, Biochem Life Sciences), citric acid (Cat # BCO0055A-1KG, Biochem Life Sciences), gentamicin (REF 15710-064, Gentamicin Reagent Solution, Gibco, Life Technologies); glycerol (Cat # 20118, SD Fine Chem. Limited). Extender was kept in water bath at 35 ℃ till use.

2.4. Semen processing

A total of 20 ejaculates were collected each from five buffalo bulls and semen was evaluated for mass and individual motility using computer assisted semen analyzer (IVOS, IMV Technologies, France). Concentration and dilution ratio was calculated by using Bovine photometer and Automatic dilutor (IMV technologies, France). Semen was extended in tris egg yolk extender at 35 ℃ till final sperm concentration at 80 million per mL was achieved. Extended semen having individual motility more than 90% was used for further processing. Packaging of semen was done in 0.25 mL French mini straws. Filling, sealing and printing of straws were done with integrated filling, sealing and printing machine attached to a Domino printer (IMV Technologies, France). Straws were kept in cold handling cabinet (IMV Technologies, France) for equilibration at 4 ℃ for 4 h. Twenty straws thus produced were earmarked for each freezing protocol mentioned as follows.

2.4. Alterations in freezing rates within critical temperature range (4 ℃ to – 60 ℃)

2.4.1. First phase: Variable freezing rates during 4 ℃ to –15 ℃

Twenty straws per protocol were frozen by following 9 different freezing protocols (P11, P12, P13, P14, P15, P16, P17, P18 and control) in the 1st phase having freezing rates -2, -5, -10, -20, -30, -40, -50, -60, and -4 ℃/min, respectively, in the temperature range of 4 ℃ to -15 ℃. In these protocols, freezing from -15 ℃ to -60 ℃ and from - 60 ℃ to -140 ℃ were kept fixed at -40 ℃/min and -50 ℃/min, respectively.

2.4.2. Second phase: Variable freezing rates during –15 ℃ to –60 ℃

Fifteen straws per protocol were frozen by following 6 freezing protocols in the 2nd phase (-15 ℃ to -60 ℃). The best protocol in terms of post thaw semen quality obtained amongst the freezing protocols of the 1st phase (P15, cooling rate at -30 ℃/min) was used as the fixed freezing rate during 4 ℃ to -15 ℃ of the 2nd phase. The 2nd phase of freezing was comprised of six protocols [P21, P22, P23, P24 (control), P25 and P26] having freezing rates at -10, -20, -30, -40, -50 and -60 ℃/min, respectively. The freezing from -60 ℃ to -140 ℃ was kept constant at -50 ℃/min in all the protocols.

2.4.3. Freezing of semen

Above mentioned freezing protocols were constructed in programmable freezer. Freezing of semen straws was carried out in programmable biofreezer, Mini Digitcool (ZH 400, IMV

technologies, France) to reach the temperature to -140 ℃. Straws were quickly taken out of the freezing chamber and plunged into the liquid nitrogen. Frozen straws of each protocol were stored in a goblet under liquid nitrogen till evaluation.

2.5. Evaluation of post thaw semen quality

2.5.1. Sperm motility

Individual and progressive motilities were evaluated through computer assisted semen analyzer (CASA; Hamilton Thorn IVOS 12.2) at Division of Buffalo Physiology and Reproduction, Central Institute for Research on Buffalo, Hisar, Haryana, India. Briefly, five straws from each protocol were thawed and 5 μL semen was placed on Leja 4 slide and individual and progressive motility were evaluated[24].

2.5.2. Viability, hypo-osmotic swelling test (HOST) and sperm morphology

Five frozen thawed semen samples from each protocol were evaluated for viability and membrane integrity through Eosin Nigrosin staining method and HOST, respectively. The sperm morphology was studied from the thin smear stained with Rose Bengal stain[25].

2.5.3. Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential was assessed by using fluorescent dye Tetramethylrhodamine methyl ester (TMRM, Life Technologies; Cat#T-668). Stock solution (10 mM) was prepared in dimethyl sulfoxide and stored at -20 ℃ till use. A working solution of 50 μM was prepared and stored at -20 ℃. Post thaw semen samples (250 μL) were taken into microcentrifuge tubes and 1 mL of phosphate-buffered saline (PBS) was added to them. The samples were given 2 washings with PBS by centrifuging at 1 000 rpm for 5 min at 37 ℃. Then, 5 μL of tetramethylrhodamine methyl ester solution was added to each sample and incubated at 37 ℃ for 90 min. After incubation, washing was done with 1 mL of PBS at 1 000 rpm for 5 min at 37 ℃ to remove all the unbound dye. The pellet was mixed well with 500 μL of PBS. On a microslide, 10 μL of washed sample and 8 μL of ProLong Gold Antifade Mountant with diaminophenyl- indole (DAPI) (Life Technologies, Cat# P36941) was taken and covered with cover slip. The slide was kept at 4 ℃ after wrapping it in aluminium foil for 10 min. The slide was examined under upright fluorescent microscope (Nikon) with DAPI filter (420-480 nm) and fluorescein isothiocyanate filter (510-580 nm). Around 100 sperms were observed for high or low fluorescence in mid piece region as an indicator of mitochondrial membrane potential and % active mitochondria was calculated.

2.6. Statistical analysis

The data analysis was performed with Statistical Package for Social Sciences (IBM SPSS statistic version 20) program. Arc sine transformation was applied on all percent data. Normality of data was determined using Shapiro–Wilk test. Homogeneity of variance was tested with Brown and Forsythe's Test. The effect of different freezing protocols on post thaw semen quality was analyzed using one-way analysis of variance. The comparison among treatment groups were made using Games Howell Post hoc test. The data were presented as mean ± SE. The significant interaction was considered at $P<0.05$.

3. Results

3.1. Evaluation of different freezing protocols of first phase (4 ℃ to -15 ℃)

The present study was conducted to evaluate the different freezing protocols on the basis of sperm cryosurvival following cryopreservation of buffalo bull semen. The data were analyzed and presented in Table 1. There were no significant differences ($P>0.05$) in percent individual motility, progressive motility, viability among the various cooling protocols applied during the first phase. However, percent membrane integrity indicated by HOST was significantly higher ($P<0.05$) in the P16 (30.80 ± 2.98) as compared to P11 (15.00 ± 2.12), P12 (15.20 ± 2.50), P18 (15.47 ± 2.41) and control (15.30 ± 1.88). The head and total sperm abnormalities were significantly lower ($P<0.05$) in P16 as

Table 1

Quality of post thaw buffalo bull semen cryopreserved with various freezing protocols.

Protocols	Individual motility (%)	Progressive motility (%)	Viability (%)	HOST	Abnormalities (%)			Active mitochondria(%)
					Head	Tail	Total	
P11	39.66±2.79^{a}	15.62±1.22^{a}	39.90±4.07^{a}	15.00±2.12^{b}	13.63±1.46^{a}	18.87±2.15^{a}	38.50±6.99^{a}	32.16±4.18^{a}
P12	37.79±3.07^{a}	15.21±1.57^{a}	41.40±3.42^{a}	15.20±2.50^{b}	10.50±0.89^{ab}	16.27±2.21^{a}	27.10±2.56^{ab}	33.98±3.09^{ab}
P13	40.09±3.98^{a}	15.99±1.97^{a}	41.93±3.27^{a}	16.73±2.33^{ab}	9.53±0.96^{ab}	20.33±2.74^{a}	30.10±3.13^{ab}	34.42±5.18^{ab}
P14	36.65±3.05^{a}	14.56±1.40^{a}	39.17±2.64^{a}	21.13±2.90^{ab}	9.57±1.18^{ab}	20.37±2.71^{a}	30.00±3.17^{ab}	45.15±3.60^{ab}
P15	32.41±3.87^{a}	16.38±2.04^{a}	41.80±3.97^{a}	21.60±3.69^{a}	8.10±0.99^{b}	20.67±2.22^{a}	28.70±2.33^{b}	42.89±5.22^{b}
P16	41.65±2.83^{a}	14.26±1.48^{a}	47.27±3.15^{a}	30.80±2.98^{ab}	11.13±1.25^{ab}	15.20±2.84^{a}	26.13±3.31^{ab}	55.88±6.12^{ab}
P17	35.81±2.82^{a}	15.34±1.49^{a}	41.20±3.10^{a}	18.23±2.94^{ab}	12.40±1.33^{ab}	17.77±2.38^{a}	29.80±2.91^{ab}	43.12±2.99^{ab}
P18	38.62±3.57^{a}	16.05±1.77^{a}	38.53±3.03^{a}	15.47±2.41^{b}	13.33±1.31^{ab}	17.13±2.51^{a}	30.50±3.11^{ab}	39.77±4.99^{ab}
Control	40.15±2.99^{a}	16.88±1.30^{a}	45.90±3.54^{a}	15.30±1.88^{b}	15.27±1.79^{a}	17.83±2.03^{a}	35.10±2.50^{a}	28.34±5.15^{a}

Values marked with different superscripts within a column differ significantly ($P < 0.05$).

compared to P11 and control. The sperm with active mitochondria as indicated by tetramethylrhodamine methyl ester staining (Figure 1 and Figure 2) in P16 was significantly higher (P<0.05) as compared to P11 and control. So, the protocol P16 with cooling rate at -30 ℃/min was considered best in the first phase (4 ℃ to -15 ℃).

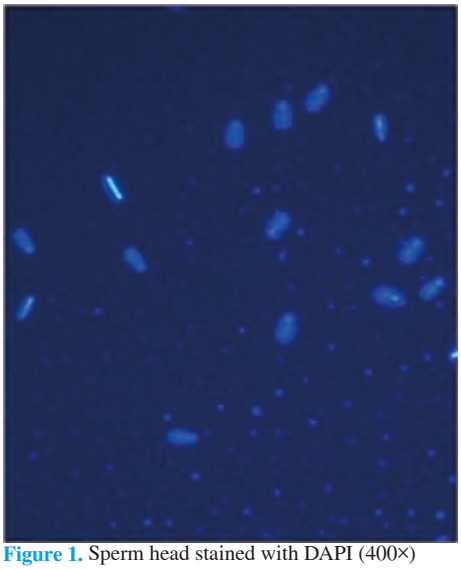

Figure 1. Sperm head stained with DAPI (400×)

Figure 2. Sperm mitochondria stained with tetramethylrhodamine methyl ester dye (Fluorescein Isothiocyanat Filter, 400×).

3.2. Evaluation of different freezing protocols of second phase (–15 ℃ to –60 ℃)

The data obtained from the second phase of freezing (-15 ℃ to -60 ℃) were analyzed and presented in Table 2. The % individual motility (41.9±3.3) in P25 was significantly higher (P<0.05) than the P22 (25.10±3.35) and control (25.07±3.03). However, progressive motility was similar in P25 and control. The % progressive motility (34.2±3.2) in P25 was significantly higher (P<0.05) than P22 (19.80±3.24). The % viability was similar among the various freezing protocols. The % HOST reactive spermatozoa in P25 (25.0±1.9) was significantly (P<0.05) higher than the P21 (8.60±0.96), P22 (12.20±1.23), P23 (15.27±1.86), P26 (13.33±1.52) and control (9.33±1.25). The head, tail and total sperm abnormalities were similar among the various groups. The percent active mitochondria was significantly higher in P25 (67.44±5.87) as compared to control (42.28±6.12). Hence, the freezing protocol P25 yielded best post thaw semen quality (Figure 3).

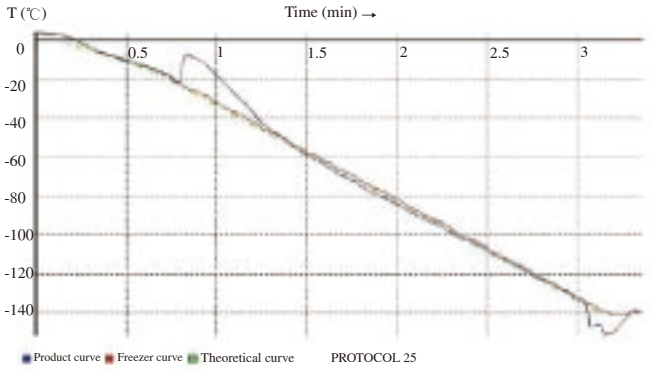

Figure 3. Best freezing curve for the cryopreservation of buffalo bull semen.

4. Discussion

This is the first report to optimize the freezing protocol for the cryopreservation of buffalo bull semen. Till date, buffalo bull semen is being frozen by following the protocol developed for cattle

Table 2

Quality of post thaw buffalo bull semen cryopreserved with various freezing protocols.

Protocols	Individual motility (%)	Progressive motility (%)	Viability (%)	HOST	Abnormalities (%)			Active mitochondria(%)
					Head	Tail	Total	
P21	29.60±3.55^{b}	23.33±3.40^{b}	41.97±2.95^{b}	8.60±0.96^{b}	9.43±0.61^{a}	17.70±1.51^{a}	27.13±1.66^{a}	34.76±4.38^{b}
P22	25.10±3.35^{b}	19.80±3.24^{a}	44.13±3.59^{a}	12.20±1.23^{b}	10.60±0.92^{a}	17.53±1.11^{a}	27.80±1.56^{a}	32.12±5.30^{b}
P23	31.33±3.63^{a}	25.73±3.10^{b}	47.27±1.86^{a}	15.27±0.86	9.60±1.77^{a}	19.43±2.02^{a}	29.03±4.87^{a}	35.91±3.85^{b}
P24	37.73±3.37^{a}	31.17±3.41	48.23±3.32^{a}	19.20±2.12^{a}	9.00±0.77^{a}	17.27±1.38^{a}	26.27±1.72^{a}	51.66±5.57^{a}
P25	41.90±3.30^{a}	34.20±3.20^{b}	52.90±3.70^{a}	25.00±1.90^{a}	8.30±0.90^{a}	17.80±1.50^{a}	26.20±1.80^{a}	67.44±5.87^{a}
P26	38.53±3.41^{a}	32.47±3.16^{b}	45.40±2.78^{a}	13.33±1.52^{b}	9.07±0.64^{a}	16.20±1.33^{a}	25.30±1.51^{a}	54.69±4.59^{a}
Control	25.07±3.03^{b}	26.50±2.79^{b}	45.47±2.50^{a}	9.33±1.25^{b}	10.53±0.76^{a}	18.27±1.28^{a}	28.47±1.41^{a}	42.28±6.12^{b}

Values marked with different superscripts within a column differ significantly (P < 0.05).

bull semen. However, buffalo sperm membrane have high content of polyunsaturated fatty acids as compared to cattle bull sperm making it highly susceptible to oxidative stress during freeze-thaw process[26,27]. Moreover, glutathione concentration, an antioxidant in buffalo semen is less as compared to cattle bull semen leading to more lipid peroxidative damage[21]. These might be the reasons for poor post thaw quality of buffalo bull semen cryopreserved using freezing protocol optimized for cattle bull semen.

In the study, individual and progressive motilities were similar in various freezing protocols of the 1st phase (4 ℃ to -15 ℃). However, in the 2nd phase (-15 ℃ to -60 ℃), the individual motility was significantly higher in P25 as compared to control. Sperm motility is a very complex phenomenon, which involves various structural (α, β tubuline; dyenine) and regulatory proteins tyrosine, calmodulin, cyclic adenosine monophosphate; protein kinase A, calcium influx and level of adenosine triphosphate (ATP) [28]. Dyenine activation is the critical regulatory point for the initiation of motility[29], which is further controlled by both cyclic adenosine monophosphate / protein kinase A and calcium signaling pathways[11]. The precursor molecules (proteins, calcium) in both the pathways have the ability to maintain sperm motility in varied physical conditions[30]. This might be the reason for similar sperm motility under various freezing protocols within 4 ℃ to -15 ℃, wherein precursor molecules might have maintained the motility under extreme and moderate freezing rates. However, in this study, significant increase in individual motility in the 2nd phase (-15 ℃ to -60 ℃) might be due to the combined ameliorative effects of moderate cooling rates in the 1st and 2nd phase. Other studies have also reported that freezing of buffalo bull semen from 4 ℃ to -120 ℃ at the freezing rates of -20 or -30 ℃/min yielded better post thaw progressive motility[31]. On the contrary, semen freezing with cooling rates between 4 ℃ to -15 ℃ at -3 or -10 ℃/min and between -15 ℃ to -80 ℃ at -10, -20 or -30 ℃/min yielded similar post thaw semen quality[32]. A lower % individual and progressive motility in protocol P22 (-20 ℃/min) as compared to P25 (-50 ℃/min) was observed. It indicates that if freezing rate of the 1st phase is lower than the 2nd phase, post thaw individual and progressive motilities will lower as in P22.

In the study, there was no significant difference (P>0.05) in percent sperm viability among various protocols of the 1st and 2nd phase. It has been observed that cryodamage to head and tail membrane occurs independently[33]. Disruption of sperm head membrane occurs more easily than the tail membrane. Further, sperm tail membrane is affected only by extreme cooling rates[33]. The differential susceptibility to membrane damage is due to the dissimilar composition of head and tail membrane. Polyunsaturated fatty acid content is higher in head as compared to tail membrane, which renders head more susceptible to cryodamage[12]. This might be the reason for similar viability at various cooling rates as head membrane could be damaged even at best cooling rate in the 1st phase (-30 ℃/min) and 2nd phase (-50 ℃/min). No similar study has been reported to compare our results.

In this study, percent hypo-osmotic swelling reactive sperm was significantly higher in the 1st phase (P15) and 2nd phase (P25) as compared to their respective controls. HOST indicates the proportion of sperm with functionally intact membrane, which is based on fluid transport across the sperm tail membrane under hypo-osmotic environment. Due to the influx of fluid, tail expands and bulges in the characteristic patterns and is considered as hypo-osmotic response[34,35]. An intact sperm tail membrane allows passage of fluid into the cytoplasmic space causing tail fibres to curl, and on the contrary, damaged tail membrane does not show any response[36,35]. As the sperm tail membrane is less susceptible to cryodamage due to lower content of polyunsaturated fatty acids[4], higher membrane disruption might have occurred only at extreme cooling rates. This suggests that moderate cooling rates in P15 and P25 caused less tail membrane damage leading to the significant differences in the hypo-osmotic swelling reactive sperm.

In the present study, head abnormalities were significantly lower in P15 as compared to control. During cryopreservation, cold shock is relatively higher during slow or fast cooling[37]. Cold shock can increase abnormal chromatin condensation and alter head morphology in human[38], boar[5], and ram sperm[39]. In the study, the cooling rate in P15 was moderate as compared to control. This might be the reason for significantly lower head abnormality in P15. The upper permissible limit for frozen-thawed sperm abnormalities is less than 25%[40]. So, sperm abnormalities in protocol P15 were within the permissible limit as compared to other protocols. In protocol P25, the proportion of sperm abnormalities were similar in all the groups. This might be due to the combined effects of both the protocols.

In the study, higher percent active mitochondria in protocol P15 and P25 as compared to their respective controls indicates that extreme cooling rates affected mitochondrial membrane activity seriously. It has been observed that cryofreezing stress inflicts detrimental changes on sperm mitochondria[41]. The function of the mitochondrial cristae in sperm is also affected by cold shock[42]. The resultant detrimental effects of cryofreezing stress and cold shock might have caused the decline in percent active mitochondria under extreme freezing rates. The study also revealed that the higher proportion of active mitochondria in protocol P15 and P25 did not correspond to similar pattern of motility. It is well known that sperm dynein proteins are distributed along the tail and require high quantum of ATP for flagellar motility. However, sperm mitochondria are limited to the mid-piece region, whereas the axonemal motor extends throughout the length of the flagellum. The mitochondrial ATP diffuses up to some distance to supply ATP in principal piece region[28] and ATP produced in mid-piece is insufficient to meet the energy requirement of axonemal dynein[10]. This suggests that mitochondrial oxidative phosphorylation is not the exclusive source of flagellar motility. Glycolysis carried out along the length of the principal piece is the most important source of ATP[28]. This corroborates our results that lower motility might be observed when high proportion of active mitochondria is present.

Overall, best post thaw semen quality was obtained with cooling rate at -30 ℃/min (P15) in the 1st phase of freezing (4 ℃ to

-15 ℃), when cooling rate in the 2nd phase (-15 ℃ to -60 ℃) was fixed at -50 ℃/min. It is well established that cooling rates beyond -60 ℃ have negligible effects on sperm[4,43], a fixed freezing rate of -50 ℃/min was applied from -60 ℃ to -140 ℃ in both the 1st and 2nd phase. In this study, various freezing rates were applied in critical temperature range (4 ℃ to -60 ℃) to explore the best freezing protocol for the cryopreservation of buffalo bull semen. Overall, higher motility, membrane integrity and mitochondrial membrane potential in protocol P25 indicate the best freezing protocol of buffalo bull semen. During freezing, several biophysical and biochemical changes take place in the semen. As the temperature drops below freezing point, extracellular ice crystals begin to form leading to increase in concentration of solutes[4]. Further, ice crystallization is slower inside the sperm as compared to surrounding medium which leads to efflux of water and sperm becomes gradually dehydrated[23]. The rate of efflux of water from sperm also depends upon the cooling rate. The slower cooling rate causes much greater dehydration and reduces the chance of intracellular ice crystal formation, thereby minimizing the physical damage[7,8]. But, simultaneously, a greater damage occurs due to increased intracellular dehydration and solute concentration. On the other hand, if the cooling rate is rapid, water has little time to move out and large intracellular ice crystals form, causing physical damage to cell membranes and other intracellular components. However, the problems of dehydration and solute concentration are less marked with rapid cooling. A successful cryopreservation would be better with an optimum cooling rate that will provide a balance between the two factors of sperm damage. On the basis of present study, it is concluded that the best post thaw semen quality of buffalo bull could be achieved by using freezing rate at -30 ℃/min from 4 ℃ to -15 ℃ and at -50 ℃/min from -15 ℃ to -140 ℃, followed by plunging of straws in into liquid nitrogen for storage.

Conflict of interest statement

The authors declare no conflict of interests (financial or non-financial) with any organization or entity.

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