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Effect of Centrifugation Regime on Cryopreservation of Beetal Buck Semen

Himsikha Chakravarty[1], Sudip Sinha[2], Dhrubajyoti Borpujari[2], Bharat Chandra Deka[2], Ranjan Kumar Biswas[2], Mitali Dutta[2], Birina Borah[3]

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ABSTRACT

Background: Goat semen has phospholipase A enzyme which makes removal of seminal plasma an important step in its preservation. This study was aimed to standardize a centrifugation regime for cryopreservation of Beetal buck semen.

Methods: A total of 36 pooled semen ejaculates were used for the experiment where initially 27 pooled ejaculates were utilized to find out the best time period out of 5, 8 and 11 minutes for each g-force *viz*., 700 × g, 1100 × g and 1400 × g using 9 pooled ejaculates per g-force. The remaining 9 pooled ejaculates were employed to find out the best centrifugation regime amongst the best time period for each g-force (8 minutes for 700 × g, 8 minutes for 1100 × g and 5 minutes for 1400 × g).

Result: The percentage of sperm motility, live sperm, intact acrosome and HOST-reacted sperm differed significantly (P<0.0001) between time periods at 700 × g, 1100 × g and 1400 × g. The sperm parameters were significantly (P<0.05) higher at 1400 × g for 5 minutes and 1100 × g for 8 minutes than at 700 × g for 8 minutes. In conclusion, adoption of a high centrifugation force for a short duration of time for washing could improve the quality of frozen spermatozoa.

Key words: Beetal buck semen, Centrifugation regime, Cryopreservation, Intact acrosome.

INTRODUCTION

Goat a 'movable wealth', is one of the earliest domesticated animals in livestock farming. It plays a pivotal role in the upliftment of rural economy of the country and serves as a continuous source of livelihood. Goat semen is different from other species in that it contains an enzyme in the seminal plasma secreted from the bulbourethral gland which was later identified as phospholipase A enzyme (Iritani and Nishikawa, 1961, 1963). It hydrolyzes egg yolk lecithin into fatty acids and lysolecithin causing sperm death by acrosome reaction (Upreti *et al*., 1999) and chromatin decondensation (Sawyer and Brown, 1995). Hence, removal of the seminal plasma is important to improve the quality of frozen-thawed buck semen. Some of the centrifugation regimes and washing solutions evaluated earlier for goat semen cryopreservation were 800 × g for 15 minutes with Tris citric acid buffer (Tuli and Holtz, 1994; Ferreira *et al.,* 2014), 600 × g for 10 minutes with Krebs-Ringer phosphate plus sodium citrate (Azeredo *et al*., 2001), 1200 × g for 15 minutes with Tris citric acid glucose (TCG) buffer (Peterson *et al*., 2007), 1500 × g for 10 minutes with TCG (Kozdrowski *et al*., 2007) and 1000 × g for 10 minutes with Ringer's lactate (Sariozkan *et al*., 2010). However, there was no unanimity regarding the best centrifugation regime for yielding superior quality semen. Therefore, this study was conducted to explore the effect of different centrifugation regimes on freezability of Beetal buck semen.

MATERIALS AND METHODS

Animals and site of experiment

Five stall fed healthy Beetal bucks aged two to four years, maintained under uniform feeding and managemental practices in Goat Research Station, Assam Agricultural University, Burnihat located at 26° North and 91.8° East were used for the study, after formal ethical approval by the Institutional Animal Ethics Committee bearing Approval no. 770/ac/CPCSEA/FVSc/AAU/IAEC/17-18/497 dated 09.08.2017. The study was conducted during the period August 2017-June 2019.

Collection of semen

Semen was collected from each buck once or twice a week with the help of a standard artificial vagina using a restrained doe as a mount (Fig 1).

Processing of semen

Semen was collected in 15 ml polypropylene graduated eppendorf tubes, diluted (1:5) with warm (35°C) Tris buffer

[1]Division of Animal and Fisheries Science, ICAR-Research Complex for NEH Region, Umiam-793 103, Meghalaya, India.
[2]Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-781 022, Assam, India.
[3]Department of Mathematical Science, Gauhati University, Guwahati-781 022, Assam, India.

Corresponding Author: Himsikha Chakravarty, Division of Animal and Fisheries Science, ICAR Research Complex for NEH Region, Umiam-793 103, Meghalaya, India.
Email: himsikhachakravarty@gmail.com

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having pH 6.8 (Deka, 1984) and placed in a beaker containing warm water (37°C) inside a vaccine carrier box. The semen sample was then evaluated for volume, mass activity and initial sperm motility. The ejaculates having volume 0.5 ml or more, mass activity (0 to 4+scale) 3+ or more and initial sperm motility 85 per cent or more were only pooled and used for the study. The tubes containing diluted semen samples were brought in the temperature controlled chamber from the Goat Research Station, Burnihat to the frozen semen laboratory at Assam Agricultural University, Khanapara within 20 minutes and centrifuged using eppendorf centrifuge - 5430 machine at different centrifugation regimes. A total of 36 pooled semen ejaculates comprising 98 ejaculates were employed for the study by adopting split sample technique to find out the suitable centrifugation regime for removal of seminal plasma wherein the effect of three 'g' forces *viz*., 700 × g, 1100 × g and 1400 × g at three time periods *viz*., 5, 8 and 11 minutes were studied in Tris extender. Initially 27 pooled ejaculates were used to find out the best time period out of 5, 8 and 11 minutes for each g-force using 9 pooled ejaculates per g-force. The remaining 9 pooled ejaculates were worked to find out the best centrifugation regime amongst the best time period for each g-force (8 minutes for 700 × g, 8 minutes for 1100 × g and 5 minutes for 1400 × g).

The sperm pellet was extended using Tris egg yolk citric acid fructose with glycerol (TEYCAFG) extender and packaged in 0.25 ml French mini straws, slowly cooled from room temperature to 5°C for 2 hours inside cold handling cabinet and subjected to equilibration. Straws were frozen over nitrogen vapour, 4 cm above the nitrogen level for 15 minutes, plunged and stored in liquid nitrogen. On the following day the frozen semen was thawed in warm water at 37°C for 30 seconds for evaluation.

Composition of tris basic solution

Ingredient	Quantity
Tris (hydroxymethyl) amino methane ($C_4H_{11}NO_3$, M.wt=121.14)	2.422 g
Citric acid	1.36 g
Fructose	1.0 g
Double glass distilled water up to	73.6 ml
Benzyl penicillin	1,00,000 IU
Streptomycin	100 mg

The pH of the solution was adjusted to 6.8 using 5 per cent citric acid solution.

Composition of tris extender (100 ml)

Tris basic solution	73.6
Glycerol	6.4 ml
Egg yolk	20.0 ml

Evaluation of semen

Each frozen semen sample was evaluated for sperm motility, live sperm, intact acrosome and HOST-reacted sperm. Sperm motility was examined under compound microscope at a magnification of 400X and recorded on visual appraisal based on the percentage of progressively motile sperm. Percentage of live sperm (Fig 2) in frozen-thawed semen was determined using Eosin-Nigrosin staining technique described by Blom (1977). The incidence of intact acrosome (Fig 3) was studied in stained smears of frozen-thawed semen using Giemsa staining technique (Watson, 1975) and the functional integrity of the sperm membrane (Fig 4) was studied as per the method described by Revell and Mrode (1994) using Hypo-Osmotic solution and calculated in percentage.

Statistical analysis

Data were analyzed using statistical software package SAS Enterprise Guide-4.3 software.

RESULTS AND DISCUSSION

Sperm motility and liveability

The mean per cent sperm motility and live sperm after freezing of washed Beetal buck semen centrifuged at 700 × g, 1100 × g and 1400 × g for 5, 8 and 11 minutes differed significantly ($P<0.0001$) between time periods. Critical difference test revealed that sperm motility and live sperm at 700 × g was significantly ($P<0.05$) higher for 8 minutes than for 11 and 5 minutes and for 11 minutes than for 5 minutes. At 1100 × g they were significantly ($P<0.05$) higher for 8 minutes than for 5 and 11 minutes and for 5 minutes than for 11 minutes while at 1400 × g the values of the parameters were significantly ($P<0.05$) higher for 5 and 8 minutes than for 11 minutes whereas there was no significant difference in sperm motility between the former two periods (Table 1).

In the present study, the post-thaw goat sperm motility recorded at 700 × g for 8 and 11 minutes was higher and for 5 minutes was lower when compared to the findings of Bispo *et al.* (2011) (54.3±6.2%) who used a centrifugation regime of 600 × g for 10 minutes. Sen (2015) recorded 51.0±1.56 per cent post-thaw sperm motility in Norduz goat semen frozen using 10 per cent egg yolk in skimmed milk following centrifugation at 600 × g for 10 minutes. Ferreira *et al.* (2014) found 13.47% post-freeze sperm motility on cryopreservation of buck semen centrifuged at a speed of 800 × g for 15 minutes and diluted in Tris citrate extender with 10 per cent egg yolk. When the mean live sperm was compared, that obtained in the experiment performed by Cabrera *et al*. (2005) after freezing Canary buck semen by centrifugation at 700 × g for 15 minutes was 17.9 per cent which was much lower than the present findings. The post-thaw sperm motility obtained at 110 0 × g centrifugation for different time periods were higher when compared with the post-thaw sperm motility (40.9±1.8%) in buck semen reported by Sariozkan *et al.* (2010) using a centrifugation regime of 1000 × g for 10 minutes and that (35.7±4.1%) reported by Rabadan *et al.* (2012) who used a centrifugation regime of 1200 × g for 10 minutes. The present values obtained after centrifugation of semen at 1400 × g for 5, 8 and 11 minutes were much higher than that obtained by Kozdrowski *et al*. (2007) using

a centrifugation regime of 1500 × g for 10 minutes who reported 28.12±6.51 per cent post-thaw sperm motility of goat spermatozoa. A post-thaw total sperm motility of 13.8±4.1 per cent and progressive sperm motility of 5.5±1.8 per cent was obtained when Ramukhithi *et al.* (2011) cryopreserved goat semen centrifuged at 1500 × g for 10 minutes and extended in 10 per cent egg yolk Tris extender. Using a higher speed of acceleration (1600 × g) for 10 minutes, Naing *et al.* (2011) obtained a post-thaw goat sperm liveability of 56.50±2.69 per cent which was lower than the present values of mean live sperm obtained after centrifuging at 1400 × g for 5, 8 and 11 minutes.



Fig 1: Collection of semen from beetal buck by artificial vagina method.

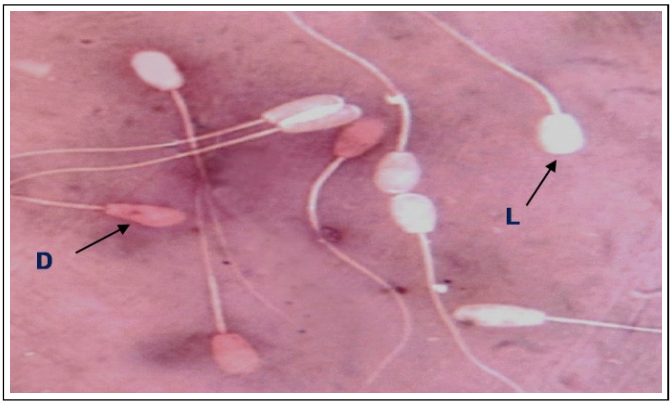


Fig 2: Live (L) and dead (D) sperm (1000 × magnification).

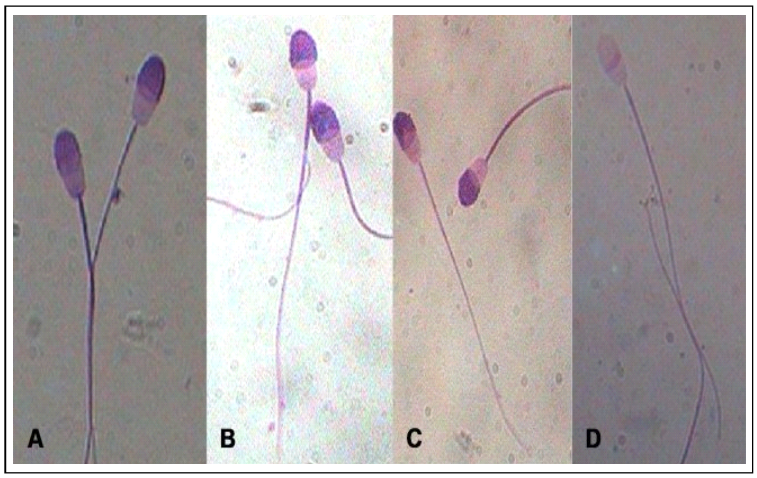


Fig 3: Spermatozoa with intact (a), swollen (b), separating (c) and entirely lost (d) acrosome (1000 × magnification).

Acrosomal normality and plasma membrane integrity using HOST

The mean per cent intact acrosome and HOST reacted spermatozoa after freezing of washed Beetal buck semen centrifuged at 700 × g for 5, 8 and 11 minutes differed significantly (P<0.0001) between time periods. Critical difference test revealed that after freezing, the mean intact acrosome and HOST-reacted sperm at 700 × g was significantly (P<0.05) higher for 8 minutes than for 11 and 5 minutes and for 11 minutes than for 5 minutes. At 1100 × g they were significantly (P<0.05) higher for 8 minutes than for 5 and 11 minutes and for 5 minutes than for 11 minutes. The incidence of intact acrosome was significantly (P<0.05) higher at 1400 × g for 5 minutes than for 8 and 11 minutes and for 8 minutes than for 11 minutes. The HOST-reacted sperm at 1400 × g after freezing was significantly (P<0.05) higher for 5 and 8 minutes than for 11 minutes while there was no significant difference in between the former two periods (Table 2).

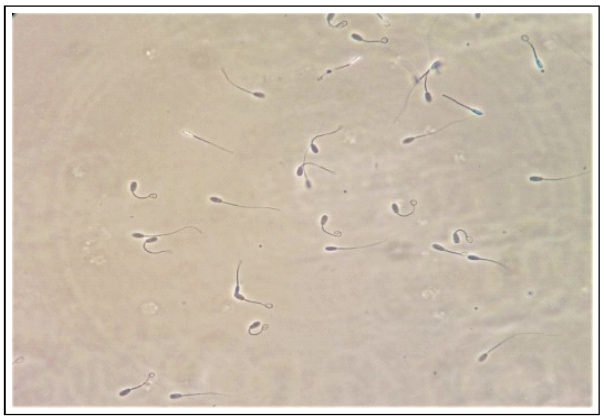


Fig 4: Host-reacted spermatozoa showing coiling of the tip of the tail (400 × magnification).

On comparison of the mean per cent intact acrosome after freezing of buck semen centrifuged at 700 × g for 5, 8 and 11 minutes, much lower post-freeze value for incidence of intact acrosome (33.2%) was obtained by Cabrera *et al.* (2005) using the same centrifugal force for a higher time (15 mins). Naing *et al.* (2011) studied the post-freeze sperm parameters by centrifuging Boer goat semen at a speed of 800 × g for 15 minutes and recorded that the per cent intact acrosome was 61.25±0.66 which was comparable with the value obtained with 700 × g for 5 minutes. Rabadan *et al.* (2012), found substantially lower post-thaw intact acrosome of 58.9±4.2 per cent on freezing of buck semen centrifuged at 1200 × g for 10 minutes as compared to our values obtained after washing semen at a speed of 1100 × g for 5, 8 and 11 minutes. The per cent plasma membrane integrity obtained after washing Beetal buck semen using a g-force of 1100 × g for 5, 8 and 11 minutes were higher than the post-freeze HOST-reacted spermatozoa (30.6±1.0%) obtained by Sariozkan *et al.* (2010) by using a centrifugation regime of 1000 × g for 10 minutes.

Centrifugation regimes

The mean per cent sperm motility, live sperm, intact acrosome and HOST-reacted sperm after freezing of washed Beetal buck semen centrifuged at 700 × g for 8 minutes, 1100 × g for 8 minutes and 1400 × g for 5 minutes differed significantly (P=0.0016, 0.0050, 0.0072 and 0.0019) between centrifugation regimes. Critical difference test showed that percentage of motile and live sperm as well as intact acrosome and HOST reacted spermatozoa after

Table 1: Per cent sperm motility and live sperm (mean†±se) after freezing of beetal buck semen centrifuged at different g-forces for 5, 8 and 11 minutes.

Period	Sperm motility			Live sperm		
(min)	700 × g	1100 × g	1400 × g	700 × g	1100 × g	1400 × g
5	52.89^{c}±0.63	63.56^{b}±0.50	63.44^{a}±0.69	59.60^{c}±0.56	67.71^{b}±0.32	67.39^{a}±0.61
8	61.89^{a}±0.45	67.33^{a}±0.67	61.89^{a}±0.42	66.01^{a}±0.39	71.35^{a}±0.60	67.19^{a}±0.68
11	58.44^{b}±0.50	58.56^{c}±0.58	54.67^{b}±0.62	63.53^{b}±0.53	64.75^{c}±0.58	61.18^{b}±0.53

†9 pooled ejaculates.

Means bearing different superscripts in a column differ significantly (P≤0.05).

Table 2: Per cent intact acrosome and host-reacted sperm (mean†±se) after freezing of beetal buck semen centrifuged at different g-forces for 5, 8 and 11 minutes.

Period	Intact acrosome			HOST-reacted sperm		
(min)	700 × g	1100 × g	1400 × g	700 × g	1100 × g	1400 × g
5	62.41^{c}±0.74	73.10^{b}±0.66	73.25^{a} ±0.73	39.86^{c} ±0.33	47.64^{b}±0.41	47.00^{a}±0.59
8	68.57^{a}±0.44	78.96^{a}±0.61	70.88^{b}±0.55	45.78^{a}±0.49	50.05^{a} ±0.37	47.08^{a} ±0.53
11	66.32^{b} ±0.27	67.63^{c}±0.73	65.34^{c}±0.64	44.23^{b}±0.55	44.66^{c} ±0.47	41.08^{b}±0.63

†9 pooled ejaculates.

Means bearing different superscripts in a column differ significantly (P≤0.05).

Table 3: Per cent sperm motility, live sperm, intact acrosome and host-reacted sperm (mean†±se) after freezing of beetal buck semen centrifuged at different regimes.

Centrifugation regime	Sperm motility	Live sperm	Intact acrosome	HOST-reacted sperm
700 × g for 8 minutes	60.78^{b}±0.72	64.68^{b}±0.91	76.67^{b}±0.64	44.29^{b}±0.78
1100 × g for 8 minutes	63.11^{a}±0.63	67.32^{a}±0.70	78.25^{a}±0.37	46.79^{a}±0.49
1400 × g for 5 minutes	64.33^{a}±0.47	68.17^{a}±0.39	79.19^{a}±0.50	47.44^{a}±0.39

†9 pooled ejaculates.

Means bearing different superscripts in a column differ significantly (P≤0.05).

freezing was significantly (P<0.05) higher at 1400 × g for 5 minutes and 1100 × g for 8 minutes than at 700 × g for 8 minutes while there was no significant difference in the parameters between the former two regimes (Table 3).

In the study it could be observed from the results that at the highest time of centrifugation *i.e*, 11 minutes, the sperm parameters studied registered the lowest values when 1100 and 1400 g-forces of centrifugation were used. It might be due to the injury caused to spermatozoa with increase in the time of centrifugation. Present findings support the observations of earlier workers who recorded poor post-freeze sperm quality on centrifugation of goat semen before freezing at 900 × g for 20 minutes (Coloma *et al.*, 2010) and 800 × g for 15 minutes (Naing *et al.*, 2011). On the other hand, when the time of exposure for centrifugation was decreased to 5 minutes, 700 × g resulted in the lowest per cent sperm motility, live sperm, intact acrosome and HOST-reacted sperm. This was in agreement with the findings of Daramola (2017) who used a centrifugation regime of 500 × g for 5 minutes and obtained poor post-thaw sperm motility after one time centrifugation in different extenders. Thus, it may be hypothesized that long duration of exposure to higher centrifugal force might inflict injury to spermatozoa whereas centrifugation at lower centrifugal force for a brief period could entail inadequate separation and removal of seminal plasma from the semen.

Taking into consideration of the gamut of centrifugation with different centrifugal forces and periods allowed, it emerged from the study that increase in centrifugal force allowing shorter time (1400 × g for 5 minutes) for centrifugation to remove the seminal plasma before freezing resulted in the highest percentage of sperm parameters. The present results were in agreement with that of Carvajal *et al.* (2004) and Naing *et al.* (2011) who found that the use of short-term centrifugation with a relatively high force contributed to obtaining better quality frozen semen. The present results were also in agreement with Shekarriz *et al.* (1995) who found that the time of centrifugation is more critical than g-force for inducing human sperm damage in the preparation of sperm for ART. Although centrifugation caused potential damage to the spermatozoa, the use of high g-force centrifugation could enhance the removal of the ejaculate contaminants in shorter time such as abnormal and dead spermatozoa (Naing *et al.,* 2011).

CONCLUSION

Adoption of a high centrifugation force for a short duration of time as in the present study could prove beneficial by conferring higher percentages of semen parameters that were in commensurate with obtaining good quality frozen spermatozoa in respect of motility, live sperm, incidence of intact acrosome and HOST-reacted sperm.

Conflict of interest: None.

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