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Osmotic Tolerance and Freezability of Beetal Goat Sperm during Breeding and Non-Breeding Seasons

Research Article

M. Akhtar[1], E. Ahmad[2*], Z. Naseer[3], M.S. Akhtar[2], T. Ahmad[2], T. Hussain[1] and M.T. Khan[2]

[1] Department of Molecular Biology, Faculty of Science and Technology, Virtual University, Lahore, Pakistan
[2] Department of Clinical Science, Faculty of Veterinary Science, Bahauddin Zakariya University, Multan, Pakistan
[3] Department of Clinical Science, Faculty of Veterinary and Animal Science, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan

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*Correspondence E-mail: ejaz.ahmad@bzu.edu.pk

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ABSTRACT

The present study was conducted to determine seasonal variations in osmotic tolerance and post-thaw quality of Beetal goat sperm. The ejaculates (n=12) were collected from mature bucks (n=3) during breeding (late summer-early fall) and nonbreeding (winter-early spring) seasons. In the first experiment, the samples were exposed to hypo-osmotic, iso-osmotic, and hyper-osmotic conditions adjusted to 50, 100, 290, 500, or 1000 mOsm/L and incubated at 37 ˚C for 15 min. After the osmotic challenge, sperm viability and plasma membrane integrity (PMI) were determined using a combined hypo-osmotic swelling test (HOST) and eosin-nigrosine staining. In experiment 2, the ejaculates were diluted with a tris-citric acid fructose (TCF) extender and frozen in liquid nitrogen following a standard protocol. After freezing, samples were thawed to assess motility, motion kinetics, PMI, and viability. The results showed a significant ($P<0.05$) impact of the interaction between osmolality and season on live-HOST +ve and total-HOST +ve sperm. The live-HOST +ve and total-HOST +ve sperm were higher ($P<0.05$) in the breeding season. Post-thaw sperm PMI, progressive and total motilities were higher ($P<0.05$) in the breeding season; while, viability and sperm motion kinetics were similar ($P>0.05$) between the seasons. In conclusion, the season impacts the osmotic tolerance and cryopreservation of Beetal goat sperm.

KEY WORDS Beetal goat sperm, freezing, osmotic tolerance, season.

INTRODUCTION

Artificial insemination (AI) by using frozen semen is routinely practiced for breeding in livestock species for improvement of productivity. AI adoption needs time to improve goat production using effective breeding protocols (Ranjan *et al.* 2021). The success of AI depends on the quality of frozen semen, which is compromised during the cryopreservation and thawing process. The cryopreservation of semen is a very complex process and inserts various stresses on spermatozoa. Throughout the freezing and thawing process, the spermatozoa are intermittently exposed to hyper-tonic and hypo-tonic media (Oldenhof *et al.* 2015). The unusual exposure to harsh osmotic conditions may result in swelling or shrinking spermatozoa due to the influx of water in hypotonic conditions or outflow of water upon exposure to hypertonic conditions. This outflow and influx of water cause damage to the sperm membrane. Therefore, it is ensured to use the isotonic media for sperm cryopreservation to minimize membrane-associated damage. The goat spermatozoa exhibit osmotic damage during cryopreservation (Xu *et al.* 2022). However, there is a paucity of

information regarding the maximum tolerance of goat sperm in hypertonic and hypo-osmotic media.

The photoperiod is another factor influencing reproductive activity in bucks (Arrebola and Abecia, 2017). Season of the year, however, seems to be the major cue affecting semen quality in goats (Qureshi *et al.* 2013). The freezability and fertilizing capacity of buck sperm collected during the breeding (autumn-winter) and nonbreeding season (spring-summer) is variable due to changes in testicular or accessory sex gland milieu (Gallego-Calvo *et al.* 2015; Ustuner *et al.* 2018; Kulaksız *et al.* 2019; Kulaksız *et al.* 2020). Further work is needed to monitor any deterioration in the semen quality by observing the osmotic tolerance and freezing capacity of buck sperm during breeding and non-breeding season. Therefore, the present study was designed to observe the osmotic tolerance and freezability of Beetal goat spermatozoa during different breeding and low breeding seasons.

MATERIALS AND METHODS

Animals and management

Three mature (2.5 to 3 years, 50 to 55 kg body weight) regular semen donors, known fertility Beetal goat bucks, were maintained at a private goat farm in Multan, Punjab, Pakistan, during the study period (March-2020 to May-2021).

The bucks were born at the same goat farm, provided optimum management during the growth phase, and were selected for breeding purposes. Bucks were housed separately from the female herd in a semi-covered shed, where bucks had sufficient loafing space. The bucks were provided the daily concentrates and mineral mixture in addition to daily grazing (6-8 hours) time and had free access to water.

Semen collection and evaluation

The ejaculates (n=12) were collected from goat bucks during breeding (late summer-early fall) and nonbreeding (winter- early spring) seasons (six ejaculates/season and two ejaculates /buck/season) by using an electro-ejaculator. The ejaculation of each buck was processed separately and considered as one complete replicate. The semen samples were shifted to the laboratory immediately after collection in the Styrofoam box.

In the laboratory, the volume and color of each sample were recorded and then placed in a water bath at 37 ˚C. Afterward, each sample was subjected to the initial evaluation. The ejaculates of minimum standard quality having motility (>70%), viability (>80%), sperm concentration (>100×10^6/mL), and morphologic abnormalities (<20%) were used in this study.

Experiment 1

First, a stock solution of fructose and sodium citrate was made, with the osmolality adjusted to 1500 mOsm/L. Then working solutions of various osmolality [hypo-osmolality (50 and 100 mOsm/L), iso-osmolality (290 mOsm/L), and hyper-osmolality (500 and 1000 mOsm/L) were then prepared from the stock solution. These solutions were maintained at 37 ˚C in a water bath. The fresh sperm samples (25 µL) were exposed to (475 µL) one of the hypo-osmotic, iso-osmotic, or hyper-osmotic conditions (adjusted at 50, 100, 290, 500, or 1000 mOsm/L). They were incubated at 37 ˚C for 15 min. The hyper-osmotic incubation samples (500 and 1000 mOsm/L) were returned to hypo-osmotic (100 mOsm/L) conditions. After the osmotic challenge, sperm viability and plasma membrane integrity (PMI) were determined using a combined hypo-osmotic swelling test (HOST) and eosin-nigrosine staining, as described previously (Küçük *et al.* 2014). Briefly, a drop from each osmotic-challenged group was mixed with a drop of eosin-nigrosine stain on a glass slide. Then a uniform smear was prepared from this mixture. The smear was air-dried and then observed under a bright field microscope at ×400 magnification. The live HOST +ve (only unstained heads with curled tails), total HOST +ve (all with curled tails), and total live sperm (all unstained heads with or without curled tails) were counted. At least 100 sperm from each slide were counted.

Experiment 2

This study used semen cryopreservation following a standard protocol described by Küçük *et al.* (2014). The semen was collected through electroejaculation from Beetal goat bucks during breeding and nonbreeding seasons. The ejaculates were diluted with TCF extender (Tris 300 mM, fructose 28 mM, citric acid 95 mM) containing egg yolk (15%) and glycerol (5%) by fixing a final concentration of 200 ×10^6 sperm/mL. The extended samples were loaded into 0.5 mL French straws, cooled to 4 ˚C, and then equilibrated for 2 hours. After equilibration, the straws were horizontally frozen in liquid nitrogen vapors (5 cm above liquid nitrogen) for 12 min and then plunged into liquid nitrogen for storage. After freezing, two straws from each replicate were thawed to assess sperm progressive and total motilities, motion kinetics (curvilinear velocity (VCL µm/s), straight-line velocity (VSL µm/s), average path velocity (VAP µm/s), linearity (LIN %), straightness (STR %), and Beat cross frequency (BCF Hz)), PMI, and viability. The sperm motilities and motion kinetics were determined through Computer Assisted Sperm Analyzer (CASA). In contrast, sperm PMI and viability were determined using a combined HOST and eosin-nigrosine staining as described in experiment 1.

Statistical analysis

The data were analyzed using SPSS statistical software. The GLM procedure with repeated measures was applied to determine the effect of fixed factors (osmolality, season, and their interaction) on plasma membrane integrity and viability parameters. One-way ANOVA was used to compare the overall effect of various osmolalities (50, 100, 290, 500, or 1000 mOsm/L) on PMI and viability parameters. The season effect on plasma membrane integrity, viability parameters, and post-thaw sperm quality parameters was compared by paired sample t-test. The level of significance was kept as $P < 0.05$.

RESULTS AND DISCUSSION

The results of Experiment 1 indicate an effect of osmolarity on ($P<0.05$) live-HOST+ve sperm rather than the season or its interaction. Season, osmolarity, and its interaction affected ($P<0.05$) the total-HOST +ve sperm. Total viable sperm were only affected ($P<0.05$) by season (Table 1). The live-HOST +ve and total-HOST +ve sperm percentages were higher ($P<0.05$) in the breeding season compared to the nonbreeding season, irrespective of the osmolarity effect (Table 2).

The lowest and highest live-HOST +ve sperm were observed ($P<0.05$) in 1000 and 100 mOsm/L challenge groups, respectively. There was no change in total-HOST +ve and total viable sperm rate in response to osmotic challenges (Table 3).

In experiment 2, the higher ($P<0.05$) post-thaw progressive and total motilities and PMI were observed in the breeding season cryopreserved semen than in the nonbreeding season. However, viability and sperm motion kinetics (VCL, VSL, VAP, LIN, STR, and BCF) were similar across the seasons (Table 4).

The present study dealt with the effects of osmotic challenge and cryopreservation on Beetal goat buck sperm during breeding and nonbreeding seasons. The observed data show that Beetal buck sperm withstands remarkably better in the breeding season under hypoosmotic conditions. Likewise, sperm characteristics of the Beetal buck were better when semen was cryopreserved in the breeding season.

This artificial modulation of osmotic stress simulates the cryopreservation challenge to sperm which experience a wide range of osmotic pressures (0-2000 mOsm) in cryopreserved media (Johnston *et al.* 2014; Ahmad *et al.* 2018). In the present model, the Beetal buck sperm were exposed to different osmotic challenges (50-1000 mOsm) across the breeding and nonbreeding season.

The previous studies described the impact of season on the semen quality of goat buck (Wang *et al.* 2015; Kumar *et al.* 2016; Ustuner *et al.* 2018; Kulaksiz *et al.* 2019) and resulted in poor fertility response to freezing-thawing process because of alteration in seminal plasma components, sperm structural configuration and molecular expression in different breeding seasons. This study appears to be the first document for goat buck sperm exposure to different osmotic media (50 to 1000mOsm) and following exposure to isotonic conditions of goat seminal plasma. Plasma membrane integrity was maintained during hypoosmotic conditions exposure, but a significant decline was observed when sperm underwent hypertonic media exposure. The hypotonic range of media also affected the buck sperm membrane integrity but prominently in nonbreeding season. The variation in sperm plasma membrane integrity in response to hypo- and hypertonic exposure and followed exposure to isotonic states indicated some irreparable damage in the sperm plasma membrane. Probably the most remarkable finding of this study was the extreme tolerance of the crocodile sperm plasma membrane in response to exposure to hypotonic media. A similar pattern of sperm plasma membrane response was observed in rams (Ahmad *et al.* 2018), rabbits (Gloria *et al.* 2021), tom (Kunkitti *et al.* 2017), crocodiles (Johnston *et al.* 2014), horses and bull (Oldenhof *et al.* 2015). However, seasonal variation in buck sperm response to different osmotic conditions directly explains the seasonal effect on sperm structural or molecular attributes. The plasma membrane's physical change might occur due to the loss of subcellular components with water flux during exposure (Johnston *et al.* 2014). Additionally, the increasing trend of testosterone for forthcoming spermatogenesis during breeding season seems an involving factor for seasonal alteration in buck sperm plasma membrane against hypo- or hypertonic response (Ahmad *et al.* 2018). Studies following the influx or outflux of different plasma membrane components in buck sperm, particularly during the non-breeding season, could explain the underlying mechanism clearly.

Cryopreservation is a process of extreme conditions where sperm are exposed to different stressors measured as a freezing loss. The season of semen cryopreservation in goats does not provide an optimal fertility rate around the year. Earlier studies were conducted to observe a relationship between season and freezing in different goat breeds (Wang *et al.* 2015; Kumar *et al.* 2016; Kulaksiz *et al.* 2019; Kulaksiz *et al.* 2020), and reasonable freezing loss had occurred in goat semen across the seasons. The current findings also show similar results of low post-thaw goat buck semen motility and PMI in the non-breeding season.

Table 1 Effect of different osmotic conditions, season, and interaction of season × osmolality on plasma membrane integrity (PMI) and viability of goat sperm (Mean±SE)

Parameters	Nonbreeding season					Breeding season					Season	mOsm	Season × mOsm
	mOsm/L					mOsm/L							
	50	100	290	500	1000	50	100	290	500	1000			
PMI													
Live HOST +ve sperm (%)	9.3±2.6	19±1.1	18.3±5.4	28.6±6.0	11.6±0.3	37.0±6.6	40.6±2.0	27±5.6	22.3±5.6	16.0±2.0	0.108	0.023	0.030
Total HOST +ve sperm (%)	25.3±3.7	26.0±4.0	33.3±10.1	40.0±8.6	19.0±3.4	59.6±2.4	67.0±1.0	49.3±4.4	46.3±4.6	34.0±3.2	0.051	0.051	0.000
Total viable sperm (%)	34.6±6.9	61.0±4.0	52.6±5.2	58.0±1.1	47.0±8.0	57.0±7.5	56.3±4.1	63.0±3.4	54.3±4.6	56.3±1.2	0.048	0.117	0.261

HOST: hypo-osmotic swelling test.

Table 2 Effect of season on plasma membrane integrity (PMI) and viability of goat sperm (Mean±SE)

PMI variables	Breeding season	Nonbreeding season	P-value
Live HOST +ve sperm (%)	28.6±2.9	17.4±2.3	0.006
Total HOST +ve sperm (%)	51.2±3.2	28.7±3.1	0.000
Total viable sperm (%)	57.4±1.9	50.6±3.2	0.088

HOST: hypo-osmotic swelling test.

Table 3 Effect of various osmotic challenges (hypertonic hypotonic and isotonic conditions) on the membrane structural integrity and viability of goat sperm (Mean±SE)

Variables	Osmolality				
	50 mOsm	100 mOsm	290 mOsm	500 mOsm	1000 mOsm
Total HOST +ve sperm (%)	42.5±7.9	46.5±9.3	41.3±6.1	43.1±4.6	26.5±3.9
Live HOST +ve sperm (%)	23.1±6.9^{ab}	29.8±4.9^{a}	22.6±3.3^{ab}	25.5±3.9^{ab}	13.8±1.3^{b}
Total viable sperm (%)	45.8±6.7	58.6±2.7	57.8±3.6	56.1±2.3	51.6±4.2

HOST: hypo-osmotic swelling test.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 4 Effect of season on post-thaw sperm quality parameters in Beetal buck (Mean±SE)

Variables	Winter	Summer	P-value
Progressive motility	8.4±0.5	13.0±0.7	0.005
Total motility	69.9±0.5	87.7±2.1	0.001
Viability	28.7±3.4	35.3±2.3	0.18
PMI	30.3±1.8	46.3±5.2	0.044
VCL	34.4±1.4	38.3±1.4	0.117
VSL	12.2±0.9	12.8±0.7	0.641
VAP	18.5±1.3	20.8±0.9	0.228
LIN	28.5±2.0	28.0±1.6	0.844
STR	53.8±1.4	51.8±1.6	0.418
WOB	49.8±2.1	51.3±1.5	0.605
ALH	1.8±0.0	2.0±0.1	0.018
BCF	4.0±0.3	4.1±0.2	0.766

Changes in seminal plasma, melatonin, and testosterone levels during spermatogenesis are major contributing factors that affect the fresh and cryopreserved goat semen quality. Because of the geographical location of the study site, the seasonality element does not have a major effect; however, fresh or post-thaw semen parameters indicate a prominent effect. In the future, the fertility trials in the field using cryopreserved semen across the seasons could provide a better layout to design hormonal, feeding, or semen extender supplementation studies in low fertility periods of semen cryopreservation.

CONCLUSION

In conclusion, the osmotic tolerance of Beetal buck sperm to hypo- and hypertonic conditions are variable during breeding and nonbreeding seasons. Moreover, cryopreserved semen during the nonbreeding season affects some post-thaw buck sperm characteristics.

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