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Research Article

DISCRETE PHASIC REDUCTION IN SPERM CRYOTOLERANCE WITHIN BREEDING SEASON IN GADDI MALE GOATS (*CAPRA HIRCUS*) IN SUB-TROPICAL HIMALAYAS

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ABSTRACT: The sperm cryotolerence during different phases of breeding season for Gaddi bucks needs to be elucidated. Gross and microscopic characteristics of fresh, pre-freeze and frozen-thawed semen samples (n=149) to different climatic variables during Phase 1 (October-December, n=94) and Phase 2 (January-February, n=55) in Gaddi bucks were examined. Gross and microscopic characteristics of fresh and pre-freeze semen during both phases were similar. Contrarily, the ability of sperm to endure cryopreservation damage was remarkably reduced during Phase 2. Frozen-thawed semen during Phase 2 exhibited significant reduction in progressive motility and live sperms, while the morphological abnormalities increased significantly ($p<0.05$). Hence, semen cryopreservation in Gaddi bucks is conducive from October to December as compared to January to February.

Keywords: Gaddi goat, Season, Sperm cryotolerance.

INTRODUCTION

Goats play a crucial role in Indian agriculture, particularly for small and marginal farmers, offering high returns with minimal investment. Gaddi goats, prevalent in the North-Western Himalayan region of Himachal Pradesh, serve as valuable sources of meat, milk, hide, manure, and wool/hair. This multi-faceted contribution enhances income, generates employment, stores capital, and improves household nutrition for farmers engaged in goat farming in the Himalayas [1].

The speed of genetic progress in goat lags behind that of large ruminants, primarily due to indiscriminate slaughter of bucks, leading to a shortage of genetically superior males for natural mating [2]. Seasonality in goat reproduction further hampers the genetic progress [3]. To address these challenges, artificial insemination (AI) is necessary, but goat spermatozoa's susceptibility to cryopreservation presents another obstacle [4, 5]. The factors mentioned *vide supra* are no different in Gaddi goats. Variations in breeds affect the semen potential [6] and cryopreservation tolerance [7]. Our previous attempts showed inconsistent cryopreservation outcomes in Gaddi goats during different phases of reproductive season (short days), prompting further investigation [8, 9]. Therefore, this study aimed to understand the seminal attributes, cryopreservation changes, and their relationship with climatic cues during different phases of breeding season in Gaddi bucks.

MATERIALS AND METHODS

The study adhered to guidelines set by the College Animal Ethical Committee (QSD/VOG/COVAS/PG/IAEC/2000/148; 2020.05.16). It focused on sexually matured Gaddi bucks during the conventional breeding season (October 2020 to February 2021; average temperature humidity index (THI), rainfall and light hours of 60.1±3.9 percent, 70.6±22.4 mm, and 10.5±0.2 h, respectively). Six goats with optimal gross seminal features were chosen from a pool of 13. They were housed at the University Livestock farm, CSK Himachal Pradesh Agricultural University, Palampur, situated at 1300 m above sea level having geographical coordinates of 32°6'7"N and 76°33'17"E, respectively. The climate is monsoon-influenced subtropical with an average annual rainfall of 1578 mm. The bucks' average age was 2.16±0.36 years, and weight was 39.1±2.82

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Kg. They grazed for five h daily (900 to 1200 h and from 1400 to 1600 h), supplemented with seasonal green fodder (maize, cowpea, berseem or jowar) and concentrates (maize-50%, wheat bran-29%, groundnut cake-10%, molasses-7%, vitamins fortified mineral mixture-2%, salt-1% and by pass fat-1% rendering crude protein-18%, ether extract-5%, total ash-12%, acid detergent fiber-17%, neutral detergent fiber-45% and TDN-77%, respectively) at the rate of 3-3.5% of body weight [10], and had continuous access to clean water. The bucks were maintained in individual pens. Before the study, all the bucks were screened for brucellosis and chlamydiosis following standard procedures [11].

A total of 179 semen ejaculates were collected using an artificial vagina (IMV Technologies, France) in the presence of an estrus doe. Each ejaculate was immediately evaluated for colour (milky to yellowish white), volume (mL), and sperm concentration (10^6/mL) and mass motility (0-5 scale) (all mentioned hereafter as fresh semen parameters). Ejaculates were selected based on the absence of gross abnormalities and a mass motility of ≥3 [12]. Thirty ejaculates did not meet these criteria and were discarded, leaving 149 for further processing.

Sperm concentration, volume of extender to be added and number of semen doses to be prepared were ascertained by examining each ejaculate in caprine photometer (IMV Technologies, 1409® France). The remaining ejaculate was mixed with Ringer's solution (1:10), centrifuged to remove seminal plasma, and the sperm-rich pellet was extended in a tris-egg yolk-glycerol extender (tris-1.12 g, citric acid-0.685g, D-Fructose-0.5g, glycerol 3 mL (all from Sigma-Aldrich, USA), egg yolk-5mL, benzyl penicillin-50000IU (Karnataka Antibiotic Pharmaceutical Limited, India), and streptomycin sulphate-50mg (Abbott Healthcare Private Limited, India); all mixed in ultrapure water to achieve a final extender volume of 50 mL) [13, 14]. Each extended sample was evaluated for progressive motility (%), live sperms (%) (Fig. 1a), morphological abnormalities (%) (Fig. 1b) and hypo-osmotic swelling (HOS) positive sperms (%) (Fig. 1c) (all mentioned hereafter as pre-freeze semen parameters) [12]. The extended semen was then cryopreserved in 0.25 mL French mini straws (IMV Technologies, France) [8, 9, 13]. After 24 h, frozen-thawed semen samples were examined for the same parameters as pre-freeze samples. Cryopreservation-induced alterations for different parameters were assessed using the formula:

$$\% \text{ charge} = \frac{\text{Pre-freeze value} - \text{Post-thaw value}}{\text{Pre-freeze value}} \times 100$$

The study's climatic variables were air temperature (T) (°C), day length (h), and temperature humidity index (THI). The THI was calculated using temperature and relative humidity as per NRC [15] :

$$THI = (1.8 \times T + 32) - [(0.55\text{-}0.0055 \times RH) \times (1.8 \times T - 26)]$$

Temperature and relative humidity were recorded daily. Temperature was noted at 0700 h, 1100 h, and 1500 h, and relative humidity at 0700 h. Daily temperatures were averaged and then compiled monthly throughout the experiment. Based on percentage change in frozen-thawed semen during the breeding season, the study period and corresponding data on semen and climate were divided into Phase 1 (October to December) and Phase 2 (January and February of the following year).

Results are presented as Mean ± S.E.M. Data for different phases were analyzed using a repeated measures general linear model. The model included effects of treatment (fresh, pre-freeze, and percentage change in frozen-thawed semen characteristics with climatic variables) during different phases and their interactions, with buck (treatment x phase) as the replicate. Differences among means were determined using Duncan's multiple range tests. The entire statistical analysis was performed using SAS (1990) [16]. Statistical significance was set at $P<0.05$, with tendencies reported at $0.05<p<0.10$.

RESULTS AND DISCUSSION

This study records a significant reduction in cryotolerance of sperms during Phase 2 of the breeding season, despite ideal fresh and pre-freeze semen parameters in Gaddi bucks. The breeding season from October to February (of the succeeding year) aligns with natural breeding-based kidding patterns (Table 1) lasting between March to July in Gaddi does [17]. Geographical latitude influences the duration of semen production, with breeds reared between 30º N and 40º N showing optimal semen production for five to seven months [18, 19]. Thus, a five-month breeding span for Gaddi bucks at 32º N is consistent with these findings.

Table 1. Kidding patterns from March to July in Gaddi does rear in Himachal Pradesh [data from a survey of 40 farmers].

Mating month	Kidding month	Kidding percentage
October	March	17.8±2.3
November	April	21.3±0.7
December	May	23.7±1.5
January	June	21.7±1.2
February	July	15.5±1.6

Table 2. Average (Mean ± S.E.M.) fresh semen indices during different months of phase 1 and phase 2 in Gaddi male goats.

Phase	Month	Fresh semen parameters		
		Volume (ml)	Concentration (10^6/ml)	Mass motility (0-5)
Phase 1	October	0.68±0.04	3689.87±207.54	3.91±0.05
	November	0.70±0.04	3491.67±201.23	3.92±0.03
	December	0.62±0.02	3511.18±277.54	3.91±0.05
Phase 2	January	0.60±0.06	2950.55±295.45	3.95±0.04
	February	0.59±0.04	3155.44±277.53	3.90±0.08

Table 3. Average (Mean ± S.E.M.) pre - freeze semen indices during different months of phase 1 and phase 2 in Gaddi male goats.

Phase	Month	Pre-freeze semen parameters			
		Progressive motility (%)	Live sperms (%)	Morphological abnormalities (%)	Hypo osmotic swelling positive sperms (%)
Phase 1	October	73.67±0.45	74.55±1.28	7.92±0.58	73.74±0.88
	November	72.57±0.57	75.43±1.42	7.41±0.48	72.98±1.38
	December	74.51±0.59	77.12±1.96	5.89±0.69 [e]	73.81±1.32
Phase 2	January	73.81±0.88	77.11±2.25	8.51±0.79 [f]	75.14±1.15
	February	71.14±0.72	74.54±1.83	7.92±0.46	78.21±1.91

[e] *vs.* [f] within the same column differ ($p<0.05$)

Table 4. Average (Mean ± S.E.M.) frozen – thawed semen indices (presented as absolute and % change to pre – freeze values) during different months of phase 1 and phase 2 in Gaddi male goats.

Phase	Month	Values	Frozen-thawed semen parameters			
			Progressive motility (%)	Live sperms (%)	Morphological abnormalities (%)	Hypo osmotic swelling positive sperms (%)
Phase 1	October	Absolute	40.11±1.01 [a]	45.87±1.49 [a]	8.71±0.49 [a]	56.89±1.45
		% change to pre-freeze	45.73±1.46 [c]	38.42±2.77 [c]	- 9.84±1.07 [c]	23.84±2.33 [c]
	November	Absolute	39.27±0.67 [a]	48.67±1.06 [a]	8.12±0.53 [a]	54.91±1.89
		% change to pre-freeze	45.88±1.24 [c]	35.45±2.48 [c]	- 9.72±1.01 [c]	24.71±3.27 [c]
	December	Absolute	39.82±0.77 [a]	48.42±1.33 [a]	6.97±0.39 [a]	54.22±1.72
		% change to pre-freeze	47.08±1.36 [c]	37.11±3.29 [c]	- 12.20±0.99 [c]	22.33±3.04 [c]
Phase 2	January	Absolute	23.82±1.25 [b]	27.66±1.86 [b]	12.34±0.40 [b]	54.22±2.21
		% change to pre-freeze	68.42±2.05 [d]	64.22±4.08 [d]	- 45.17±1.00 [d]	27.81±3.22
	February	Absolute	22.21±1.21 [b]	28.91±2.52 [b]	11.12±0.41 [b]	52.41±1.61
		% change to pre-freeze	68.17±1.91 [d]	61.28±4.31 [d]	- 40.75±0.81 [d]	32.97±2.55 [d]

[a]vs.[b] and [c]vs.[d] within the same column differ ($p<0.01$).

The variability and vitality of buck semen were investigated, revealing notable insights. Interestingly, fresh semen parameters remained consistent between Phase 1 and Phase 2 ejaculates (Table 2), surpassing benchmarks set by previous studies [20, 21]. However, seasonal influences were evident, with the ejaculates exhibiting significantly ($p<0.05$) reduced morphological abnormalities during December compared to January (Table 3). Unlike pre-freeze semen, the percentage decrease in progressive motility and live sperms as

Table 5. Salient interactions between sperm indices at different stages of processing with climatic variables during different months of phase 1 and phase 2 in Gaddi male goats.

Breeding phase	Stage of semen evaluation	Detail of interaction (x) between semen and climatic variables	Level of significance
Phase 1	Pre-freeze	Morphological abnormalities x T	*
		Morphological abnormalities x THI	*
		Morphological abnormalities x day length	*
Phase 2	Fresh / pre-freeze	Volume x THI	*
		Morphological abnormalities x THI	*
	Frozen-thawed	Progressive motility x T	**
		Progressive motility x THI	**
		Live sperms x T	***
		Live sperms x THI	**
		Morphological abnormalities x THI	**
		HOS positive sperms x THI	**

*, **, *** depict significant interactions at $0.05<p<0.10$, $p<0.05$ and $p<0.01$, respectively

well as the percentage increase in morphological abnormalities in the frozen-thawed semen were significantly different during different months in Phase 2 *vs.* Phase 1 (Table 4). Moreover, in spite of recording the highest value of HOS positive sperms in pre-freeze semen during February (Table 3), its percentage decline in frozen-thawed semen was the maximum in the same month (Table 4). It's acknowledged that a loss of up to 50% of motile sperm post-freezing is typical [22]. In Phase 1, the observed changes in frozen-thawed semen parameters were within this range, notably better than previous reports on certain Indian goat breeds (Jamunapari and Jakhrana) raised in different locations [23, 24]. However, Phase 2 demonstrated much higher percentage changes ($p<0.01$), rendering the cryopreserved semen unsuitable for storage or use. Recent studies pinpoint optimal semen quality in Barbari bucks during November and December [25], although disparities between fresh and frozen-thawed semen were evident, especially in Phase 2, indicating significant challenges in maintaining viability during cryopreservation (Table 3,4).

The study's climatic trends, depicted in Fig. 2, reveal declining patterns through Phase 1, followed by gradual increases in Phase 2. Amongst different climatic cues, day length has been most important in regulating seasonality in the goats [8], while it remained relatively stable, the THI and T variations significantly impacted semen quality (Table 5). The semen quality deteriorates significantly with an increase in THI in male goats [26, 27]. Lower THI levels (<71) in November and December correlated with the highest semen quality in Barbari bucks. Surprisingly, despite non-stressful conditions in January and February, semen quality deteriorated, particularly in frozen-thawed samples [25]. This highlights the parallel decline in fresh and cryopreserved semen quality. In contrast, we recorded that despite normal fresh and pre-freeze semen attributes, cryopreserved semen quality drastically declined in Phase 2. Contrary to Ranjan et al. [25], we observed THI levels <71 in both phases. This suggests that changing day lengths may influence semen quality, as seen in Iranian Markhoz bucks [11]. Artificial light simulating day length reduction has been used to induce cyclicity in nonbreeding seasons [28]. Factors like breed, location, and genetic variation also impact semen quality [29]. Climatic variables had no effect on fresh semen attributes in Phase 1. THI emerged as the primary variable influencing semen quality across both phases, as depicted in Table 5.

Morphological abnormalities were affected by all three climatic variables in pre-freeze semen during Phase 1. However, during Phase 2, the percentage change in progressive motility, live sperms, and morphological abnormalities in frozen-thawed semen showed significant interaction ($p<0.05$) with THI. This exclusive interaction of morphological abnormalities with climatic variables throughout the study suggests increased sensitivity of the seminiferous epithelium, known to regulate sperm morphology [30]. Sperm abnormalities are considered as indicators of seasonal effects on sperm quality [31]. Cryopreservation damages

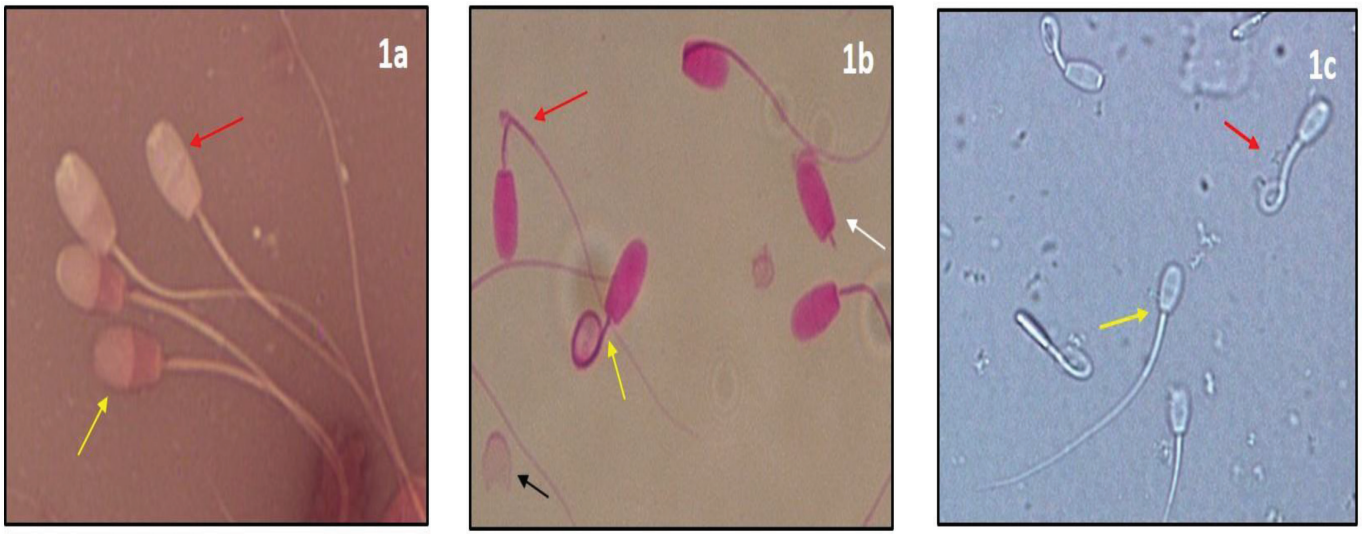

Fig. 1. Microscopic representation of Gaddi goat semen analysis. [1a depicts live (red arrow) and dead (yellow arrow) (Eosin-Nigrosin stain, ×1000); 1b depicts morphological abnormalities (red arrow: bent tail; yellow arrow: coiled tail; white arrow: detached head; black arrow: detached acrosome) (Rose Bengal stain, ×1000); 1c depicts hypo-osmotic swelling (HOS) test (red arrow: HOS reacted sperm; yellow arrow: HOS non-reacted sperms) (×400)].

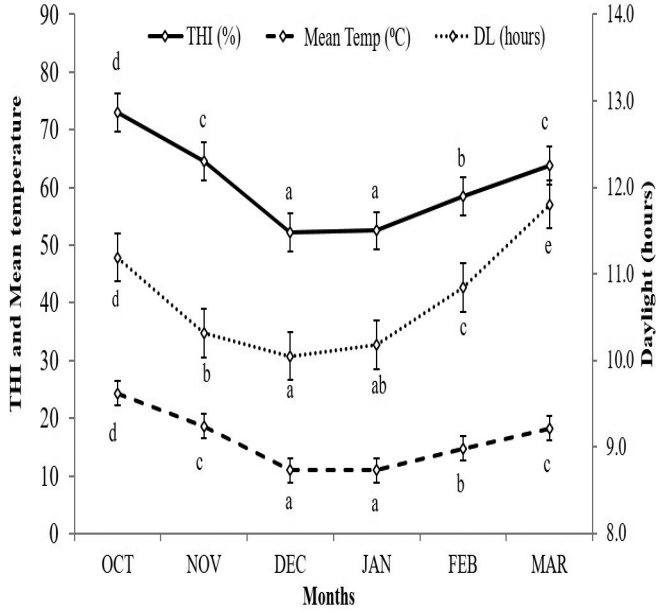

Fig. 2. Month wise changes in the climatic variables (Mean ± SEM) [Phase 1 (October to December) and Phase 2 (January to February) of breeding season in Gaddi male goats. Different superscripts (a-d) within the same variable indicate significant difference ($p<0.05$)].

sperm membranes, including both surface plasma and mitochondrial membranes [32], but the exact mechanisms are not fully understood. It's believed that phospholipids, especially phosphatidylcholine and phosphatidylinositol, which make up most of the sperm membrane, enhance sperm cryotolerance [33]. Seasonal changes in sperm fragility could contribute to variations in sperm cryotolerance during different phases. For example, seminal plasma heparin-affinity proteins with phospholipase A2 activity, which hydrolyze phospholipids into toxic compounds (unsaturated fatty acid and lysophospholipids) for spermatozoa [34], were significantly higher (4.4-folds) during the non-breeding season compared to the breeding season in bucks [7]. These proteins had a more intense adverse effect on sperm viability during the nonbreeding season [7]. While we cryopreserved seminal plasma-free ejaculates, potential changes in sperm fragility due to altered sperm phospholipids: cholesterol ratio, influencing sperm cryotolerance during different phases, require further investigation.

CONCLUSION

To summarize, we found that fresh ejaculates from Gaddi bucks in the Himalayas experienced reduced sperm cryotolerance in the final two months (January and February) of the breeding season at 32°N. As a result, semen collection for cryopreservation should be limited to the first three months (October to December) of the breeding season. Additionally, temperature humidity index emerged as the primary climatic variable affecting most seminal interactions, regardless of semen collection phase or processing stage.

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