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Effects of seasons on enzymatic changes and cholesterol efflux in relation to freezability in Tharparkar bull semen

J. S. Rajoriya[1], J. K. Prasad[1], S. K. Ghosh[1], P. Perumal[2*], Anuj Kumar[3], Shobhana Kaushal[4], Mahak Singh[1]

[1]*Division of Animal Reproduction, Indian Veterinary Research Institute(ICAR), Izatnagar, Bareilly, Uttar Pradesh, India*

[2]*Animal Reproduction Laboratory, National Research Centre on mithun (ICAR), Jharnapani, Nagaland – 797 106*

[3]*Department of Gynaecology, DUVASU, Mathura, Uttar Pradesh, India*

[4]*Division of Animal Genetics, Indian Veterinary Research Institute(ICAR), Izatnagar, Bareilly, Uttar Pradesh, India*

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ABSTRACT

Objective: To assess the effect of different seasons on sperm motility, viability, total sperm abnormality, acrosomal and plasma membrane integrity, antioxidant profiles such as superoxide dismutase (SOD) and catalase (CAT), enzymatic profiles such as aspartate amino transaminase (AST), lactic acid dehydrogenase (LDH) and biochemical profiles such as total cholesterol in Indian breed, Tharparkar bull. **Methods:** Total numbers of 60 ejaculates from 3 bulls were collected through artificial vagina method twice a week during summer and winter season (30 ejaculates from each season). The semen samples were pooled and diluted with the standard TEYC extender and these seminal, biochemical parameters were studied at fresh, pre- freeze and post thaw stage of semen cryopreservation. **Results:** Seminal parameters such as volume, plasma membrane integrity and biochemical parameters such as SOD at fresh semen, acrosomal integrity, LDH, AST and total cholesterol at pre-freeze level and acrosomal integrity, SOD at post thaw stage of cryopreservation were differed significantly ($P< 0.05$) between the seasons. But most of other seminal, biochemical parameters showed no significant difference between the seasons in this pride bull. Forward progressive motility was positively correlated significantly with livability ($P< 0.01$), acrosomal ($P< 0.01$) and plasma membrane ($P< 0.01$) integrity and negatively correlated significantly with total sperm abnormality ($P< 0.05$) at fresh, pre- freeze and post thaw stage of cryopreservation of semen in both winter and summer seasons. Similarly Forward progressive motility was positively correlated significantly with SOD ($P< 0.05$) at pre- freeze level in winter season and at fresh semen in summer season and negatively correlated significantly with AST ($P< 0.05$) at fresh semen in winter season. **Conclusion:** It can be concluded that cryopreservation of Tharparkar bull semen in summer and winter season do not show any definite pattern in relation to seminal and biochemical profile changes and the semen can be cryopreserved in both seasons throughout the year in this prestigious Indian breed of cattle.

1. Introduction

Indigenous breeds of cattle are integral part of traditional agriculture and are progressively diluted due to crossbreeding programme and mechanization of agriculture in India. Indigenous cattle contribute 50 percent of milk production in India and are able to withstand in the extreme conditions. Tharparkar is one of the most important dual purpose indigenous breed and its population in this country is about 5 lakhs. They are well adapted to harsh environmental conditions and are highly resistant to many tropical diseases with good heat tolerance ability. Due to unplanned breeding and crossbreeding programme, number of Tharparkar cattle population is rapidly decreasing, such that this breed is considered as "insecure" according to FAO expert panel. Under these circumstances, it is imperative to improve and conserve this valuable germplasm.

*Corresponding author: P. Perumal, Scientist, Animal Reproduction Laboratory, National Research Centre on mithun (ICAR), Jharnapani, Nagaland – 797 106.
E-mail: perumalponraj@gmail.com

Season is one of the important factors that have influenced the variation in semen quality[1-3] and fertility[4]. Seminal and biochemical parameters are significantly influenced by seasons. Heat tolerance and disease resistance capacity of *Bos indicus* are better than *Bos taurus* bulls characterized by lower values of sperm abnormalities.

Cryopreservation induces damage to spermatozoa are classified into cold-shock, osmotic stress[5] and oxidative stress[6]. Oxidative stress is one of the important causes of sperm damage and elicited greater interest in recent years[6, 7]. Normally mammalian seminal plasma contains antioxidants such as reduced glutathione, glutathione reductase, superoxide dismutase (SOD) and catalase (CAT) that counteract the toxic effect of reactive oxygen species (ROS) such as superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2). But the quality of semen deteriorated during this cryopreservation process due to dilution, freezing and thawing procedure.

Various functional integrity tests are associated with fertility of bulls[8, 9]. Although semen analysis is a valuable diagnostic tool to assess the fertility status of the bull, conventional parameters used for evaluation of semen have limited application because they only help to assess the structural integrity of the cell[10]. Prediction of potential fertility of a bull on the basis of a single assay is not reliable[11]. However certain functional tests of sperm cells and enzymatic tests of seminal plasma are of great value and could be applied for better evaluation of bovine semen at pre-freeze and post-thaw levels[12]. The leakage of intracellular substances, particularly enzymes into the extra cellular fluid as a consequence of sperm cell damage or increased membrane permeability is currently being used as valuable marker of sperm cell integrity and its fertilizing ability.

A very few studies including routine physico-morphological and functional tests have been done to assess the semen quality at pre-freeze and post thaw level in Tharparkar bull. No systematic study was conducted on enzyme leakage and estimation of antioxidant enzymes in the Tharparkar bull semen. Therefore the present study was designed to study the effects of different seasons on seminal parameters, enzymatic and biochemical profiles of Tharparkar bull semen.

2. Materials and methods

2.1. Animals and semen collection

Three apparently healthy Tharparkar bulls, approximately 4 to 6 years of age, were selected with good body condition (score 5-6) and maintained under uniform feeding, housing and lighting conditions at Germplasm Centre, Indian Veterinary Research Institute, Bareilly, India, is located at an altitude of 564 feet above the sea level and at latitude of 28℃ north and a longitude of 79℃ east. The climate touches both the extremes of cold and hot weather experienced in the country and the relative humidity ranges between 15% and 85%. Each experimental animal was offered *ad libitum* drinking water and concentrate: 1 kg/100 kg b.w, green fodder: 25 kg, dry roughage: 6 kg. Concentrate mixture consists of 30 parts of maize, 30 parts of soy bean meal, 37 parts of wheat bran which are fortified with mineral mixture and salt daily. Semen from Tharparkar bulls was collected using artificial vagina (AV) method (40 cm long and 6.5 cm in diameter) twice a week between 08.00 to 09.00 hrs, in morning before feeding following standard practice in both winter (November to January) and summer (May to July) season. During the study, all the experimental protocols met the Institutional Animal Care and Use Committee regulations.

2.2. Semen processing and evaluation

A total of 60 ejaculates (10 each from 3 bulls for each season), were collected via AV method and pooled each other to reduce the individual bull effect. Immediately after collection, the samples were kept in a water bath at 37 ℃ and evaluated for volume, colour, consistency, mass activity, initial individual motility, livability, total morphological abnormality, plasma membrane and acrosomal integrity and total seminal plasm protein as per standard procedure. Moreover the reaction time was also observed. Similarly, biochemical profiles such as SOD, CAT, LDH, AST and cholesterol content were estimated. After the preliminary evaluations, samples were subjected to the initial dilution with pre-warmed (37 ℃) Tris egg yolk citrate extender (TEYC) (Tris-hydroxymethyl aminomethane 3.028% (w/v), sodium citrate 1.655% (w/v), fructose 1.250% (w/v) and egg yolk 20% (v/v); 100 000 IU penicillin G (sodium salt) and 100 mg dihydrostreptomycin were added in 100 mL of buffer). The diluted samples were allowed to study the seminal, biochemical and antioxidant profiles at pre- freeze and post thaw stage of cryopreservation.

Sperm motility was assessed by analyzing four to five fields of view of sample placed on a pre-warmed slide (37 ℃) under pre-warmed cover slip (37 ℃) using bright-field optics (Nikon, Eclipse 80i; magnification 400×). The concentration of spermatozoa (million/ml) in the neat semen was determined using Sperm Quality Analyzer [13].

The count of live spermatozoa was determined using eosin-nigrosin stain [5% (w/v) nigrosin water soluble, 0.6% (w/v) eosin yellow water soluble, and 3% sodium citrate dihydrate; filtered and pH adjusted to 7.0 by adding few drops of 0.1M NaH_2PO_4 or 0.1M Na_2HPO_4] according to a previously described method [14] using bright-field optics (Nikon, Eclipse 80i; magnification 1 000×). Spermatozoa (eosin-nigrosin stained; 200 per sample) were also evaluated under bright-field optics (Nikon, Eclipse 80i; magnification 1 000×) for morphological abnormalities. The acrosomal integrity (percent normal acrosome) based on acrosomal damage in fresh, pre-freeze and post thaw level was studied in Giemsa-stained smears according to the method described by Watson[15].

The hypo-osmotic swelling test (HOST) was used as a complementary test to the viability assessment protocol to evaluate the functional integrity of the sperm plasma membrane. HOST relies on the resistance of the membrane to loss of permeability under stress condition of swelling in a hypo osmotic medium[16]. Sperm cells with resistant membranes exhibited swelling around the tail such that the flagella become curled and the membrane maintained a swollen bubble around the curled flagellum. HOST was performed after slight modification of the method described by Jeyendran *et al.* [17] in fresh, pre-freeze and post thaw

samples to assess the functional integrity of the sperm tail membrane which gives an idea of spermatozoal membrane integrity. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

2.3. Estimation of SOD activity

The SOD activity of the seminal plasma was estimated using the method as described by Madesh and Balasubramanian [18] with some modifications. The reaction mixture consisted of 100 μL seminal plasma, 60 μL 1.25 mM MTT, 1280 μL PBS and 15 μL 1 mM pyrogallol. Pyrogallol solution was prepared freshly every time to prevent auto oxidation. In blank, enzyme/seminal plasma were replaced with same amount of PBS. The reaction of formation of formazan crystals by reduction of MTT was terminated by addition of 1.5 mL DMSO and the readings were taken spectrophotometrically at 570 nm using Double beam UV-VIS Spectrophotometer (DBS; Model-UV5704SS, ECIL, India). One unit of SOD was defined as the amount of protein required to inhibit the MTT reduction by 50%. The total SOD activity was expressed in units per mg of protein present in seminal plasma.

2.4. Estimation of catalase activity

Activity of catalase enzyme was estimated by spectrophotometric method as described by Bergmeyer [19] and were expressed as mM H_2O_2 utilized /min /mg protein. Briefly, in a test tube, 2.0 mL phosphate buffer and 10 μL seminal plasma (1:10 dilution) were added, the contents were mixed and transferred to the cuvette. 1.0 mL of H_2O_2 was added directly into the cuvette and the optical density was recorded every 30 sec for 2 min at 240 nm against distilled water taken as blank. Catalase activity was calculated using following formula

$$\frac{\Delta OD/time}{0.067} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times \frac{1}{\text{mg of protein}}$$

ΔOD: change in optical density per unit time, and 0.067-volume fraction.

2.5. Estimation of biochemical profiles

Biochemical profiles such as AST and LDH were estimated using commercially available diagnostic kits (SPAN Diagnostics Ltd, Surat, India) and total seminal plasma protein was estimated with the standard procedure described by Lowry *et al.* [20].

2.6. Statistical analysis

The results were analysed statistically after arcsine transformation of percentage data by using SPSS 15 (SPSS, Chicago, IL, USA). Means were analysed by one-way ANOVA followed by the Tukey's post hoc test to determine significant differences between the fresh, pre- freeze and post thaw stage of cryopreservation and Student's '*t*' test was used to assess the difference between the winter and summer season for different seminal parameters at fresh, pre- freeze and post thaw stage of cryopreservation. The data were expressed as mean ± SEM.

3. Results

In the present study, total of 60 ejaculates (10 each from 3 bulls for each season) from Tharparkar bulls were evaluated for their physico-morphological, antioxidant and biochemical profiles at fresh, pre-freeze and post thawed stage in two different seasons (winter and summer).

The mean reaction time was recorded as (107.75±26.94) in winter and (157.63±17.98) second in summer season. There was a significant ($P<0.05$) difference between the two seasons (Table 1) was observed.

3.1. Physio-morphological characters

3.1.1. Fresh semen evaluation

The ejaculate volume ranged from 2 to 4.5 and 2.4 to 6.5 mL in winter and summer season, respectively. The mean volume in summer season was significantly ($P<0.05$) higher in comparison to winter ejaculates (Table 1). Similarly plasma membrane integrity of sperm (Table 2) was also significantly ($P<0.05$) differed between the seasons and was higher in winter season. Other seminal parameters such as mass activity, sperm concentration, progressive motility, acrosomal integrity and total abnormal sperm percentage were non-significantly differed between the seasons (Table 2).

Table 1

Physico-morphological seminal attributes of fresh ejaculates during winter and summer season in Tharparkar bulls.

Seminal attributes	Season	
	Winter	Summer
Reaction time (sec.)	107.75±26.94[a]	157.63±17.98[b]
Volume (mL)	3.56±0.22[b]	4.45±0.18[a]
Mass activity (Grade)	4.03±0.11	3.97±0.13
Concentration(x10^6/mL)	1 093.73±71.26	936.51±67.52
TSPP (mg/dL)	9.99±0.38	9.68±0.51

Within rows means with different letters (a, b) differ significantly ($P<0.05$).

Table 2

Physico-morphological seminal attributes during winter and summer season in Tharparkar bulls at fresh, pre-freeze and post thaw stage.

Seminal attributes	Stages of cryopreservation	Season	
		Winter	Summer
Forward progressive Motility (%)	Fresh	84.00±3.03	83.00±2.16
	Pre-freeze	75.67±1.04	74.33±1.24
	Post thaw	51.33±1.04	49.53±1.09
Livability (%)	Fresh	88.23±1.82	88.60±1.71
	Pre-freeze	80.70±2.86	81.73±3.95
	Post thaw	70.03±1.88	69.30±0.96
Total sperm abnormality (%)	Fresh	9.47±0.43	9.30±0.35
	Pre-freeze	11.93±0.49	12.17±0.48
	Post thaw	14.90±0.61	15.20±0.45
HOST (%)	Fresh	81.43±1.49[a]	78.20±1.94[b]
	Pre-freeze	69.57±1.94	68.73±1.85
	Post thaw	51.13±1.22	48.97±1.20
Acrosomal integrity (%)	Fresh	90.77±1.68	91.47±1.61
	Pre-freeze	83.70±2.85[a]	86.57±3.76[b]
	Post thaw	72.63±1.21[a]	75.83±0.91[b]

Within rows means with different letters (a, b) differ significantly ($P<0.05$).

3.1.2. Pre-freeze semen evaluation

Pre-freeze semen evaluation results revealed a significant ($P<0.05$) difference was observed in acrosomal integrity between the winter and summer seasons (Table 2), whereas other seminal parameters such as forward progressive motility, livability and plasma membrane integrity were revealed non-significantly differed between seasons. But total sperm abnormality was non- significantly higher in summer season than in winter season semen in Tharparkar bull (Table 2).

3.1.3. Post thaw semen evaluation

Similar to pre-freeze semen evaluation, post thaw semen evaluation revealed that there was significant ($P<0.05$) difference in acrosomal integrity between the winter and summer ejaculates (Table 2). But other seminal parameters such as forward progressive motility, livability and plasma membrane integrity revealed non-significantly higher values in winter month semen than summer month semen, whereas total sperm abnormality was higher summer season than winter season semen in Tharparkar bull.

3.2. Biochemical characteristics

Biochemical analysis of fresh semen revealed that there was significant ($P<0.05$) difference in SOD and AST activity between the winter and summer months, but other biochemical profiles of semen CAT, LDH, total cholesterol and total protein were not significantly different between winter and summer semen ejaculates in Tharparkar bull (Table 3). Similarly at pre-freeze stage of semen cryopreservation, LDH, AST and total cholesterol content were significantly ($P<0.05$) differed between the seasons. But SOD and CAT differed significantly ($P<0.05$) at post thaw stage between the seasons in Tharparkar bull, whereas other parameters non-significantly differed between the seasons at different stage of cryopreservation.

Table 3

Biochemical profiles of Tharparkar bull seminal plasma during winter and summer season in at fresh, pre-freeze and post thaw stage of semen preservation.

Seminal attributes	Stages of cryopreservation	Season	
		Winter	Summer
SOD (U/mL)	Fresh	137.45±10.52[a]	158.87±2.47[b]
	Pre-freeze	119.27±3.59	118.11±4.09
	Post thaw	77.86±2.98[a]	69.14±1.80[b]
CAT (U/mg protein)	Fresh	1.01±0.06	1.11±0.08
	Pre-freeze	0.51±0.07	0.66±0.08
	Post thaw	0.20±0.08[a]	0.35±0.03[b]
LDH (IU/L))	Fresh	274.49±14.22	244.96±13.95
	Pre-freeze	440.63±20.78[a]	508.12±24.88[b]
	Post thaw	929.04±21.06	905.10±29.78
AST (IU/L)	Fresh	19.69±1.26[a]	30.69±1.44[b]
	Pre-freeze	46.97±1.48[a]	53.63±1.16[b]
	Post thaw	137.22±8.14	152.04±4.66
Total cholesterol (μ g/10^8 sperm)	Fresh	30.75±1.95	27.34±1.24
	Pre-freeze	23.09±1.53[a]	19.85±0.86[b]
	Post thaw	12.65±0.74	11.86±0.49

Within rows means with different letters (a, b) differ significantly ($P<0.05$)

3.3. Correlation among different seminal attributes and enzyme activities at fresh pre-freeze and post thaw level

3.3.1. Fresh semen evaluation

In fresh semen, there was significant positive correlation between the progressive motility with mass activity, livability, acrosomal and plasma membrane integrity of sperm and significant negative correlation was observed between the forward progressive motility and total sperm abnormality in the winter season ejaculates (Table 4). Similarly, there was significant positive correlation between the progressive motility with livability, acrosomal, plasma membrane integrity and cholesterol content of semen and significant negative correlation was observed between the plasma membrane integrity and total sperm abnormality in the summer month ejaculates in Tharparkar bulls (Table 7).

Biochemical parameters such as AST and total protein were significantly negatively correlated with forward progressive motility and mass activity in winter month ejaculates (Table 4), whereas summer ejaculates have significant positive correlation between SOD and forward progressive motility and significant negative correlation between the cholesterol content and mass activity, livability and acrosomal integrity (Table 7).

3.3.2. Pre- freeze semen evaluation

Correlation analysis revealed that there was significant positive correlation between the forward progressive motility with livability, acrosomal and plasma membrane integrity of sperm and significant negative correlation was observed with total sperm abnormality in the winter month ejaculates (Table 5). Similarly, significant positive correlation between the progressive motility with livability, acrosomal and plasma membrane integrity of semen of summer month ejaculates were observed (Table 8). Biochemical parameter such as SOD was significantly positively correlated with forward progressive motility and acrosomal integrity in winter month ejaculates (Table 5).

3.3.3. Post thaw semen evaluation

Similar to fresh and pre-freeze stage, there was significant positive correlation between the forward progressive motility with livability, acrosomal and plasma membrane integrity of sperm in the winter month ejaculates (Table 6). Significant positive correlation between the forward progressive motility with livability, acrosomal integrity and significant negative correlation in the forward progressive motility and livability with total sperm abnormality were observed in the summer month ejaculates (Table 9).

Table 4

Correlation coefficient (*r*-value) among different fresh seminal parameters and enzymatic constituents in winter semen of Tharparkar bull.

Seminal attributes	Concentration	Mass activity	Individual motility	Livability	Acrosomal integrity	HOST	Total sperm abnormality	TSPP	Catalase	LDH	SOD	AST	Total cholesterol
Volume	0.12	0.23	0.19	0.16	0.15	0.15	−0.13	−0.18	0.05	0.03	−0.23	−0.04	−0.04
Concentration		0.83**	0.57**	0.52**	0.44*	0.13	−0.08	−0.31	−0.27	0.13	−0.03	−0.33	0.14
Mass activity			0.75**	0.69**	0.66**	0.19	−0.29	0.49**	−0.05	0.17	0.10	−0.43*	0.12
Individual motility				0.85**	0.81**	0.44*	−0.38*	0.36*	0.05	−0.05	0.30	−0.46*	0.15
Livability					0.97**	0.51**	−0.27	−0.17	0.06	0.04	0.23	−0.19	0.11
Acrosomal integrity						0.52**	−0.32	−0.19	0.02	0.00	0.25	−0.18	0.14
HOST							−0.48**	−0.00	0.00	−0.02	0.07	0.15	0.28
Total sperm abnormality								0.21	−0.16	0.06	−0.16	−0.06	−0.03
TSPP									−0.23	−0.13	−0.19	0.25	−0.11
Catalase										0.16	0.13	0.16	−0.03
LDH											−0.12	−0.19	−0.26
SOD												−0.14	0.09
AST													0.10
Total cholesterol													1.00

**Correlation is significant at the 0.01 level ($P< 0.01$). *Correlation is significant at the 0.05 level ($P< 0.05$)

Table 5

Correlation coefficient (*r*-value) among different pre-freeze seminal parameters and enzymatic constituents in winter semen of Tharparkar bulls

Seminal attributes	Livability	Acrosomal integrity	HOST	Total sperm abnormality	Catalase	LDH	SOD	AST	Total cholesterol
Individual motility	0.93**	0.82**	0.77**	−0.56**	0.02	−0.30	0.36*	−0.30	0.07
Livability		0.83**	0.70**	−0.56**	−0.09	−0.28	0.24	−0.27	−0.07
Acrosomal integrity			0.59**	−0.34	−0.07	−0.22	0.46**	−0.23	0.07
HOST				−0.46*	0.29	−0.09	0.22	−0.28	0.02
Total sperm abnormality					0.07	0.10	0.04	0.21	0.20
Catalase						0.00	0.10	−0.03	0.05
LDH							−0.10	0.13	−0.01
SOD								−0.05	−0.07
AST									0.26
Total cholesterol									1.00

**Correlation is significant at the 0.01 level ($P< 0.01$). *Correlation is significant at the 0.05 level ($P< 0.05$).

Table 6

Correlation coefficient (*r*-value) among different post thaw seminal parameters and enzymatic constituents in winter semen of Tharparkar bulls.

Seminal attributes	Livability	Acrosomal integrity	HOST	Total sperm abnormality	Catalase	LDH	SOD	AST	Total cholesterol
Individual motility	0.47**	0.79**	0.58**	−0.32	0.07	−0.16	0.20	−0.05	−0.11
Livability		0.32	0.38*	−0.19	−0.06	−0.17	0.03	−0.18	0.05
Acrosomal integrity			0.44*	−0.27	0.23	−0.06	0.04	−0.26	0.02
HOST				−0.38*	−0.12	−0.17	0.26	−0.07	0.05
Total sperm abnormality					0.06	0.16	−0.11	0.13	−0.06
Catalase						−0.14	−0.33	0.17	−0.08
LDH							0.10	−0.20	−0.15
SOD								−0.11	0.00
AST									−0.07
Total cholesterol									1.00

**Correlation is significant at the 0.01 level ($P<0.01$). *Correlation is significant at the 0.05 level ($P<0.05$).

Table 7

Correlation coefficient (r-value) among different fresh seminal parameters and enzymatic constituents in summer semen of Tharparkar bulls

Seminal attributes	Concentration	Mass activity	Individual motility	Livability	Acrosomal integrity	HOST	Total sperm Abnormality	TSPP	Catalase	LDH	SOD	AST	Total cholesterol
Volume	0.23	0.32	0.28	0.32	0.28	0.24	0.19	0.11	0.02	0.06	0.10	0.18	0.22
Concentration		0.54**	0.52**	0.50**	0.47**	0.52**	0.00	0.03	0.02	0.03	0.06	0.01	0.13
Mass activity			0.81**	0.84**	0.78**	0.70**	−0.06	0.06	0.12	−0.15	0.08	−0.16	−0.46**
Individual motility				0.93**	0.90**	0.84**	−0.31	0.15	0.01	−0.12	0.36*	0.00	0.41*
Livability					0.96**	0.81**	−0.27	0.14	0.03	−0.08	0.09	−0.03	−0.49**
Acrosomal integrity						0.76**	−0.31	0.14	0.06	−0.05	0.02	−0.01	−0.44*
HOST							−0.34*	0.12	0.14	−0.20	0.10	−0.26	0.34
Total sperm abnormality								0.07	−0.26	0.01	−0.05	0.02	−0.26
TSPP									0.24	−0.21	0.05	−0.16	0.15
Catalase										0.03	0.34	0.32	0.05
LDH											0.13	0.09	0.21
SOD												−0.39*	0.02
AST													0.21
Total cholesterol													1.00

**Correlation is significant at the 0.01 level ($P< 0.01$). *Correlation is significant at the 0.05 level ($P< 0.05$).

Table 8

Correlation coefficient (r-value) among different pre-freeze seminal parameters and enzymatic constituents in summer semen of Tharparkar bulls.

Seminal attributes	Livability	Acrosomal integrity	HOST	Total sperm abnormality	Catalase	LDH	SOD	AST	Total cholesterol
Individual motility	0.84**	0.67**	0.63**	−0.08	0.12	0.16	0.00	0.19	0.17
Livability		0.80**	0.73**	−0.11	0.14	0.06	0.01	0.21	0.05
Acrosomal integrity			0.64**	0.01	0.04	0.11	0.02	0.21	0.10
HOST				0.09	0.01	0.02	0.22	0.15	0.07
Total sperm abnormality					−0.31	0.06	0.15	0.20	−0.11
Catalase						0.03	0.01	−0.21	0.12
LDH							0.08	−0.12	0.00
SOD								0.01	0.15
AST									0.26
Total cholesterol									1.00

**Correlation is significant at the 0.01 level ($P< 0.01$). *Correlation is significant at the 0.05 level ($P< 0.05$).

Table 9

Correlation coefficient (r-value) among different post thaw seminal parameters and enzymatic constituents in summer semen of Tharparkar bulls.

Seminal attributes	Livability	Acrosomal integrity	HOST	Total sperm abnormality	Catalase	LDH	SOD	AST	Total cholesterol
Individual motility	0.66**	0.68**	0.30	−0.36*	0.08	0.28	0.18	−0.24	0.00
Livability		0.87**	0.24	−0.47**	0.04	0.14	0.17	−0.14	0.24
Acrosomal integrity			0.20	0.32	0.09	0.05	0.04	−0.07	0.17
HOST				0.05	0.01	0.14	0.00	−0.11	0.35
Total sperm abnormality					0.08	0.08	−0.08	0.15	−0.07
Catalase						0.21	0.31	0.00	0.02
LDH							−0.31	0.20	−0.19
SOD								−0.04	0.16
AST									−0.10
Total cholesterol									1.00

**Correlation is significant at the 0.01 level ($P< 0.01$). *Correlation is significant at the 0.05 level ($P< 0.05$).

4. Discussion

4.1. Physico-morphological characteristics in fresh semen

The motive of this present research was to study the physico-morphological characteristics along with the concentration of seminal plasma protein, SOD, catalase, LDH, AST and total cholesterol in relation to semen freezability in different seasons in Tharparkar bulls.

4.1.1. Reaction time

In the present study, reaction time in Tharparkar bulls varied from 58 to 307 seconds (1–5 minutes), while other workers reported the variation in reaction time from 5.10 to 10.43 minutes[21]. The lower reaction time in present study might be due to differences in climatic changes, and training and management of bulls.

4.1.2. Ejaculate volume

A significant higher semen volume in summer season (4.45±0.18 mL) in comparison to winter season (3.56±0.22 mL) was observed in present study. A similar finding with higher volume of semen was reported during summer season followed by winter season[22]. In contrast to present finding, Sinha and Prasad[23] reported higher volume in winter (5.6 mL) than summer (5.01 mL) in Tharparkar bulls. Ejaculate volume varies from breed to breed and within a breed from bull to bull[24]. The semen volume may be influenced by various factors like body weight, scrotal size, age, pre-coital stimulation, frequency and season of semen collection[25]. The lower volume in present study might be due to and seasonal effect.

4.1.3. Sperm concentration

In present study, mean values of sperm concentration did not show any significant difference between winter and summer season. Seasonal variations in the concentration of spermatozoa of *Bos taurus* and Zebu bulls are not significant[26]. Several authors reported similar sperm concentration in Tharparkar semen [27]. In present study, sperm concentration varied between winter and summer season and this variation may be due to frequency of semen collection from different bulls.

4.1.4. Mass activity

The mass motility of fresh semen was examined in both season immediately after collections. No significant difference in mass activity was recorded in semen ejaculate of winter and summer season. Similar reports were observed by other authors in Tharparkar bull[27].

4.1.5. Initial progressive motility

The initial motility of semen was not influenced by winter and summer season in present study. However, season appears to have some control over the initial motility especially winter and spring season [28]. Humid hot season was not desirable for the production of highly motile spermatozoa. Initial progressive motility in Tharparkar bull semen in present study was in agreement with Rao and Rao[24]. However, Rafiq [27] has obtained lower motility percentage. Similar result of mass and initial progressive motility in the present finding in both winter and summer season in an indication that season does not have significant effect on these parameters.

4.1.6. Percent live spermatozoa

The percent live spermatozoa observed in winter and summer semen ejaculates were in agreement with Rao and Rao[24]. Non-significantly higher sperm livability was observed in the winter season as lower live percentage was observe during humid hot season in Hariana bulls [28].

4.1.7. HOST positive spermatozoa

Season had significant effect on HOST positive spermatozoa as evidenced by significantly higher HOST positive spermatozoa observed in winter season as HOST asses the functional and physiological aspect of the membrane permeability. The higher value of HOST positive in the present study indicates higher percentage of membrane integrity of the spermatozoa. The higher percentage of HOST positive spermatozoa in Tharparkar bull semen than in most of the crossbred bull semen [29, 30] is as indicative of superiority of Tharparkar bull semen over the crossbred.

4.1.8. Acrosomal integrity

Acrosome can be detached from the sperm head under the influence of different physical and chemical factors[30]. Optimum fertility depends on the acrosome being structurally and functionally intact [30]. In the present study, no significant change was observed between the seasons.

4.1.9. Total sperm abnormality

In the present study, non-significant difference was in total abnormal sperm percentage between the seasons. But no literature is available to compare with Tharparkar bulls. Similar report was observed in zebu and buffalo bulls[25]. Moreover, Tomar *et al.* [28] observed that there was no seasonal variation in the abnormal percentage of live sperms in Indian cattle. No seasonal variation in relation to sperm abnormality in Tharparkar bulls indicates it superiority and better adjustment in both the extremes of temperature variation in comparison to other breeds/ crossbred.

4.2. Pre-freeze and post thaw seminal characteristics

4.2.1. Per cent motile spermatozoa

In present study, mean post thaw motility in winter and summer season did not differ significantly. Similar report was reported in Tharparkar bull semen [27]. But in other Indian breed such as Sahiwal bull showed significant variation between the summer and winter season [22]. At pre-freeze stage significant positive correlation of sperm motility with live percentage, acrosomal integrity and HOSTwas observed in both winter and summer season. While negative correlation was observed with sperm abnormality in winter season.

At post thaw stage, it was also significant positively correlated with live percentage in both winter and summer season. However, positive correlation with HOST was observed in winter season. This indicates better quality of semen possibly due to higher number of live spermatozoa and also because of intactness of sperm cell membrane.

4.2.2. Live spermatozoa

There was no significant effect of winter and summer season on pre-freeze and post thaw livability of spermatozoa in Tharparkar bull semen. Present research finding of sperm livability at pre-freeze level was in agreement with Rafiq[27]. However, it was higher in respect to post thaw livability.

4.2.3. HOST positive spermatozoa

The integrity of the sperm plasma membrane, motility and acrosomal integrity are essential for the fertilizing capacity of spermatozoa[31]. Percent HOST positive spermatozoa in winter and summer season at pre-freeze level which was in agreement with the finding of Rafiq [27]. But no significant difference was observed in the present study. HOST positive spermatozoa at pre-freeze and post thaw in winter season and pre-freeze stage in summer season was significantly

correlated with motility and acrosomal integrity probably because of higher number of intact acrosome and normal membrane integrity of the spermatozoa in both the seasons of semen collection.

4.2.4. Acrosomal integrity

In present study, significant variation in acrosomal integrity was observed between the seasons at pre-freeze and post thaw stage which was similar as reported in the previous study by Verma [32] in crossbred bull. The difference in percent intact acrosome from pre-freeze to post thaw stage between winter and summer semen might be due to higher percentage of intact acrosome spermatozoa at fresh stage and often superiority of sperm resistance in summer season in comparison to other breeds of cattle. To our knowledge no reference is available at present regarding acrosomal integrity in different seasons in Tharparkar bulls.

4.2.5. Total sperm abnormality

Though the percent abnormal spermatozoa was no significant correlation between summer and winter season but it was well within the permissible range [33].

4.3. Biochemical characteristics

4.3.1. Total seminal plasma protein

Proteins in the seminal fluid influence various functions of the sperm such as motility, capacitation, acrosome reaction, DNA integrity and interaction with oocyte [34]. The levels of total seminal plasma protein in fresh semen in the present study were similar in both winter and summer seasons. Therefore, this parameter would not be an indicator for the seasonal variation in semen of Tharparkar bull at initial stage. Similarly, Singh *et al.* [35] reported a positive association of the protein values in the semen with its freezability. The reason could be the great importance of protein for the motility and the survival of spermatozoa during storage [34].

4.3.2. Cholesterol content

Cholesterol is known to be one of the major components of seminal plasma [36]. Several studies have demonstrated that cholesterol influx reduces of spontaneous acrosome reaction [37]. However, its efflux mimics the capacitation and acrosome reaction [38]. The cholesterol content of bull spermatozoa varies between spermatozoa and wide variation between the bulls as well as between ejaculates. But, no literature available on cholesterol content in Tharparkar bull semen and spermatozoa. A significant reduction of cholesterol content after cryopreservation recorded in the study was in agreement with the reports of Srivastava *et al.* [39]. Cholesterol content was significantly negatively correlated with mass activity, IPM, live percentage and acrosomal integrity which is a desirable relationship in summer this also reflects the superiority and resistance of spermatozoa in the summer season.

4.3.3. Enzyme activity

4.3.3.1. Antioxidant enzyme activities

In the present study, the antioxidant, dehydrogenase and transaminase enzyme activities were measured at fresh, pre-freeze and post thaw level to ascertain the protective action as well as leakage of enzyme during preservation of semen at ultra low temperature. No literature is available for the level of antioxidants (SOD and CAT) in Tharparkar bull seminal plasma. Most of the studies are related to incorporation of these enzymes as additives in cryopreservation process in crossbred bulls [30].

Mean SOD activity was significantly higher in summer in comparison to winter season. At pre-freeze level there was no significant change in the concentration of SOD activity. However, at post thaw stage significantly higher SOD level in winter season was observed. The reason for decrease in SOD activity during freezing may be due to the utilization of SOD in neutralizing the ROS level produced by the dead spermatozoa. In the present observation, there was a decline in SOD activity after freeze thaw and the extent of the reduction was not uniform. Similar findings were reported in human and bull semen [40]. Cryopreservation significantly reduced sperm SOD activity by 50% [41] in HF bull semen and a similar observation was found in the present study. The variation may be due to the different assay methods applied or units of expression of sample used and breed variation. The pre-freeze SOD activity in the winter season was significantly positively correlated with percent PFM and acrosomal integrity.

SOD and catalase are on the front line of defense mechanisms against the toxic effect of reactive oxygen species. SOD spontaneously dismutates (O^{2-}) anion to form O_2 and H_2O_2, while catalase converts H_2O_2 to O_2 and H_2O. SOD activity in summer season was significantly negatively correlated with AST activity in the present finding which are in agreement with Gebreselassie [30]. Such a relationship between SOD and AST is desirable for better freezability of the semen. In present study there was no significant difference in catalase activity between seasons at fresh stage. However, there was significantly higher catalase activity at post thaw seminal plasma in summer season.

4.3.3.2. Dehydrogenase and transaminase enzyme activity

Dehydrogenase and transaminase enzyme activity were measured in seminal plasma at fresh, pre-freeze and post thaw level in both winter as well as summer season to find out the level of enzyme leakage due to membrane damage during cryopreservation. LDH is an enzyme of almost universal distribution in the body which catalyses the reversible transamination of pyruvate to lactate. In semen, it is chiefly located in the mid-piece region [42].

In present study, there was significant difference from pre-freeze to post thaw in LDH activity in winter and summer season. The reason may be due to damage of sperm plasma membrane during cryopreservation and loss of LDH from the cell. LDH activity at pre-freeze and post thaw level was less in comparison to the works reported by Dhami [42] in crossbred bulls, this indicates more intactness of sperm plasma membrane of Tharparkar bull spermatozoa during freezing and thawing process.

The post thaw LDH activity in the winter and summer ejaculates have no any significant correlations with either of the parameters at post thaw level. Significant correlation between LDH activity of semen and its post thaw survivability/freezability and fertility [43] was observed.

AST activity was not influenced by seasonal changes in Tharparkar bull semen. Significant higher AST activity at pre-freeze stage was perhaps due to the variation in the concentration of this enzyme in the neat seminal plasma. Higher activity of AST at post thaw seminal plasma clearly indicated that much of the enzyme leaked out in the extracellular fluid following deep freezing of semen due to structural damage and increase cell membrane permeability. This was in agreement with Verma [32] in cattle semen.

It was concluded from the present study that most of seminal and biochemical parameters were showed no significant difference between the seasons. This indicates no seasonal effect on seminal and biochemical profiles of Tharparkar bull semen and the semen can be cryopreserved throughout the year in this prestigious Indian breed.

Conflict of interest statement

We declare that we have no conflict of interest.

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