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Studies on effect of different seasons on expression of HSP70 and HSP90 gene in sperm of Tharparkar bull semen

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ABSTRACT

Objective: To assess the effect of different seasons on expression of HSP70 and HSP90 genes of spermatozoa 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 semen samples were allowed to study the expression of HSP 70 and HSP 90 genes of spermatozoa with commercially available kit. **Results:** No significant difference was observed in spermatozoal mRNA expression of HSP 70 and HSP 90 during winter and summer season in this bull semen. But the mRNA expression of both HSP 70 and HSP 90 during summer season was found non-significantly higher in comparison to winter season. **Conclusion:** It was concluded from the present study that there was no significant difference in the mRNA expression of HSP 70 and HSP 90 between the winter and summer season, presence of similar type of stress resistant spermatozoa in Tharparkar bull semen and the semen can be cryopreserved throughout the year in this prestigious Indian breed.

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.

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* (*B. indicus*) are better than *Bos taurus* (*B. Taurus*) bulls characterized by lower values of sperm abnormalities.

Seminal plasma, which is a complex mixture of secretions originating from the testis, epididymis and accessory glands contain factors that modulate the fertilizing ability of the sperm. Seminal plasma factors also have an influence on sperm storage. There is evidence revealing that seminal plasma prevents premature capacitation of sperm and protects sperm from peroxidative damage. Some accessory

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sex gland proteins bind to the spermatozoa membrane and affect its function and properties[5]. Seminal plasma of bulls contains several proteins[6], some of which are on the surface of ejaculated sperm[7]. The potential influence of seminal plasma proteins on male reproduction came to attention because of the studies showing that their expression is associated with fertility and freezability indices of dairy bulls[8, 9], beef bulls[10] and horses[11].

The synthesis of a group of proteins called heat shock proteins (HSPs) results during heat stress[12] and these HSPs protect cells from toxic effects of heat and other stresses [13]. The induction of HSPs is remarkably rapid and intense as an emergency response. HSP expression has also been correlated with resistance to stress and thresholds for HSP expression are correlated with levels of stress they naturally undergo[14]. Up regulation of the synthesis of a number of these proteins upon environmental stress establishes a unique defense system to maintain cellular protein homeostasis and to ensure survival of the cell. This increase in expression is transcriptionally regulated. Wu[15] reported that dramatic up regulation of the HSPs is a key part of the heat shock response and is induced primarily by heat shock factor (HSF).

These HSPs interfere with several heat shock processes within cell organelles and proper functioning are translocated to different compartments following stress induced synthesis. The role of HSPs has been identified in most of the livestock species[16]. Lymphocytes isolated from large animals and heat stressed in vitro and respond by synthesizing heat stressed proteins[16]. This cellular response may be an important mechanism by which animals are able to protect cells from heat stress. The increased HSP 70 expression has positively correlated to DNA damage detected in mice sperm[17]. Further, no study has been conducted on heat shock protein (HSP 70 and HSP 90) preventing heat stress to spermatozoa. Therefore the present study was designed to study the effects of different seasons on expression of HSP 70 and HSP 90 of spermatozoa of Tharparkar bull semen.

2. Material 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) 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 BW, 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 routine seminal parameters as per standard procedure.

2.3. Expression of HSP70 and HSP90 gene

The study was conducted with use of commercially available kits such as RevertAid™ cDNA synthesis Kit (Fermentas, USA), Block PCR kit (New England Biolabs Inc), DyNAmoTM HS SYBRR Green qPCR kit (Finnzymes, Finland).

2.3.1. Primer sequences

To amplify the genes, a set of gene specific primers were designed from the published sequence. These primers were synthesized by the IDT (Integrated DNA Technologies) using Beacon = and the details have been given in the Table 1.

2.3.2. Isolation of total RNA using Trizol reagent

Dissolve the sperm pellet with 100 -200 μL PBS (1×) then

Table 1
Gene transcripts, primer sequences and resulting fragment size.

Target	Sequence of nucleotide	Fragment size (bp)	EMBL/Reference
HSP70	For: 5′-GACGACGGCATCTTCAAG -3′; Rev: 5′-GTTCTGGCTGATGTCCTTC -3′	132	FJ975769.1
HSP90	For: 5′-GCATTCTCAGTTCATTGGCTATCC- 3′ Rev: 5′-GTCCTTCTTCTCTTCCTCCTCTTC- 3′	190	NM_001012670.1
Beta Actin	For: 5′-AGTTCGCCATGGATGATGA-3′ Rev: 5′- TGCCGGAGCCGTTGT-3′	54	NM_001009784.1

EMBL- accession number or reference of published sequence

transfer to 1.5 mL centrifuge tube (if fresh) or take out the stored sperm from -20 ℃ and thaw at room temperature for 10-15 min; Add 1 mL heated Trizole (60 ℃) reagent, mix properly and aspirate by using 26 G needle and syringe (25-30 times) and keep for 30 min. at room temp (20 ℃); Add 200 μL chloroform and mix gently by rotate the tube for 15-30 times and slight vortex for 30 s then centrifuge at 12 000 rpm at 4 ℃ for 15 min; Transfer upper layer into fresh micro-centrifuge tube and add 0.5 mL isopropanal then mix by inverting the tube 20 times and incubate at room temperature for 10 min; Then centrifuge at 12 000 rpm at 4 ℃ for 15 min and discard the supernatant; Wash the pellet with 70% ethanol, repeat centrifugation as before and remove ethanol; Air-dry the RNA pellet for 20 min and re-suspend in 10-20 μL of RNase/DNase free water dissolve by keeping at 60 ℃ for 10 min; RNA samples were stored at -80 ℃.

2.3.3. Quantification of RNA

The purity of total RNA was checked using the spectrophotometer by Nanodrop reading. Quantification of RNA was done spectrophotometrically. 1 μL of total RNA was used and absorbance at 260 nm and 280 nm wavelengths were recorded against nuclease free water as blank. RNA samples showing the OD 260: OD 280 value more than 1.8 would be expected to contain no protein and taken for further use.

2.3.4. Confirmation of RNA by gel electrophoresis

The quality and integrity of the total RNA was checked using denaturing agarose gel (1%) electrophoresis and visualization under UV light. Two intact bands of 28 s, and 18s with smearing indicated good quality and intactness of RNA.

2.3.5. Synthesis of first strand cDNA

The first strand cDNA was synthesized from the isolated total RNA. RT-PCR was done using reverse transcription system (FERMENTAS, USA) following manufacturers instruction. Reverse transcription was carried out in 20 μL reaction mixtures. Calculation was done by using the concentration of total RNA from Nanodrop reading (ng/ μL) to take 100 μg of total RNA for each reaction and dissolved in nuclease free water to make final volume 11 μL. One microgram of random hexamer primer was added and then incubated at 65 ℃ for 5 minutes. Snap cooled in ice and following mixture was added

Components of reaction mixture: 5× Reaction Buffer (4 μL), dNTP mix. (10 mM, 2 μL), Ribonuclease Inhibitor (20 Units/ μL, 1 μL), Reverse transcriptase enzyme (200 units/ μL, 1 μL).

Reaction mixture was mixed to RNA-primer complex and spinned, followed by incubation at 25 ℃ for 5 minutes and 42 ℃ for 60 minutes. Reaction was stopped by incubating for 5 min at 70 ℃ and finally at 4 ℃ forever. The cDNA is stored at -20 ℃ for long term use.

2.3.6. Confirmation of cDNA with Beta Actin primers

The integrity of the cDNA was checked by PCR with β-Actin primers. The amplification of 54 bp β-Actin gene fragment from the cDNA indicated that the cDNA was made from the RNA extracted from sperm cells and was of good quality.

2.3.7. Optimization of end point PCR

End point PCR conditions were optimized to amplify bull spermatozoa HSP70 and HSP90 gene sequences in gradient thermo cycler. Factor specific primers were used for the amplification of genes. The annealing temperature was standardized using cDNA prepared from mRNA of Tharparkar bull spermatozoa by PCR. The reaction was carried out at different annealing temperatures, primer concentrations, $MgCl_2$ concentration, template DNA and Taq polymerase. The optimum temperature of 58 ℃ for HSP70 and HSP90 were found to be most suitable for annealing for respective primers and was used in subsequent polymerase chain reaction. The concentration of different component which were found suitable for the optimum amplification are as follows: cDNA template(1.0 μL), PCR-H_2O (18.30 μL), 10× Buffer (2.50 μL), Mgcl2 (1.50 μL), dNTP Mix (10 mM, 0.50 μL), Primer For (10 μM, 0.50 μL), Primer Rev (10 μM, 0.50 μL), Roche Taq Polymerase, 0.20 μL).

The above reactants (total volume, 25.0 μL) were added to a nuclease free thin walled 0.2 mL microcentrifuse tube pre-chilled on ice. The contents were gently vortexed and then spun down to collect at the bottom of tube by brief centrifugation.

The reaction was carried out in a thermal cycler using the following cycling parameters that have been found optimum for amplification of gene fragments.

Step I: 95 ℃ for 5 min for initial denaturation; Step II: 95 ℃ for 30 sec for denaturation; Step III: 58 ℃ for HSP70, HSP90 for 30 sec for annealing; Step IV: 72 ℃ for 30 sec extension; Step: II to IV repeated for 35 cycles; Step V: 72 ℃ for 10 min for final extension

2.3.8. Agarose gel electrophoresis

The confirmation of amplification of specific RT-PCR amplicon was done by agarose gel electrophoresis (appendix). 2% agarose was mixed with 40 mL 1X TBE buffer

and melted in a microwave oven. When the molten gel had cooled to about 60 ℃, ethidium bromide (1 μL) was added to final concentration of 0.5 μg/mL. The gel was mixed thoroughly by gentle swirling and then poured into the gel casting tray fitted with the comb. The gel was allowed to solidify and the comb was removed. The PCR product were mixed with 1X gel loading dye (final concentration) and loaded into the wells. For the comparison, a 50 bp molecular weight marker was gel electrophoresed in parallel to the RT-PCR amplicon. The gel was run at a voltage of 5 V/cm till the running dye crossed at least two third of the gel. The bands were visualized under UV light and recorded on a gel documentation system (GELDOC, USA).

2.3.9. Real time PCR

Quantitative Real-time PCR was performed with DyNAmoTM HS SYBRR Green qPCR kit and Stratagene M×3 000 P (Agilent technology USA) spectroflouremetric thermal cycler operated by MxPro™ QPCR software. Reaction setup was performed in area separate from nucleic acid preparation or PCR product analysis. Pipetting was done with sterile filter tips. Exposure of light to the qPCR mastermix was minimized. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. No template control (NTC) was put for gene quantification for checking the contamination in the reaction components other than the cDNA. To ensure the cDNA samples were not contaminated with genomic DNA, reactions were set up using 10 ng of non-reverse transcribed RNA in place of cDNA. Failure to generate a detectable signal signified the samples as DNA free. In negative control, only the real time master mix and primers were added. For reaction set up optically clear caps were used. 1 μL of cDNA was taken. Following master mix was prepared: (Total volume: 19.0 μL).

Nuclease free water (8.0 μL), Primer forward (0.5 μL), Primer reverse (0.5 μL), SYBR green mix (10 μL),

Touching of the optical surface of the caps without gloves was avoided. Strips were centrifuged before starting the cycling programme to spin down the solution to the bottom of the tubes and to remove any possible bubbles. Three segmented qPCR amplification programme was used as given in Table 2.

The amplification and denaturation data was acquired.

Table 2
Thermal cycler protocol of real time PCR.

Segment	Thermal profile	Time	No. of cycles	Comments
Segment 1	95 ℃	15 min	1 cycle	Hot start PCR
Segment 2	95 ℃	10 sec	35 cycles	Denaturation
	58 ℃ for HSP70 and HSP90	30 sec		Annealing
	72 ℃	30 sec		Extension
Segment 3	95 ℃	1 min	1 cycle	Dissociation curve analysis
	65 ℃	30 sec		
	65 ℃-95 ℃	2 degrees per min		
	95 ℃	30 sec		

After the run has ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the "SYBR Green (with Dissociation Curve)" method of the real time machine (Stratagene MxPro3005 (Agilent technologies, USA).

2.3.10. Gene expression analysis

The mRNA expression of the housekeeping gene Beta Actin was not statistically regulated. Thus, it is assumed that equal amounts of mRNA were used in each sample. In order to obtain the mRNA expression differences, the crossing points (CP) was not subtracted from a control group, but from the value 40, so that a high "40-CP" value indicated a high gene expression level and vice versa.

3. Results

3.1. RNA isolation and quantification

Total RNA was isolated from sperms using Trizol reagent by standard protocol. The integrity of total RNA was checked on 1.0% agarose gel using 1× TBE as electrophoresis buffer. Total RNA was in good yield in all the samples. The bands of 28s RNA and 18s RNA reflected the high quality of extracted total RNA (Figure 1).

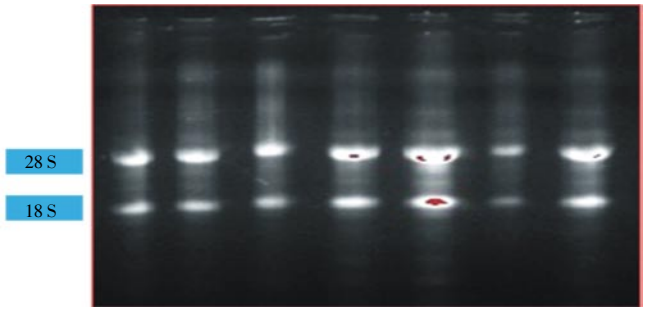

Figure 1. Gel showing integrity of total RNA sample.

The purity and concentration of total RNA was checked using Nanodrop. Isolated RNA samples were free from the protein contamination as the OD 260: OD 280 values were more than 1.8. The concentrations of the RNA samples were in the range of 35-65 ng/μL.

3.2. cDNA synthesis and its confirmation

One hundred nanogram (100 ng) of total RNA was directly used for cDNA synthesis in thermo cycler using cDNA synthesis kit (Fermentas, USA) as per manufactures instructions. cDNA integrity was checked by Beta Actin gene amplification with already published primer whose reaction conditions were already known. After running on 2% agarose gel, single band of 54 bp was visualized (Figure 2). In order to ensure the amplification of specific fragment with higher yield, the PCR protocol was optimized with respect to reaction conditions as well as cycle parameters.

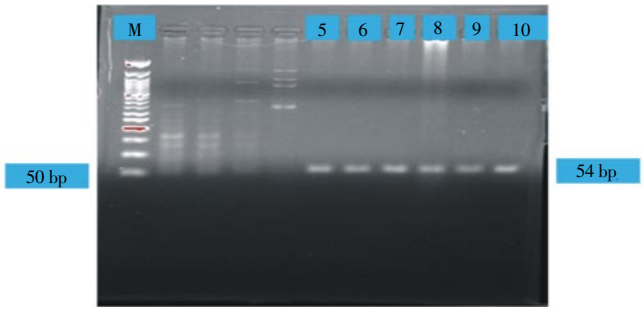

Figure 2. RT-PCR amplification of Beta Actin gene.
Lane M: Molecular weight Marker (50 bp),
Lane 5 to 10: 54 bp PCR product.

3.3. Optimization of end point PCR protocol

Primers were synthesized using BEACON software. Details of primers have been given in Table 2. Gradient PCR conducted in special cyclers that allow different temperature profiles to be programmed for each cavity in the cycler. Gradient PCR used to optimize PCR conditions with respect to the primer annealing temperature. All samples were treated equally, but different annealing temperatures were used. The reaction conditions were optimized using different combinations of the primers, $MgCl_2$ and dNTPs for all the genes.

In agarose gel, the PCR efficiency analyzed investigating intensity and integrity of the product bands. It was observed that annealing temperature of 58 ℃ for HSP70 and HSP90 gave best results. The optimized concentrations that gave the best results were 1.5 mM of $MgCl_2$, 200 μM of dNTPs and 10 pMole of each primer. Finally PCR was carried out in 25 μL volume of reaction mixture containing optimized concentrations of $MgCl_2$, dNTPs and primers, and 1 μL cDNA as template, 1X PCR assay buffer and 1 unit of Taq DNA polymerase.

3.4. Amplification of HSP70 and HSP90 genes

Following PCR, the amplicon length was checked using high resolution agarose gel electrophoresis. As expected a single and specific band of 132 bp for HSP70 (Figure 3), 190 bp for HSP90 (Figure 4) was amplified from the cDNA.

3.5. Real time PCR

Real time PCR was optimized using different dilutions of templates of cDNA. 1.0 μL of the template gave good results for all genes. After standardization, real time PCR was performed for each gene taking all the samples. After the run has ended, cycle threshold (Ct) values and amplification plot for HSP70 (Fig.3), HSP90 (Figure 4) were acquired by using the "SYBR Green (with Dissociation Curve)" method of the real time machine (Stratagene M× 3005P QPCR System, Agilent Technologies, USA). The PCR-product was identified by the characteristic melting curve of HSP70, HSP90 (Figure 5, 6). The melting curve showed only one peak.

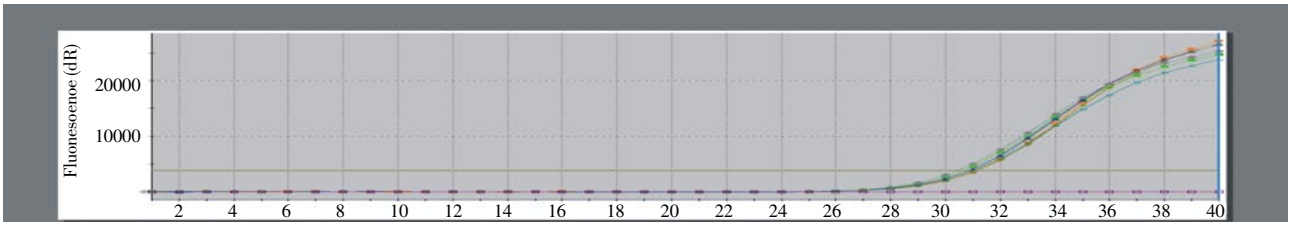

Figure 3. Amplification plot of HSP 70 mRNA expression.

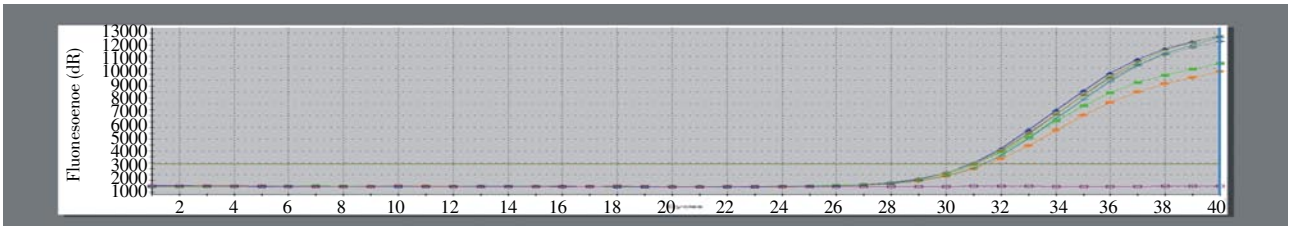

Figure 4. Amplification plot of HSP 90 mRNA expression.

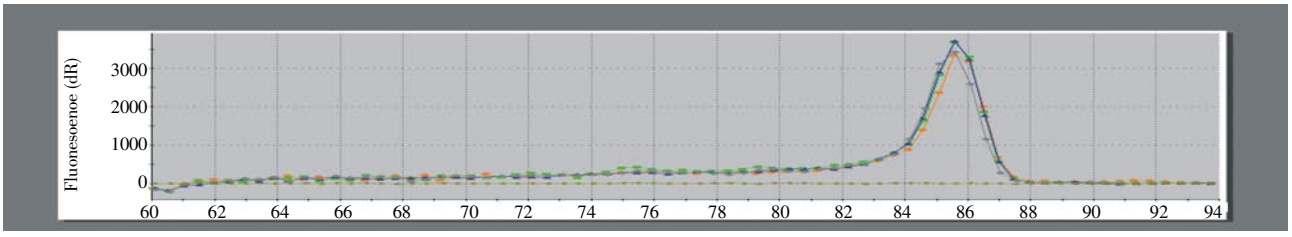

Figure 5. Dissociation curve of HSP 70 mRNA expression.

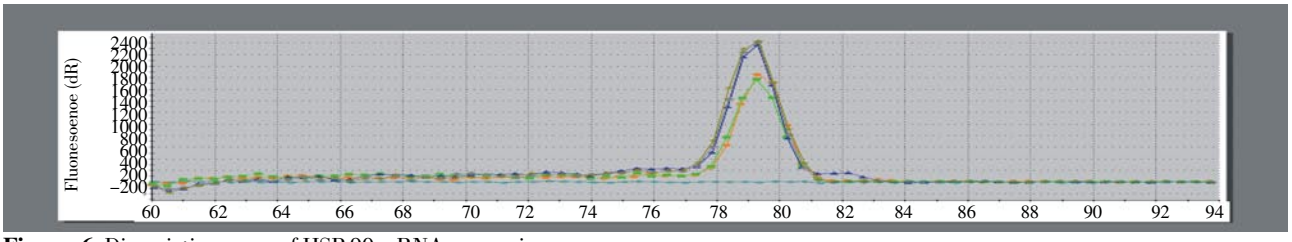

Figure 6. Dissociation curve of HSP 90 mRNA expression.

3.6. Expression of mRNA for HSP70 and HSP90

The expressions of mRNA for HSP70 and HSP90 in sperm of Tharparkar bull during winter and summer are presented in (Figure 7 & 8). The mRNA expression during summer season was found non-significantly higher in comparison to winter season.

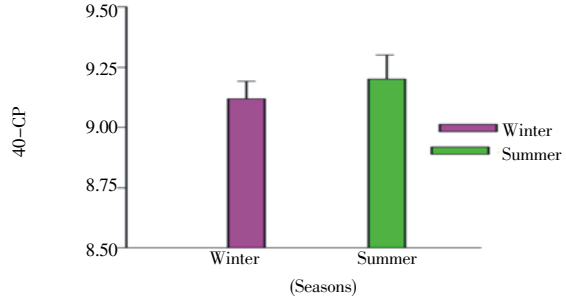

Figure 7. HSP70 mRNA expression in sperms of Tharparkar bulls.

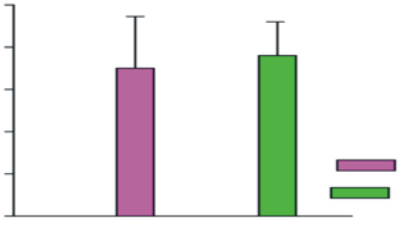

Figure 8. HSP90 mRNA expression in sperms of Tharparkar bulls.

4. Discussion

4.1. HSP70 and HSP90 expression

Most of the livestock species experiences stress of varying degrees due to thermal challenges beyond their comfort zone and are able to cope with these environmental stressors through behavioural and physiological measure such as sweating, panting, drinking water or shivering or by regulating their metabolic rates. Comfort zone is a range of environmental temperature within which body temperature is maintained constant with a minimal effort from thermoregulatory mechanism and within which the sensation of cold or heat is absent. Despite animal's physiological and behavioural response to ameliorate the discomfort of thermal stress, there might be some molecular mechanisms to maintain their cellular homeostasis. Further, there are numerous intrinsic mechanisms in the cell which protect it from deleterious effects of environmental stress and maintain homeostasis, release of HSPs is one those mechanisms. HSP is considered as potential indicator of animal adaptation to harsh environmental stress and its expression has been correlated with resistance to stress[14]. HSPs participate in numerous functions including folding of newly synthesized proteins, transport of proteins into cell compartments, disaggregation of protein complexes and others functions [18]. HSPs account for 1%-2 % of total protein in unstressed cells

which increased to 4%-6% of cellular proteins when cells are heated[19]. In the present investigation, the effects of thermal stress on the mRNA expression of HSP70 and HSP90 in spermatozoa of Tharparkar bull were studied during winter and summer seasons.

4.2. HSP70

HSP70 is mostly found in the cytosol and nucleus. Its functions are protein folding, cyto-protection and as molecular chaperones. In the present investigation, it was observed that HSP70 mRNA expression in Tharparkar bull's sperms during summer season was found non-significantly higher in comparison to winter season. In previous studies, heat stress induced HSP70 expression was observed in the bovine lymphocytes[16, 20], in myocardium[21], in lung cells [22] and in hepatocytes and liver[23]. Cao *et al*. [24] reported that higher heat stress level led to higher concentration of HSP70 in the testis and epididymis of mice. HSP 70, which is a constitutively expressed member of the family, is shown to be especially important in murine spermatogenesis[25]. The increased HSP 70 expression has positively correlated to DNA damage detected in sperm[17]. Heat shock factor (HSF)-1 is activated and consequently HSP 70 expression is increased in spermatids exposed to heat[26].

4.3. HSP90

HSP90 is mostly found in the cytosol, endoplasmic reticulum and nucleus. Its main functions are protein translocation and regulation of steroid hormone receptors. In the present study, it was noticed that during summer season, HSP90 mRNA expression was found non-significantly higher in comparison to winter season in Tharparkar bull sperms. Earlier study reported that HSP 90 expression was increased due to heat stress in human blood lymphocytes[27], in T cells [28], rat myocytes[29], in heart, liver and kidney of broilers [30], in murine embryonic fibroblast cells[31], lung, heart, spleen, liver, and brain of human[32].

It was concluded from the present study that there was no significant difference was observed in the mRNA expression of HSP 70 and HSP 90 between the winter and summer season indicates, presence of similar type of stress resistant spermatozoa in 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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