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Effect of melatonin on the expression profile of HSP60 and HSP70 in Beetal and Assam Hill Goat exposed to direct sunshine during summer in Assam

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Abstract
The present investigation was carried out to evaluate the effect of melatonin on mRNA expression of HSP60 and HSP70 genes in the peripheral blood mononuclear cells (PBMC) in Beetal goats in Assam with reference to native breed of goat i.e., Assam Hill Goat (AHG) that were exposed to direct sunshine in summer. The study included 18 AHG and 18 Beetal goats of uniform age group (5-6 months), which were subdivided into three groups containing 6 animals in each group. One group was kept indoor while other two groups of each breed were exposed to direct sunshine @ 6 hours/day for a period of 30 days. Melatonin was fed @ 6 mg/goat to one of the two sunshine exposed groups. Blood samples were collected from all the animals before and after the exposure at weekly interval and real-time polymerase chain reaction was applied to investigate mRNA expression of HSP60 and HSP70 genes. The relative expression of HSP60 and HSP70 mRNA was found to be significantly higher (P <.0.01) in the sunshine exposed groups of Beetal goats in comparison to AHG. In the melatonin fed group, the expression was significantly higher than the group without melatonin of either Beetal or AHG. The relative HSP60 and HSP70 mRNA expression in both the sunshine exposed groups of either Beetal or AHG increased significantly from day one to day 21 and then decreased at 28 days. The expressions were significantly higher in the melatonin fed group in comparison to the group without melatonin in different days after day one.

Keywords: direct sunshine, HSP60, HSP70, melatonin, summer, Assam Hill Goat, Beetal

1. Introduction

Goats, which are considered poor man's cow plays an important role in the economy of poor livestock owners in our country. They are primarily raised on grazing resources under extensive management system in different parts of the country. In Assam being the sub-topical region of the country, animals such as goats are regularly exposed to extreme hot and humid weather conditions. This causes extreme stresses to animals leading to various physiological changes and may lead to decreased growth and loss of production in the animals.

At cellular level, the ability to survive and adapt to thermal stress involves biochemical responses and gene expression [1-2]. Activation of heat shock transcription factor 1 (HSF 1) and subsequent increased expression of heat shock proteins (HPSs) is integral to cellular response to heat stress [3]. HSPs are multigene families that range in molecular size from 10 to 150 kilo daltons (kDa). Some HSPs such as HSP90 (each named according to its mass in kDa) are detectable in significant levels in unstressed cells, increase in abundance following a suitable stimulus, while others such as HSP70 exists in both constitutively expressed and inducible forms that is activated by stressful stimuli [4]. This 70 kDa HSP assist the folding of proteins upon translation in the cytosol. HSPs expression acts as a potential indicator of animal adaptation to harsh environmental stress [5].

Melatonin (N-acetyl 5-methoxytryptamine), best known for its role in seasonality of reproduction, plays an important role in relieving thermal stress by influencing cardiovascular system and evaporative heat loss [6]. It also interacts with other hormones such as thyroxine to reduce the thermal stress and modify adrenal functions to relieve heat stress [7].

Melatonin has also been reported to modulate gene expression of oxidative enzymes in body, HSP60 in heat stressed pancreatic cells [8], HSP70, HSP90 and HSP40 in rat liver cells under oxidative stress [9].

To overcome the detrimental effect of climatic conditions, especially solar radiation, the basic strategy is to alter the surrounding environment by using sheds, fans and evaporative cooling [10] However, owing to the sub-tropical climate and poor economic conditions of the farmers, most of the goats in Assam are reared under extensive system, and it becomes necessary to find novel ways to counteract the adverse effect of solar radiation. Therefore, we have undertaken the present investigation with two objectives – first to compare the mRNA expression of HSP60 and HSP70 in the peripheral blood mononuclear cells (PBMC) in newly introduced Beetal goats in Assam with reference to native breed of goat i.e., Assam Hill Goat (AHG) during peak summer and second to evaluate the effect of melatonin on the expression profile of the above genes in both the breeds of goat.

2. Materials and methods

2.1. Animals and experimental design

Thirty six healthy goats consisting of 18 Beetal goats and 18 Assam Hill Goat of uniform age group of 5-6 months old, which were maintained in the Goat Research Station, Burnihat, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-22, was selected. Each breed of goat were subdivided into three groups (T-1 T-2 and T-3) containing 6 animals in each. One group was kept indoor (T-1) while other two groups (T-2 and T-3) of each breed were exposed to direct sunshine for a period of six hours from 8:00 hrs to 14:00 hrs in a fenced open paddock. Melatonin (Meloset tablet 3 mg, Aristo Pharmaceuticals Pvt. Ltd., Mumbai) was fed @ 6 mg/goat/day to one of the two sunshine exposed groups (T-3) before the exposure of sunshine. The study was conducted for a period of 30 days in the month of June. Blood samples were collected from the animals before and after the sunshine exposure at one week interval

The animals were maintained under standard managemental system of the research station during the study period. They were fed mixed grasses, tree leaves and a concentrate feed containing 91.14% DM, 12.56% crude protein (CP), 8.0% crude fiber (CF), 1.28% ether extract (EE), 73.74% nitrogen free extract (NFE) and 4.42% total ash on a dry matter basis. Water was given *ad lib* to the control groups and once daily during the treatment period and then *ad lib* to the treatment groups. Prophylactic measures against goat diseases like goat pox, paste des petits ruminants, enterotoxaemia, endo- and ecto-parasitic infestations were carried out as per approved schedules and all animals were kept under close clinical observation.

2.2. Temperature and humidity index (THI)

Temperature-Humidity Index was calculated for the entire period of study using the data obtained from the Automatic Weather Station of Assam Agricultural University, installed in the campus of College of Veterinary Science, Khanapara, Guwahati-22.

The formula used for calculating THI is

THI= 0.4[Dry bulb temperature (ºF)+ Dew point temperature (ºF)] +15

2.3. Collection of blood and isolation of PBMC

Blood samples were collected from each of the animals of different groups using EDTA as anticoagulant at standard concentration by jugular vein puncture under sterile conditions. After collection, the samples were brought to the laboratory immediately and processed for isolation of RNA from the peripheral blood mononuclear cells (PBMC). All precautions were taken to minimize the effect of ribonuclease activity while processing. The PBMCs were isolated by density gradient centrifugation method using Hisep (HiMedia, Mumbai). Briefly, the blood was layered slowly over the Hisep at 1:1 ratio to produce a clean interface between the two layers in a 15 ml sterile conical centrifuge tube at room temperature and centrifuged at 400 x g for 30 min in a swinging bucket rotor. After centrifugation, the white opaque mononuclear fraction from the interface between the plasma and the Hisep was collected and washed with sterile of PBS (pH 7.4) to get a clean cell pellet of PBMC. In situations, when it was not possible to isolate the RNA on the same day, the PBMC was put into RNA later (Invitrogen, USA) and kept at -80 °C for isolation of RNA on the next day.

2.4. Extraction of total RNA

The PBMC pellet was re-suspended in 500 µl of DEPC-PBS (pH 7.4) and transferred to a 2 ml nuclease free (DEPC treated) microcentrifuge tube. Total RNA was isolated using Trizol reagent (Invitrogen, USA) using standard protocol. Briefly, 1 ml of Trizol reagent was added to the PBMC suspension and vortexed vigorously. Then 200µl of chloroform added to the homogenized lysate, vortexed and kept at room temperature for 15 minutes. The tubes were then centrifuged at 12000 x g for 15 minute at 4 ºC and the aqueous phase on the top were transferred to a fresh eppendorp tube (DEPC treated). An equal volume of isopropanol was added to the tubes, mixed by vortexing and kept at room temperature for 10 minutes for precipitating the RNA. The tubes were centrifuged again at 12000 x g for 10 minute at 4 ºC to pellet the precipitated RNA. The supernatant was discarded carefully without disturbing the pellet. The RNA pellet was washed using 70% ethanol by centrifuging at 7500 x g for 5 minutes. The RNA pellet was air dried and dissolved in 20 µl of nuclease free water by keeping at 55-60 ºC in a digital heat block (Eppendorp, Germany) and stored at -80 ºC till further use. The concentration and purity of the extracted total RNA was measured by Nanodrop spectrophotometer. The quality and integrity of the RNA was checked using denaturing agarose gel (1%) electrophoresis and visualization under UV light (Gel Doc XR+, BioRad, USA). Two intact bands of 28s and 18s with smearing indicated good quality and intactness of RNA

2.5. Synthesis of first strand cDNA

A constant amount of RNA (1 µg) was reverse transcribed to produce cDNA using RevertAid Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) following manufacturers instruction. In brief, one µg of total RNA was dissolved in nuclease free water to make a final volume 11µl and then one microlitre of random hexamer primer was added. The mixture was incubated at 65 ˚C for 5 minutes in a thermal cycler (S1000, BioRad, USA). It was then snap cooled in ice and following mixture was added: 4 µl 5X reaction buffer, 2 µl dNTP mix. (10 mM), 1 µl ribolock and 1 µl reverse transcriptase (200 units/ µl). The mixture was again incubated at 25 ˚C for 5 minutes followed by 42 ˚C for 60 minutes. Finally, the reaction was stopped by incubating for 5 min at

70 °C and cooled down to 40 °C. The cDNA was stored at -20 °C for further use. The integrity of the cDNA was checked by PCR with β-actin primers.

2.6. Primers

The primers for HSP60 (forward: 5'-ACT GGC TCC TCA TCT CAC TC-3', reverse: 5'-CTG TTC AAT AAT CAC TGT CCT TCC-3') and beta actin genes (forward 5'-AGT TCG CCA TGG ATG ATG A-3' and reverse 5'-TGC CGG AGC CGT TGT-3') amplification were as per Dangi *et al*. (2012) For amplification of the HSP70 gene a pair of new primers: forward -5'-GAC GGA GAC AAG CCT AAA G-3' and reverse- 5'-CTC GGC GAT CTC TTT CAT C-3' were designed from the published sequences (Acc. No FJ975769) with the help primer designing software of Integrated DNA Technologies, USA as the primers reported did not worked in our condition [11]. These primers gave a PCR product of 108 bp in size. The primers were also procured from Integrated DNA Technologies, USA.

2.7. Quantitative real-time PCR

Quantitative real-time PCR was performed with QuantiTect SYBR Green PCR Kit (Qiagen, USA), and real-time thermal cycler (StepOnePlus, Applied Biosystem, USA) operated by StepOne® software v 2.2.2 software. The PCR reaction was performed in a total 20 µ1 volume reaction mixture containing 7µl of nuclease water, 10 µl SYBR Green master mix, 0.5 µ1 of each of the primers (0.125 µM each) and 2 µl of cDNA. For reaction set up 96 well plates and optically clear adhesive covers were used. 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. Beta actin gene was taken as housekeeping gene. The PCR conditions were as follows- initial denaturation at 95 °C for 10 mins, denaturation at 95 °C for 10 sec, annealing at 60 °C for 30 sec and extension at 72 ^{0}C for 30 sec for 40 cycles and the last cycle was at 95 °C for 1 min followed by 30 sec at 60 °C. Then a gradual increment from 60-95 °C @ 3 °C/min with continuous fluorescence measurement for the melting curve analysis. Finally, the reaction was held at 95 °C for 1 min before cooling to 4 °C. After the run has ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the "SYBR Green (with melting Curve)" method of the real time PCR machine. Confirmation of amplification of specific RT-PCR amplicon was done by agarose gel electrophoresis. The PCR products were visualized under UV in the gel documentation system.

2.8. Calculation of relative expression

Relative quantification of target genes among the treatment groups were performed by carrying out real time PCR against an endogenous control gene (Beta-actin). $2^{-\Delta\Delta Ct}$ method has been used to obtain the RQ (relative quantification) values. Threshold cycle (Ct) value for each sample has been obtained by running a triplicate experiment. For each real time assay treatment group of animals was compared against a control group (T-1). The quantification values obtained for target genes in control group were set to 1 for obtaining the RQ values.

2.9. Statistical analyses

The statistical significance of differences in mRNA expressions of the examined factors was assessed by Paired t-test using SPSS 16.0 software. Differences were considered significant if $P<0.05$.

3. Results and discussion

3.1. Temperature Humidity Index

The month wise mean Temperature-Humidity Index (THI) ranged from 81.46 in May to 83.44 in August with an overall mean of 83.0 during summer

3.2. Expression of HSP60

The mean relative expression of HSP60 mRNA in sunshine exposed (T-2) and sunshine exposed plus melatonin fed (T-3) group of Beetal goat were 3.08±0.04 and 3.45±0.03, respectively in comparison to the control group (1.0) and the corresponding values for AHG were 2.74±0.03 and 2.89±0.03, respectively (Table 1). The analysis of variance revealed that the expression was significantly ($P<0.01$) higher in both T-2 and T-3 groups of Beetal goats in comparison to AHG. Further, the relative expression of HSP60 mRNA was significantly ($P<0.01$) higher in T-3 group of both the breeds in comparison to T-2 groups.

The day wise relative HSP60 mRNA expression in the T-2 and T-3 groups of Beetal during summer is presented in Table 185 and analysis of variance in Table 186. The expression in the T-2 group increased significantly from day one (1.44±0.18) to day 21 (4.42±0.15) and then decreased at 28 days (3.27±0.40). Similarly in the T-3 group the expression increased significantly from day one (1.58±0.21) to 4.81±0.28 at day 21 an then decreased (3.85±0.18) at day 28 (Figure 4.18). The relative HSP60 mRNA expression was significantly ($P<0.01$) higher in the melatonin fed group (T-3) in comparison to T-2 group in different days except at day one.

3.3. Expression of HSP70

The relative expression of HSP70 mRNA in sunshine exposed (T-2) and sunshine exposed plus melatonin fed (T-3) groups of Beetal goats were 2.36±0.03 and 2.87±0.04, respectively in comparison to the control group (1.0) and the corresponding values for AHG were 1.76±0.03 and 1.98±0.03, respectively (Table 1, Figure1). The expression was significantly higher in both T-2 and T-3 groups of Beetal goats in comparison to AHG. Again the relative expression of HSP70 mRNA was significantly higher in T-3 group of both the breeds in comparison to T-2 groups.

Daywise relative HSP70 mRNA expression in theT-2and T-3 groups of Beetal and AHG is presented in Table 3 and Figure 2. The expression in the T-2 group increased significantly from day one (1.67±0.04) to day 21 (3.11±0.03) and then decreased at 28 days (2.76±0.03) in Beetal goats. In AHG, it also increased significantly from day one (1.22±0.03) to day 21 (2.35±0.05) and then decreased at 28 days (2.05±0.04). Similarly in the T-3 group of Beetal goats, the expression increased significantly from day one (1.62±0.02) to day 21 (3.84±0.03) and then decreased (3.61±0.04) at day 28. In the T-3 group of AHG, the expression increased significantly from day one (1.40±0.04) to day 21 (2.64±0.03) and then decreased at day 28 (2.18±0.03).The relative HSP70 mRNA expression was significantly higher in the melatonin fed group (T-3) in comparison to T-2 group in different days except at day one in Beetal and day one and day 21 in AHG.

Overall relative HSP60 and HSP70 mRNA expression were significantly higher in melatonin treated group (T-3) than the only sunshine exposure group (T-2). Similarly, expression was significantly higher in Beetal than in AHG. Considering the days of observation, it was also found to vary significantly; the expression was higher at day 7, 14, 21 and 28 of treatment. However, the highest expression was at day 21

Fig 1: Relative expression of HSP60 mRNA of Beetal and AHG in different treatment groups at different days

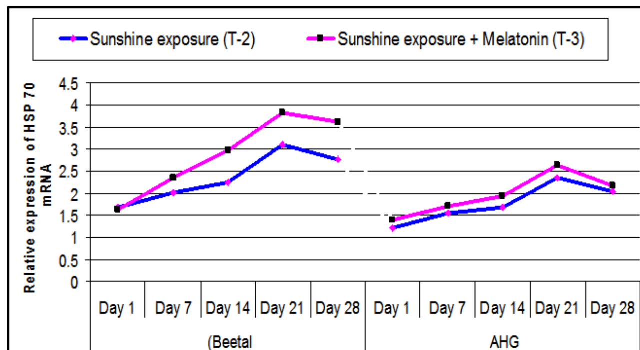

Fig 2: Relative expression of HSP70 mRNA of Beetal and AHG in different treatment groups at different days

Table 1: Relative expression of HSP60 and HSP70 mRNA (mean±SE) in Beetal and AHG in different treatment groups

Group	HSP60		HSP70	
	Beetal	AHG	Beetal	AHG
T2	$3.08_B^b \pm 0.04$	$2.74_A^b \pm 0.03$	$2.36_B^b \pm 0.03$	$1.76_A^b \pm 0.03$
T3	$3.45_B^a \pm 0.03$	$2.89_A^a \pm 0.03$	$2.87_B^a \pm 0.04$	$1.98_A^a \pm 0.03$

Different superscripts in the same column differ significantly. Different subscripts in the same raw either under HSP60 or HSP70 also differ significantly.

Table 2: Relative expression of HSP60 mRNA (mean ± SE) in Beetal and AHG goats in different treatment groups at different days

Group/ Breed	Days									
	1		7		14		21		28	
	Beetal	AHG	Beetal	AHG	Beetal	AHG	Beetal	AHG	Beetal	AHG
T-2	$1.44_E^a \pm 0.18$	$1.32_E^a \pm 0.18$	$2.53_D^b \pm 0.20$	$2.20_D^b \pm 0.20$	$3.76_B^b \pm 0.14$	$3.26_B^a \pm 0.14$	$4.42_A^b \pm 0.15$	$4.08_A^a \pm 0.15$	$3.27_C^b \pm 0.40$	$2.92_C^b \pm 0.40$
T-3	$1.58_E^a \pm 0.21$	$1.36_D^a \pm 0.21$	$2.78_D^a \pm 0.23$	$2.43_C^a \pm 0.23$	$4.17_B^a \pm 0.27$	$3.38_B^a \pm 0.27$	$4.81_A^a \pm 0.28$	$3.99_A^a \pm 0.28$	$3.85_C^a \pm 0.18$	$3.27_B^a \pm 0.18$

Mean values with different superscripts in a column and subscripts within the same breed of goat in a row differ significantly

Table 3: Relative expression of HSP70 mRNA (mean ± se) in Beetal and AHG goats in different treatment groups at different days in summer

Group/ Breed	Days/Breed									
	1		7		14		21		28	
	Beetal	AHG	Beetal	AHG	Beetal	AHG	Beetal	AHG	Beetal	AHG
T-2	$1.67 \pm 0.04_E^a$	$1.22 \pm 0.03_E^b$	$2.02 \pm 0.03_D^b$	$1.54 \pm 0.03_D^b$	$2.25 \pm 0.04_C^b$	$1.68 \pm 0.03_C^b$	$3.11 \pm 0.03_A^b$	$2.35 \pm 0.05_A$	$2.76 \pm 0.03_B^b$	$2.05 \pm 0.04_B^b$
T-3	$1.62 \pm 0.02_E^a$	$1.40 \pm 0.04_E^a$	$2.35 \pm 0.04_D^a$	$1.71 \pm 0.03_D^a$	$2.97 \pm 0.03_C^a$	$1.96 \pm 0.03_C^a$	$3.84 \pm 0.03_A^a$	$2.64 \pm 0.03_A^a$	$3.61 \pm 0.04_B^a$	$2.18 \pm 0.03_B^a$

Mean values with different superscripts in a column differ significantly. Different subscript in a raw either in Beetal goats or in AHG in different days differ significantly.

4. Discussion

The THI has been widely used as an indicator of thermal stress in livestock [12]. THI value of 70 or less are considered comfortable, 75-78 stressful and values greater than 78 cause extreme distress and animals are unable to maintain thermoregulatory mechanism or normal body temperature [13]. Therefore, in the present study the calculated value of THI in summer was found to be stressful for the animals.

Although, goats are known to be adapted to harsh environment, they suffer from heat and cold stress beyond their comfort zone, which is considered to be 13-27 ^{0}C for Indian goats [14]. Further there are evidences for deleterious effects on goat productivity, when goats had been exposed to over temperatures of 34-36 ^{0}C [15]. Since, browsing of goats in open fields during most of the day hours makes them susceptible to environmental stress due to prolong exposure to direct sunlight and may influence their physiological processes in accordance to the degree of stress. Temperature-Humidity Index values calculated in this experiment were in agreement with the values recorded [16] in which mean THI value of 69.5 is greater than 79 in pre-summer and summer-stressed Beetal goats, respectively in Punjab. THI values in summer seasons as 80.9±2.1 in Hissar, Haryana [17].

In mammalian cells, non-lethal heat shock produces changes in gene expression and in the activity of expressed proteins, resulting in what is referred to as a cell stress response [18, 2].

This response characteristically includes an increase in thermo tolerance, which is temporally associated with increased expression of HSPs. The HSP60 is mostly found in the mitochondria, it helps in refolding of proteins and prevents aggregation of denatured proteins. In the present study, relative expression of HSP60 mRNA was significantly higher ($P< 0.01$) in the T-2 (only sunshine exposure) and T-3 (sunshine plus melatonin fed) groups of both the breeds as compared to the control group (T-1). There seems to be no report on the effect of direct solar radiation (sunshine) on the level HSP60 expression in goats. However, our findings corroborates with the findings of [11], who also recorded significantly higher HSP60 mRNA expression in summer season in all age groups of tropical (Barbari) and temperate (Pashmina) region goats in comparison to winter season. Our finding were also substantiated by the report of [19], who observed significant increase in the HSP60 mRNA expression in Barbari goats exposed to thermal stress in a climatic chamber. Heat shock response is a homeostatic mechanism that protects cell from damage by upregulating the expression of genes that code for HSP60 during heat stress [20] Furthermore, in previous investigations up-regulation of HSP60 expression in rat myocytes [21] and in human umbilical venous endothelial cells [22] was observed during heat stress. Increased HSP60 mRNA expression during summer season in goats could evoke its transcription in PMBC to prevent cell from damaging effect of solar radiation like denaturation of proteins and HSP60 helps in refolding of proteins and prevents aggregation of denatured proteins.

In the present investigation, the relative expression of HSP60 mRNA was higher in the T-2 and T-3 groups of Beetal in comparison to the AHG and the values were significantly ($P<0.01$) higher in both the groups of Beetal. This may be due to the fact that AHG are more adapted to solar radiation effect than the Beetal or the Beetal goats are more prompt in mitigating the stressful effect of solar radiation. Besides, the level of expression was significantly ($P<0.01$) higher in the melatonin fed groups of both the breeds of goat. Significantly higher ($p<0.05$) relative expression of HSP60 in Barbari goats, which were treated with melatonin and exposed to thermal stress in a psychrometric chamber [19]. Thus melatonin might play a protective role in stress due to solar radiation by increasing availability of HSP60. The HSP60 mRNA expression increased significantly from day one to around day 21 in both the breeds of goats during summer and then declined in both the treatment groups. This may indicate that goats reacted to the solar radiation vigorously during this period and afterwards they developed their natural resistance for the stress and reduced the HSP60 expression.

The HSP70 is mostly found in the cytosol and nucleus, its functions are protein folding, cytoprotection and molecular chaperons. In the present study, relative expression of HSP70 mRNA in PMBC was significantly higher in the T-2(only sun-shine exposure) and T-3 (sunshine plus melatonin fed) groups of both the breeds in summer as compared to the control group. No reports are available on the effect of direct solar radiation (sun-shine) on the level HSP70 expression in goats. However, our findings are in agreement with the findings of [11], who also recorded significantly higher HSP70 mRNA expression in summer season in all age groups of tropical (Barbari) region goats in comparison to winter season. Our finding are also substantiated by the report of [19], who observed significant increase in the HSP70 mRNA expression in Barbari goats exposed to thermal stress in a psychromatic chamber. Similarly, previous studies also documented induction of HSP70 by heat stress in the bovine lymphocytes [23, 24, 25], in lung cells [26] in the hepatocytes and liver [27, 28], in myocardium [29], in the kidneys of goats [30], thereby indicating that heat shock proteins provides protection from toxic effects of thermal stress. Increased HSP70 mRNA expression during summer season in goats could evoke its transcription in PBMC to prevent cell from damaging effect of solar radiation like denaturation of proteins.

The relative expression of HSP70 mRNA was significantly higher in all the treatment groups in the Beeetal than AHG in summer and they differed significantly. This may be due to the fact that AHG are more adapted to solar radiation effect than the Beetal goats or the Beetals are more efficient in mitigating the stressful effect of solar radiation. Besides the level of expression were significantly higher in the melatonin fed groups (T-3) of both the breeds of goat in comparison to T-2 groups. Similarly, [19] reported significantly higher ($p<0.05$) level of HSP70 relative expression in Barbari goats, which treated with melatonin and exposed to thermal stress in psychromatic chamber. Thus melatonin may play a protective role in stress due to solar radiation by increasing availability of HSP70.

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