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Effect of melatonin supplementation on heat shock proteins expression profile in buffalo calves under summer stress

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Abstract

A study was undertaken to investigate the effect of melatonin supplementation on heat shock proteins (HSPs) (HSP 60, HSP70, HSP 90 and HSP 110) expression profile in buffalo calves under summer stress. Twelve healthy Murrah buffalo male calves of 6 month to 1 year age group were taken for the study. Buffalo calves were divided into control (CG) and treatment (TG) group. In TG, Melatonin (18mg/50 kg BW) was injected subcutaneously, two times at 1st and 20th day. Blood samples were collected on 0th, 7th, 14th, 21st, 28th, 35th and, 42nd days. The relative expression of genes like HSP 60, HSP 70, HSP 90 and HSP 110 was studied in peripheral blood mononuclear cells. Between the groups, the mRNA expression of HSP70, 90 and 110 differed significantly ($P < 0.05$) at some time points. In conclusion, melatonin have a dwindling effect on expression of HSPs, suggesting a possible role of these in heat stress amelioration.

Keywords: Melatonin, HSPs, buffalo calves, heat stress

Introduction

The world's second most important milch animal is known as Buffalo (*Bubalus bubalis*). The importance of buffalo to the dairy industry, food security and rural livelihood by providing milk, meat and draught power in tropical and subtropical countries hardly needs emphasizing as the species is an important livestock resource in Asian continent with over 95% of world's buffalo population [1]. Significant percentage of Indian population depends on animal husbandry for livelihood. Buffaloes have poor heat tolerance than cattle due to many physiological and genetic reasons (less sweat glands, black colored skin) and so are more pronounced to thermal stress. Under excesses thermal loads of heat and work, buffaloes employ moderate levels of sweating and resort to open mouth panting [2].

Heat stress is a major constraint on animal productivity in tropical conditions. High ambient temperature accompanied by high air humidity causes an additional discomfort and enhances the stress level which in turn results in depression of the physiological and metabolic activities of animal. Reactions of homeotherms to moderate climatic changes are compensatory and are directed at restoring thermal balance [3].

During heat stress, heat increment exceeds heat loss modifying the homeostatic functions. Heat stress elicits an integrative physiological and endocrinal modulation altering overall metabolism and helping the animal to sustain during the stressful period. Various in-depth studies on heat and nutritional stress on animals, severely compromising thermoregulatory functions which intern affect the productive potential of animals [4, 5].

The synthesis of a group of proteins called HSPs results during heat stress [6] and these HSPs protect cells from toxic effects of heat and other stresses [7]. The induction of HSPs is remarkably rapid and intense, against an emergency response. HSP expression has also been correlated with resistance to stress and thresholds for HSP expression are correlated with levels of stress [8]. Up regulation of the synthesis of a number of these proteins upon environmental stress establishes a unique defence system to maintain cellular protein homeostasis and to ensure survival of the cell. This increase in expression is transcriptionally regulated.

Melatonin (N-acetyl-5-methoxytryptamine), an indolamine synthesized from tryptophan in the pineal gland, has been shown to be associated with the regulation of seasonal reproduction in photoperiodic species. Melatonin has been shown to be a highly effective antioxidant and free radical scavenger [9]. Therefore, the present experiment was designed to study the effect of melatonin supplementation on physiological parameters, hormonal parameters and expression profile of HSP 60, HSP 70, HSP 90 and HSP 110 in buffalo calves under summer stress.

Materials and Methods

Animals and experimental design

Twelve healthy Murrah buffalo bull calves between 6 to 12 months of age group belonging to tropical region were selected for the study purpose. The experiment trial was conducted up to 6 week (42 days) period. Animals were divided into two groups viz., control (CG) and treatment (TG) group (n= 6). TG received melatonin @ 18 mg/50 kg body weight, subcutaneously (s/c), on 1st and 20th day of experiment [1]. Blood samples were collected by jugular veinipuncture under sterile conditions at 7 days interval on 1st, 8th, 15th, 22nd, 29th, 36th and 42nd days. Each blood sampling include: 2.5 ml heparinised blood for mRNA isolation and 3

ml blood of whole blood for serum isolation. Serum was collected after keeping the vial at slant for an hour and then centrifuging it at 2000 RPM for 10 minutes. Separated serum was stored in micro centrifugation tubes at -20°C for further analysis. Heparinized blood was used for mRNA isolation. Serum melatonin (ng/ml) concentration was determined using radioimmunoassay kits (LDN Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany). Analytical sensitivity of kit was 2.3pg/ml. The intra-and inter-assay coefficients of variation were found to be 10.96% and 10.73%, respectively.

Primers

Published primers were used for the study. The sequences and expected PCR Product length are shown in Table 1.

PBMCs isolation

PBMCs were isolated by density gradient centrifugation method using Histopaque 1077 (Sigma, USA). Briefly, the blood was layered carefully onto the Histopaque to produce a clean interface between the two layers. Further it was centrifuged at 2000 rpm for 30 min at room temperature. The white opaque mononuclear fraction from the interface was collected. The cells were washed thrice in PBS (pH 7.4) and finally the cell pellet was obtained.

Table 1: Gene Transcripts, primer sequences & efficiency and resulting fragment size

Target	Sequence of nucleotide	Fragment size (bp)	Efficiency (%)	EMBL/Reference
HSP60	For: 52'-ACTGGCTCCTCATCTCACTC -32'; Rev: 52'-CTGTTCAATAATCACTGTCCTTCC -32';	148	108	Dangi <i>et al.</i> , 2012 [20]
HSP70	For: 52'-GACGACGGCATCTTCAAG -32'; Rev: 52'-GTTCTGGCTGATGTCCTTC -32';	132	109.5	Dangi <i>et al.</i> , 2012 [20]
HSP90	For:52'- GCATTCTCAGTTCATTGGCTATCC -32'; Rev: 52'- GTCCTTCTTCTCTTCTCCTCTTC -32';	190	100.2	Dangi <i>et al.</i> , 2012 [20]
HSP110	For: 52'-CACAGCCCCAGGTACAAACT-32' Rev: 52'-TCCCTAACTGCCAGACCAAG-32'	204	99.9	Dangi <i>et al.</i> , 2015 [46]

EMBL – Accession number or reference of published sequence

Total RNA extraction and quality determination

The PBMC pellet was re-suspended in 500 µl of DEPC-PBS and transfer to 2 ml nuclease free (DEPC treated) microcentrifuge tube. Total RNA was isolated using Trizol reagent (Invitrogen, USA) following standard protocol. The RNA was dissolved in nuclease free water and the purity of RNA was verified by optical density (OD) absorption ratio OD 260 nm/OD 280 nm between 1.8 and 2.0 using nanodrop spectrophotometer. 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 28s, 18s with smearing indicated good quality and intactness of RNA.

Synthesis of First Strand cDNA

The first strand cDNA was synthesized from the isolated total RNA. RT-PCR was done using reverse transcription system (SsoFast™Eva Green®Supermix kit) 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 1 µg of total RNA for each reaction and dissolved in nuclease free water to make final volume 11 µl. 1 µg of random hexamer primer was added and then incubated at 65°C for 5 minutes. Snap cooled in ice and following mixture was added:

Table 2: The Components and reaction mixture

Components of reaction mixture	Quantity
5X 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 °C for 5 minutes and 42 °C for 60 minutes. Reaction was stopped by incubating for 5 min at 70 °C and finally at 4°C forever. The cDNA is stored at -20 °C for long term use.

Real Time PCR

Quantitative Real-time PCR was performed with SsoFast™ Eva Green®Supermix kit (Bio-Rad, USA) and Agilent Stratagene MX3005P Real-Time qPCR (Agilent technology, USA) spectrofluometric thermal cycler operated by MxPro™ qPCR software. The annealing temperature of each determined factors was standardized in qPCR using cDNA synthesized from goat mRNA and Eva Green®Supermix kit. Reaction setup was performed in area separate from nucleic acid preparation or PCR product analysis. Pipetting was done with sterile filter tips. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. No template control (NTC)

was placed 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. 0.5 μ l (20 ng) of cDNA was taken. Following master mix was prepared:

Table 3: Master Mix (MM)

Components	Volume
Forward primer (0.2mM)	0.25 μ l
Reverse primer (0.2mM)	0.25 μ l
SsoFast™ Eva Green @Supermix	5 μ l
Nuclease free water	4.0 μ l

Maximal efforts had been taken to maintain the optical surface clean. Strips were tapped to remove any possible bubbles and centrifuged before starting the cycling programme to spin down the solution to the bottom of the tubes. Beta actin and GAPDH were taken as housekeeping genes. The amplification and denaturation data was acquired. After the run has ended, cycle threshold (Ct) values, amplification plot and dissociation curve for all determined factors were acquired by using the “Eva green (with dissociation curve)” method of the MxPro3005 Stratagene in real time (Agilent technology, USA) machine.

Agarose Gel Electrophoresis

The confirmation of amplification of specific RT-PCR amplicon during optimization and expression of each determined factors was done by agarose gel electrophoresis. 2% agarose was mixed with 30 ml 1X TBE buffer added with 5ml of double distilled water and heated in a microwave oven for 1-2 min until gel mixture becomes crystal clear. Then the gel mixture was poured into the gel casting tray fitted with the comb. The gel was allowed to solidify and after complete solidification, the comb was removed. The PCR product (around 5 μ l) were mixed with 0.5 μ l of 10X Saf Runner C-Me™ Gel Loading dye and loaded into the wells. For the comparison, a 50 bp molecular weight marker premixed with dye was gel electrophoresed in parallel to the RT-PCR amplicons. The gel was ran 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).

Calculation of relative expression

Optical data were collected at end of each extension step and relative expression of PCR product was determined by the equation ^[10] given below:

$$\text{Ratio} = \frac{[E_{\text{target}}]^{\Delta\text{Ct target (control-sample)}}}{[E_{\text{ref}}]^{\Delta\text{Ct ref (control-sample)}}$$

Where, ratio is the relative expression, E_{target} is the real time efficiency of target gene transcript, E_{ref} is the real time efficiency of housekeeping gene transcript.

Data analysis

Data obtained was analysed statistically by one way ANOVA followed by Tukey's b test within group between the days and independent t test for between groups with the help of SPSS 17.0 software.

Results

Changes in mRNA expression of HSP 60, HSP 70, HSP 90 and HSP 110 have been depicted in Fig. 1A-D. Between the groups, the mRNA expression of HSP70, 90 and 110 differed significantly ($P < 0.05$) at some time points.

Expression of HSP60: The results of expression of HSP60 of control and treated animals has been given in fig. 1-A. Within the control group and between the control and melatonin treated group on different days, the relative mRNA expression of HSP60 did not differ significantly ($P > 0.05$) whereas, in melatonin treated group mRNA expression of HSP60 found significantly ($P < 0.05$) higher on 14th day than 35th and 42nd days of the experiment.

Expression of HSP70: The results of expression of HSP70 of control and treated animals have been given in fig.1-B. In control group the relative mRNA expression of HSP70 of buffalo calves were found significantly ($P < 0.05$) higher on 7th, 14th, 21st, 28th and 35th days than 0th and 1st days of experiment. In melatonin treated group mRNA expression of HSP70 of buffalo calves did not differ significantly ($P > 0.05$) on different days of the experiment. Between the groups relative mRNA expression of HSP70 of buffalo calves were found significantly ($P < 0.05$) different on day 21st, 28th and 35th days of experiment.

Expression of HSP90: The results of expression of HSP90 of control and treated animals have been given in fig.1-C. In control group the relative mRNA expression of HSP90 of buffalo calves were found significantly ($P < 0.05$) higher on 21st day than the 1st and 7th days of experiment. In melatonin treated group mRNA expression of HSP90 of buffalo calves were found significantly ($P < 0.05$) higher on 14th day than 28th and 35th day of the experiment. Between the groups relative mRNA expression of HSP90 of buffalo calves were found significantly ($P < 0.05$) different on day 21st, 28th and 35th days of experiment.

Expression of HSP110: The results of expression of HSP110 of control and treated animals have been given in fig.1-D. In control group the relative mRNA expression of HSP110 of buffalo calves were did not differed significantly ($P > 0.05$) on different days of experiment. In melatonin treated group mRNA expression of HSP110 of buffalo calves were found significantly ($P < 0.05$) higher on 1st day than 21st day of experiment. Between the groups relative mRNA expression of HSP110 of buffalo calves were found significantly ($P < 0.05$) different on day 1st and 14th days of experiment.

Melatonin concentrations in serum have been depicted in Table 4. In control group the concentration of melatonin in serum was significantly ($P < 0.05$) higher at 21st, 35th and 42nd day than the 14th and 28th day of the experiment. The melatonin supplemented group had significantly ($P < 0.05$) higher level of melatonin concentration on 35th day than the other days of the experiment. The concentration of melatonin in serum was significantly ($P < 0.05$) different at 7th, 14th, 21st, 28th, 35th and 42nd days of the experiment between the groups.

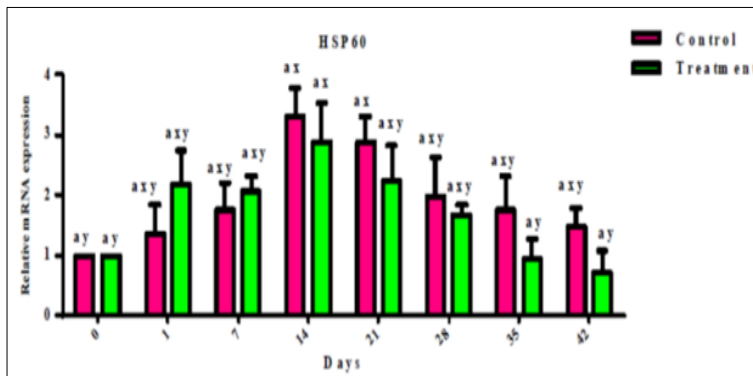


Fig 1: Relative mRNA expression in PBMCs during summer; HSP60 (1-A). The bar bearing different superscripts (a, b) differ significantly ($P < 0.05$) between control and treatment group. The bar bearing different superscripts (x, y, z) differ significantly ($P < 0.05$) during different days in same group during study period

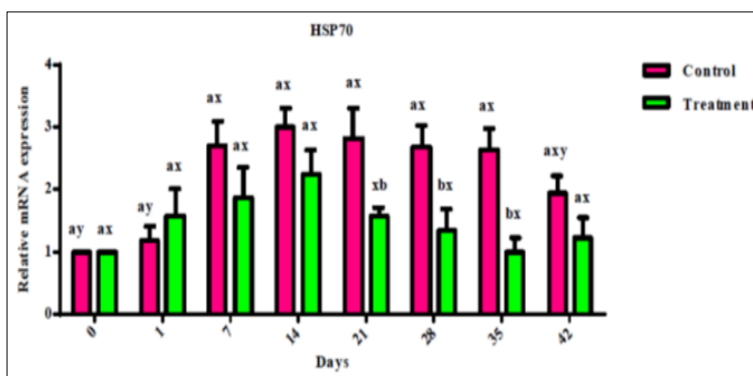


Fig 2: Relative mRNA expression in PBMCs during summer; HSP70 (1-B). The bar bearing different superscripts (a, b) differ significantly ($P < 0.05$) between control and treatment group. The bar bearing different superscripts (x, y, z) differ significantly ($P < 0.05$) during different days in same group during study period

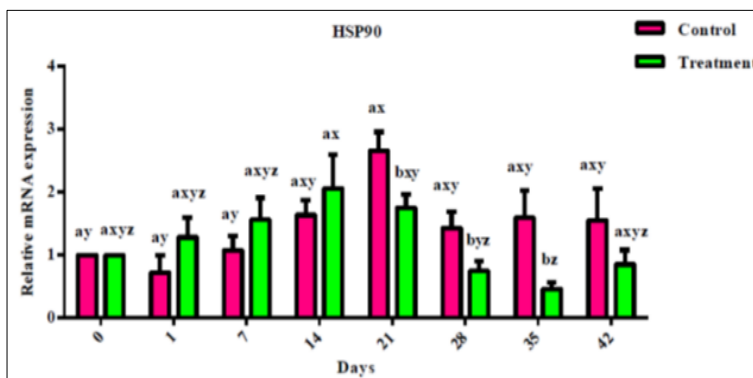


Fig 3: Relative mRNA expression in PBMCs during summer; HSP90 (1-C). The bar bearing different superscripts (a, b) differ significantly ($P < 0.05$) between control and treatment group. The bar bearing different superscripts (x, y, z) differ significantly ($P < 0.05$) during different days in same group during study period

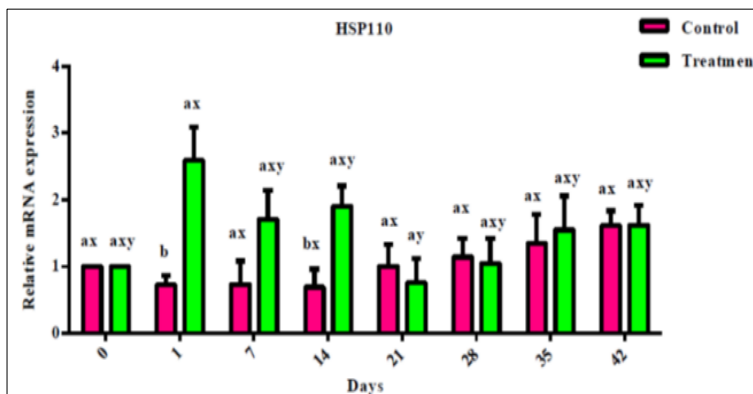


Fig 4: Relative mRNA expression in PBMCs during summer; HSP110 (1-D). The bar bearing different superscripts (a, b) differ significantly ($P < 0.05$) between control and treatment group. The bar bearing different superscripts (x, y, z) differ significantly ($P < 0.05$) during different days in same group during study period

Table 4: Melatonin (ng/ml) concentration of control and melatonin supplemented animals during summer

Parameter	Group	0 day	7 day	14 day	21 day	28 day	35 day	42 day
Melatonin (ng/ml)	Control	35.1±4.26 ^{abA}	36.4±8.13 ^{abB}	25.1±6.71 ^{bB}	45.5±4.82 ^{aA}	25.28±0.95 ^{bB}	43.59±5.06 ^{aB}	40.60±3.24 ^{aA}
	Treatment	26.0±8.6 ^{eA}	79.6±4.01 ^{dA}	246.2±11.8 ^{cA}	22.0±5.01 ^{eB}	308.9±13.4 ^{bA}	511.9±15.3 ^{aA}	28.31±2.9 ^{eB}

Bars bearing different superscript a, b, c, d, e differ significantly ($P < 0.05$) in the same group between days and superscript 'A' and 'B' denotes significant ($P < 0.05$) difference between the groups during study period

Discussion

Heat stress is defined as a group of conditions due to over exposure to or over exertion in excess environmental temperature and animal is unable to dissipate sufficient amount of heat to maintain homeothermy. The thermal comfort zone for most animal ranges between 4 °C and 25 °C and temperatures exceeding 25 °C result in heat stress. Under thermal stress, a number of physiological and behavioral responses vary in intensity and duration in relation to the animal genetic make-up and environmental factors through the integration of many organs and systems *viz.* behavioral, endocrine, cardio-respiratory and immune system [11, 12, 13]. Melatonin functions as an antioxidant by scavenging free radicals, stimulating antioxidative enzymes, enhancing the mitochondrial oxidative phosphorylation efficiency and reducing leakage of electron (thereby lowering free radical generation), augmenting the efficiency of other antioxidants [14, 15, 16, 17].

Animal's physiological and behavioral response to ameliorate heat stress, at cellular level there is intrinsic molecular mechanism to maintain cellular homeostasis. It is widely accepted that changes in gene expression are an integral part of the cellular response to thermal stress. Although the HSPs are perhaps the best-studied examples of genes whose expression is affected by heat shock, it has become apparent in recent years that thermal stress also leads to induction of a substantial number of genes not traditionally considered to be HSPs. Some of these genes are affected by a wide variety of different stressors and probably represent anonspecific cellular response to stress, whereas others may eventually found to be specific to certain types of stress [18]. In mammalian cells, nonlethal 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. This response characteristically includes an increase in thermotolerance (i.e. the ability to survive subsequent, more severe heat stresses) that is temporally associated with increased expression of HSPs. Thermal-induced changes in gene expression occur both during hyperthermia as well as hypothermia. Gene expression changes include activation of heat shock transcription factor 1 (HSF1), increased expression of heat shock proteins (HSP) and decreased expression and synthesis of other proteins [19]. Animal's adaptation to harsh environmental condition and resistance to stress has been correlated with expression of HSPs [20]. HSPs play important role in folding of newly synthesized proteins, transport of proteins into cell compartments, disaggregation of protein complexes and others functions [21]. Expression of many HSPs including HSP32, HSP40, HSP60, HSP70, HSP90, HSP110 and many others are found to be increased during hyperthermic stress [22, 23, 20, 24]. Homeostatic mechanism that protects cell damage from heat stress involves regulation of expression of genes that code for HSP 60 during heat stress [25]. Increased HSP60 mRNA expression during summer season in goats could evoke its transcription in PBMCs to prevent cell from damaging effect of heat stress like denaturation of proteins and HSP60 helps in refolding of proteins and prevents aggregation of denatured proteins [20].

The significant increase in expression of HSP 60 in both control and treatment group following heat exposure at 35°C and 40 °C can be attributed to antiapoptotic role played by the HSP 60 along with its role in protein folding and preventing aggregation of denatured proteins [24]. In the present study in control group between days and in control and treatment group on different days, the relative mRNA expression of HSP60 did not differ significantly ($P > 0.05$) whereas, in melatonin treated group mRNA expression of HSP60 found significantly ($P < 0.05$) higher on 14th day than 35th and 42nd days of the experiment. This may be due to modulating effect of melatonin on its expression under heat stress. Given the anti apoptotic role of HSP 60 in stressed cells, it can be concluded that melatonin may play protective role in heat stress by increasing availability of HSP 60. Our study is in agreement with the previous study conducted by [24] who have demonstrated increased expression of HSP 60 in heat stressed AR42J pancreatic cells following melatonin treatment. Among all the HSPs, HSP 70 is most temperature sensitive and is positively correlated with thermotolerance. It is found in cytosol and nucleus and play important role in the folding of proteins, refolding of misfolded proteins, degradation of unstable proteins, maintenance of structural proteins, translocation of proteins and prevention of protein aggregation [27]. [24] Reported that significant increase in expression of HSP 70 following heat stress. Similarly in our study in control group the relative mRNA expression of HSP70 of buffalo calves were found significantly ($P < 0.05$) higher on 7th, 14th, 21st, 28th and 35th days than 0th and 1st days of experiment. In melatonin treated group mRNA expression of HSP70 of buffalo calves did not differ significantly ($P > 0.05$) on different days of the experiment. Between the groups relative mRNA expression of HSP70 of buffalo calves were found significantly ($P < 0.05$) different on day 21st, 28th and 35th days of experiment. Stress induced increase in expression of HSP 70 in bovine lymphocytes [28, 29], in kidneys of goats [30], in myocardium [31], in lung cells [32] and in hepatocytes and liver [33] thereby indicating that heat shock proteins provide protection from toxic effects of thermal stress. [19] Have observed increased HSP 70 expression in bovine mammary epithelial cells exposed to heat stress at 42°C for first 4 h followed by decreased expression in subsequent hours.

HSP 90 plays an important role in protein translocation and regulation of steroid hormone receptors. Its presence in cytosol, ER and nucleus is in compliance with its function. Like other HSPs, it also contributes to cellular response to heat stress. In present study in control group the relative mRNA expression of HSP90 of buffalo calves were found significantly ($P < 0.05$) higher on 21st day than the 1st and 7th days of experiment. In melatonin treated group mRNA expression of HSP90 of buffalo calves were found significantly ($P < 0.05$) higher on 14th day than 28th and 35th day of the experiment. Between the groups relative mRNA expression of HSP90 of buffalo calves were found significantly ($P < 0.05$) different on day 21st, 28th and 35th days of experiment. Similarly, [24] observed that increased expression of HSP 90 with increased thermal exposure.

Similar findings under heat stress have been reported in human lymphocytes [34], in T cells [35], in rat myocytes [36], in heart, liver and kidney of broilers [37], in murine embryonic fibroblast cells [38], in lung, heart, spleen, liver and brain of human [39]. Recently *in vitro* studies also showed the increased expression of HSP90 in bovine PBMCs at 2 h post heat stress [40, 41]. Previous studies also reported increased HSP90 expression due to heat stress in human blood lymphocytes [34] and T-cells [35]. Increased HSP90 expression during summer season in comparison to winter season could be due to the fact that ambient heat stress stimulated and quickly initiated the transcription of HSP90 mRNA and translation of HSP90 protein to protect cells from heat stress. The mRNA expression of HSP60, HSP90 and ubiquitin were higher during peak summer season as compared to peak winter season in both tropical and temperate region goats which might play an important role in thermal stress tolerance against harsh environmental conditions [20]. HSP110 is a major HSP of mammals and of eukaryotic cells in general. Unlike other major HSPs, which have been intensively studied for many years, HSP110 has only been cloned and studied within the last few years. The HSP110 family has been found to be distantly related to the HSP70 family in sequence [42] and HSP110 has been observed to express functional similarities as well as differences compared with the HSP70 family. One important property of HSP110 is its ability to bind to and chaperone full-length proteins during heat shock with high efficiency [43]. In present study in control group the relative mRNA expression of HSP110 of buffalo calves were did not differ significantly ($P > 0.05$) on different days of experiment. In melatonin treated group mRNA expression of HSP110 of buffalo calves were found significantly ($P < 0.05$) higher on 1st day than 21st day of experiment. Between the groups relative mRNA expression of HSP110 of buffalo calves were found significantly ($P < 0.05$) different on day 1st and 14th days of experiment. HSP110 relative mRNA expression level remained unchanged during the first 4 h, and thereafter, it increased significantly ($P < 0.05$) and reached the peak at 6 h [44]. HSP110 expression was significantly ($P < 0.05$) highest ($P < 0.05$) in long term heat stress acclimation condition [45].

Conclusion

On the basis of experimental finding it may be concluded that the melatonin treatment during the summer stress may help in the ameliorating the deleterious effects of summer stress in buffalo calves and enables animals to maintain its cellular homeostasis and melatonin also have a dwindling effect on expression of HSPs, suggesting a possible role of these in heat stress amelioration.

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