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ORIGINAL ARTICLE

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In-vivo Thermal Stress Induces Melatonin Receptors and Heat Shock Proteins Expression in the Spleen of Mice in a Time and Temperature Dependent Manner.

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Heat shock proteins (Hsps) responses against stress conditions. Melatonin completes its stress relieving activities via its MT1 and MT2 receptors. The present study delineates the expression pattern of Hsp70/Hsc70 and MT1/MT2 receptor proteins along with the AANAT gene expression in the splenic tissue of mice subjected to hyperthermic stress in a temperature dependent and time dependent manner. *In vivo* thermal stress resulted increase in expression of Hsp70, Hsc70 and MT2 receptors proteins in both temperature dependent and time dependent manner. Optimum heat exposure at 43^0C and maximized Hsps expression was observed after 5 hours of heat exposure. Heat stress caused increase in AA-NAT gene expression of the splenic tissue resulted in the synthesis of melatonin which might act as signal molecule for upregulating the activity and rise of the stress responsive genes and proteins like: Hsp70/Hsc70, whereas simultaneous increase of MT2 expression shows its possible involvement in such mechanism.

Key words: Thermal stress, heat shock proteins, melatonin receptors, AA-NAT gene expression, time and temperature dependent study in mice

The potentiality of hyperthermic stress for damaging the cellular activity is a well-known fact. Activation of apoptosis process in rapid manner is a suggested possible mechanism through which heat stress causes the cellular loss (Kondo *et al.*, 2000; Khan and Brown, 2002). Recently a report on focusing the improvement of Arrhenius model of cell death concerning the temperature dependent time delay matter was documented (Pearce, 2015). But before the completion of cellular damage via the programmed cell death process, anti-apoptotic protein performs their activity for the protection of the cell. Heat shock proteins (Hsps) are the molecular chaperones in the cellular system which works against the apoptotic process (Gabai *et al.*, 1997; Kondo *et al.*, 2000; Li *et al.*, 2000). Expression of heat shock protein as a result of thermal shock is a natural phenomenon by which cell protects it-self. Stress at higher temperature causes aggregation of heat shock proteins with different polypeptides for their structural refolding trapped in the aggregates (Liberek *et al.*, 2008). Among various family members, inducible heat shock protein 70 (Hsp70) plays the vital role in the structural refolding process by actively participating with other chaperones (Kalmar and Greensmith, 2009). Another constitutive family member, heat shock cognate 70 (Hsc70) protein is a sensitive biomarker against the various physiological and environmental assaults (Mukhopadhyay *et al.*, 2003). Both of these heat shock protein protect other cellular proteins from unfolding, or refold the denatured proteins, or drive them for proteasomal degradation (Torigoe *et al.*, 2009).

Melatonin, a pineal gland product has multidimensional functional capability in different physiological functions of organisms (Reiter, 1991; Hardeland and Fuhrberg, 1996). Melatonin plays a significant role in circadian rhythm (Berra and Rizzo, 2009), and influences the cardio vascular system (Paulis *et al.*, 2012; Lochner *et al.*, 2013) and immune system activity (Maestroni, 1993; Guerrero and Reiter, 2002). It also works as an anti-stress hormone in the physiological system (Maestroni and Conti, 1991a; Maestroni and Conti, 1991b, Brotto *et al.*, 2001). Melatonin performs its functions either non-receptor mediated or receptor mediated action mechanisms. Melatonin is also recognized as an anti-stress hormone, due to its ability of scavenging the free radicals generated from stress conditions (Reiter *et al.*, 2001; Tan *et al.*, 2003; Berra and Rizzo, 2009; Garcia *et al.*, 2014). Melatonin by involving MT1 and MT2 receptors also mediates its functions (previously identified as Mel1a and Mel1b) (Dubocovich, 1995; Reppert, 1997; Browning *et al.*, 2000). The involvement of melatonin in thermoregulation process after heat stress is well reported (Dawson *et al.*, 1996; McLellan *et al.*, 1999; Aoki *et al.*, 2006). Melatonin also works to improve the negative impacts of heat stress (Gharib *et al.*, 2008).

The expression of melatonin receptors and heat shock proteins in various tissues of the physiological system were documented in different studies in various vertebrate groups (Stolte *et al.*, 2009; Ozacmak *et al.*, 2009; Dang *et al.*, 2010; Lollo *et al.*, 2013). But, lacking of information remains in mammals which can delineate the immune system interactions to cope up against the stress condition generated effects. The primary lymphoid organ thymus remains most active in new-borns, but along with the increase of adulthood it becomes sedentary, whereas the secondary lymphoid organs like: spleen, tonsils, lymph nodes, peyer's patches etc. works throughout life cycle. Therefore, the responsibility of encountering pathogens and antigens mainly relies on the secondary immune organs of the body. We considered spleen for our study because of its role as a main secondary lymphoid immune organ, which covers the maximum percentage of immune activity throughout the entire life cycle in mammals. Although different reports suggested that heat stress causes the rise of melatonin secretion (Abbas *et al.*, 2007; Sejian *et al.*, 2008), but till date, heat stress generated change of melatonin receptors expression pattern was not evaluated. Therefore, we evaluated the changes of expression pattern of melatonin receptors after the hyperthermic stress condition along with the increase of time and temperature within the splenic tissue of adult mice.

The present study was focused to observe the changes of melatonin receptors (MT1 and MT2) expression pattern along with heat shock proteins

(Hsp70 and Hsc70). Heat shock protein expression is considered as a sensitive biomarker for determining or evaluating the stress conditions when *in vivo* heat stress was provided to mice groups. So, heat shock protein expression (Hsp70 and Hsc70) along with the melatonin receptors (MT1 and MT2) were investigated in the mice groups exposed to different temperature gradients. Secondly, after hyperthermic stress along with the increase of time, how heat shock protein expression level changes along with melatonin receptors is also an important query which should be understood for delineating the recovery process of spleen cells from the adverse effects of heat stress.

MATERIALS AND METHODS

Animal Procurement and Maintenance:

Healthy male laboratory Swiss Albino mice were randomly selected from mice colony. Healthy mice colony was housed at ambient laboratory conditions (under 12L: 12D cycles and 25^0C-27^0C temperature). Mice were kept in groups of five in polycarbonate cages (43cm x 27cm x 14 cm) to avoid the population stress. Mice were fed regularly with mice feed and water *ad libitum.*

All the experiments on the animals were conducted in accordance with institutional practice and within the framework of the revised Animal (Specific Procedure) Act of 2007 of Govt. of India on animal welfare. The study was approved by institutional animal ethics committee (IAEC) with ethical clearance no. TU/IAEC/2014/IX/2-3.

Experimental Design:

The experimental design was divided into two subsets. Five mice were kept in each group of each experimental subset. Experimental design of the two subsets are interrelated or interlinked. The first subset consists of 6 mice groups exposed to thermal stress at 43^0C temperature for 45 minutes in an isolated thermal chamber with proper ventilation. Then the mice groups subjected to heat stress were sacrificed at 1hour intervals after heat exposure. First group sacrificed after 1hour (hr) and then subsequently remaining groups at 2hr, 3hr, 4hr, 5hr and 6hr of heat exposure, respectively. In the second experimental subset, we divided the mice into three groups subjected to different temperatures. In this subset, the first mice group was treated as control group (Con) without any kind of stress and second and third mice group was exposed to thermal or heat stress (Heat) at 41^0C and 43^0C temperature for 45 minutes in the same manner as described earlier. The mice groups subjected to heat stress in the second experimental subset were sacrificed after 5 hours of heat exposures, which we observed as the correct time of sacrifice from the result of first experimental subset. It was also noted that higher mortality was there in the mice group which were exposed to temperature above 43^0C, whereas at 41^0C and 43^0C temperature no mortality was noted during the thermal exposure period and post stress period. Therefore, only 41^0C and 43^0C temperature were considered for the study of thermal stress in the concerned mice strain. All experimental groups were sacrificed after anaesthesia and the spleen was dissected out immediately on ice. Spleen of each mouse of each group was divided and immediately stored at -40^0C for western blot analysis and the second part was fixed in aqueous Bouin's fluid for immunohistochemical studies. A third part of spleen from the second experimental subset was also immediately processed for RNA isolation and subjected to reverse-transcriptase-PCR for analysing the AANAT gene expression level.

Western Blot Analysis:

Western blot analysis was performed to assess the expression of Hsp70, Hsc70 proteins and melatonin receptors MT1, MT2 in the spleen of experimental mice. Spleen tissues were homogenized and lysed in RIPA buffer [1% (v/v) NP-40, 0.1% w/v sodium dodecyl sulphate (SDS) in PBS containing aprotinin, sodium orthovanadate and phenylmethylsulphonylfluoride (PMSF)] and then quantified by Lowry method (1951). Aliquots containing 100 ↄg proteins were resolved by 10% (w/v) SDS polyacrylamide gel electrophoresis followed by electro transfer to nitrocellulose membrane (Santa Cruz Biotech, USA). Immune detection was carried out by using anti-Hsp70, anti-Hsc70, anti-Mel 1AR, anti-Mel 1BR [Hsp70; ab79852 and Hsc70; ab 1427, rabbit polyclonal, Abcam, USA; Mel1AR (MT1); sc-13186 and Mel1BR (MT2); sc-13177, goat polyclonal, Santacruz Biotech, USA, diluted 1:200] and ✔-actin

antibody (sc-130656, rabbit polyclonal Santacruz Biotech, USA, diluted 1:500) diluted in PBS contained 5% skimmed milk and 0.01% Tween-20 followed by incubation with horseradish peroxidase conjugated secondary antibodies (goat anti-rabbit IgG for Hsp70, Hsc70 and ✓-actin antisera; diluted 1:1000 and rabbit anti-goat IgG for Mel1AR and Mel1BR antisera; diluted 1:1000). The immune interactions were detected by using Super Signal West Pico Chemiluminescent Substrate (#34080, Thermo Scientific, Rockford, USA). Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation, MD, and USA). Values were expressed as ratio of the density of the specific signal to ✓-actin signal and expressed as the % control value (Treeck *et al.*, 2006). Each sample corresponds to tissue from a single animal and at least five gels corresponding to each subunit and experimental conditions were analysed.

Immunohistochemistry:

Immunohistochemical studies of experimental tissues (spleen) were done following the procedure adopted by Savaskan *et al.*, (2002). Paraffin sections (6 µm) fixed on 1% gelatine coated slides were deparaffinised and rehydrated with alcohol grades. The sections were placed in PBS for 30 minutes and endogenous peroxide activity was blocked by 0.3% H_2O_2 in methanol for 30 minutes at room temperature (25^{0}C). Sections were washed thrice with phosphate buffered saline (PBS: 0.1M Na_2HPO_4, NaH_2PO_4, 0.9% NaCl, pH=7.4) and were placed in blocking solution (horse blocking serum, diluted 1:200 in PBS, PK -6200, Vector Laboratories, Burlingame, CA). Sections were incubated with primary antibodies [Hsp70; ab79852 and Hsc70; ab1427, rabbit polyclonal, Abcam, USA; Mel1AR (MT1); sc13186 and Mel1BR (MT2); sc13177, goat polyclonal, Santacruz Biotech, USA, diluted 1:200] overnight at 4^{0}C. Next day, sections were washed thrice with PBS and incubated with biotinylated secondary antibody (Vectastain ABC Universal Kit, PK-6200, Vector Laboratories, Burlingame, CA, dilution 1:1000). Same sections were again washed thrice with PBS and incubated with preformed AB (Avidin-Biotin) reagent for 30 minutes. The antigens were visualized using the 0.03% peroxidase substrate 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Chemicals, St. Louis, USA) in 0.01M Tris-Cl (pH=7.6) and counterstained with Ehrlich's haematoxylin. The sections were dehydrated and mounted with DPX. Microphotographs of the stained sections were taken under 40X objective of Leica microscope DM4000. To test the specificity of the used antibodies, the primary antibodies were not added in control sections which were treated as negative control and incubated with same dilution of normal serum for overnight at 4^{0}C. Next morning the immunohistochemical protocol was followed under the same conditions. The immunohistochemical studies were interpreted by following the semiquantitative analysis method described by Rezzani *et al.* (2005).

Reverse-Transcriptase-PCR of AANAT (rate limiting enzyme of melatonin biosynthesis):

Total RNA isolation:

Expression of AANAT gene was studied by semi-quantitative PCR using S-1000 thermal cycler (Biorad, USA). Total RNA was isolated from fresh spleen tissue using PureZOL™ (Biorad, USA) following the manufacturer instruction. The isolated RNA was quantitated by spectrophotometry and its integrity was verified on 1% formaldehyde agarose gel electrophoresis containing 2.2M formaldehyde, 8mM sodium acetate, 1mM EDTA and 20 mM MOPS.

Reverse Transcription Reaction:

Reverse transcription reaction was done for cDNA synthesis of RNA sample using iScript™ cDNA synthesis kit (Biorad, USA) according to the manufacturer instruction. 1 µg total RNA was used for 20 µl reaction mixture and incubated at 25 °C for 5 min. Mixture was then incubated at 42 °C for 30 min and then incubated at 85 °C for 5 min. After chilling on ice the cDNA was subjected for PCR.

Polymerase Chain Reaction:

PCR was done using GoTaq® Green Master Mix (Promega, USA) and specific oligonucleotide primer as per manufacturer instruction. The primers used for AANAT PCR were 5'-CCTTGCAGTCAGGAGTCTCA-3' (forward) and 5'-AACTCTGAGGTCCCAAGTGG-3' (reverse) give a 211bp PCR product. GAPDH

amplification was done for control expression. The primers used for GAPDH PCR were 5'-AACTTTGGCATTGTGGAAGG-3' (forward) and 5'-ACACATTGGGGGTAGGAACA-3' (reverse) give a 223bp PCR product. All the mice primers used were synthesized by Imperial Life Science, India. 5µl of RT product amplified in total mixture volume of 25µl with 0.4µM of each primer and 12.5µl of 2X GoTaq Green Master Mix. The samples were denatured at 94 °C for 2 min and were further amplified as following: (for both AANAT and GAPDH 35 cycles were performed, 94°C 15 sec, 60°C 30 sec and 72°C 40 sec) with a final extension of 10 min at 72°C. The size of amplification product on the gel was assessed by comparison with 100 bp DNA ladder (StepUp™ 100 bp DNA ladder, MBD, 13J, Merck-Millipore, Bangalore, India). All PCR experiments included negative control, in which template cDNA was omitted. PCR products were electrophoresed on ethidium bromide containing 2% agarose gel. Bands were visualized in a UV-transilluminator and photographed (MiniLumi, DNR Bioimaging System, Israel). The AANAT relative amount was expressed in terms of optical density relative to GAPDH.

Statistical Analysis:

Statistical analysis of the data was performed using SPSS 17.0 (SPSS Corp., USA) programme with one way ANOVA followed by Tukey's multiple range tests for multiple comparisons. The differences were considered significant when $p<0.05$.

RESULTS

Western blot Analysis:

We performed western blot analysis to assess the expression of Hsp70, Hsc70 and melatonin receptor (MT1 and MT2) proteins in the immune organ, spleen of laboratory Swiss albino mice at the translational level. Hsp70 and Hsc70 proteins were detected as a single band corresponding to 70kDa, whereas melatonin MT1 and MT2 receptors proteins were detected as a single band in between 35–40 kDa, which precisely corresponded to the predicted molecular mass of the receptor (Ahmad and Haldar 2010).

Heat shock protein 70 (Hsp70, inducible) expression in spleen:

Increasing trend in expression pattern of Hsp70 protein was observed in experimental mice groups sacrificed at 1hr, 2hr, 3hr, 4hr, 5hr and 6hr after the heat exposure to 43^0C temperature (Fig.1). Hsp70 protein expression also increased significantly ($P<0.01$) at both 41^0C and 43^0C exposed mice groups in comparison with the control of the second experimental subset (Fig.2). The mice group exposed to 43^0C showed significant ($P<0.01$) higher expression of Hsp70 protein than the 41^0C group.

Heat shock cognate 70 (Hsc70, constitutive) expression in spleen:

Expression of Hsc70 protein was also showed similar trend of increase in the experimental mice groups sacrificed at 1hr, 2hr, 3hr, 4hr, 5hr and 6hr after the heat exposure at 43^0C temperature (Fig.3). Significant ($P<0.01$) increased expression of Hsc70 protein was also observed in 41^0C and 43^0C experimental groups compared to control of the second experimental subset (Fig.4). Significant ($P<0.01$) higher expression of Hsc70 was noted at 43^0C than the 41^0C exposed group of mice. It was noted that within 5hr and 6hr, the increasing expression pattern of both proteins (Hsp70 and Hsc70) was stabilized and maximum expression was noted in mice of 5 hr group of the first experimental subset.

Melatonin receptor MT1 expression in spleen:

MT1 receptor protein showed consecutive decrease in expression pattern in the 1hr, 2hr and 3hr mice groups and then again increased to 5hr group (Fig.5). Significant ($P<0.01$) decrease was also noted in MT1 protein expression at 41^0C and 43^0C temperature exposed mice groups in comparison with control group of the second experimental subset (Fig.6). The mice group exposed to 43^0C showed significant ($P<0.01$) decreased expression than the 41^0C exposed mice and control one.

Melatonin receptor MT2 expression in spleen:

In contrast to MT1 receptor protein expression, MT2 receptor protein showed increased expression pattern along with the increase in time after the heat exposure such as in 1hr, 2hr, 3hr, 4hr, 5hr and 6hr groups (Fig.7). MT2 receptor protein also significantly ($P<0.01$) increased in the mice groups exposed to 41^0C and 43^0C

temperature in comparison with the control mice group of the second experimental subset (Fig.8). Mice groups exposed to 43^{0}C showed significant (P<0.01) higher expression than the 41^{0}C group. Interestingly, MT2 receptor protein showed similar pattern of change in the expression as it was noted in heat shock proteins (Hsp70 and Hsc70).

Immunohistochemistry:

We performed immunohistochemistry for observing and localizing Hsp70, Hsc70 protein and melatonin MT1 and MT2 receptors in the splenic tissue of the experimental mice groups. In negative control sections no reaction were detected. The enlarged view of microphotographs showed the specific binding of MT1 and MT2 receptors and extra and intra cellular Hsp70 and Hsc70 protein in the splenic tissue of the experimental groups. The time gap of the spleen tissue collection from the subject exposed to different temperatures was 5 hours and therefore, the immunohisochemical localization of 1 hour and 5 hour was provided here from the first experimental subset to understand the actual difference of proteins in the splenic tissue.

Heat shock proteins (Hsp70 and Hsc70):

Immunohistochemical study showed immunoreactivity of both Hsp70 and Hsc70 proteins in the splenic tissue of each group after the hyperthermic stress condition (43^{0}C). Increased immune reactivity of Hsp70 (Fig.9, B1) and Hsc70 (Fig.9, B2) was noted in the splenic tissue of 5 hour group of mice in comparison to 1 hour group of mice (Fig.9, A1, A2) sacrificed after the heat exposure. In a similar way, strong immunoreactivity of Hsp70 (Fig.10) and Hsc70 (Fig.11) was observed in the extra and intra cellular space of splenic tissue of the 43^{0}C exposed mice group compared to the 41^{0}C exposed and control mice group of second experimental subset.

Melatonin receptors (MT1 and MT2):

After hyperthermic stress at 43^{0}C, MT1 showed similar immunoreactivity in the splenic tissue of mice groups sacrificed at 1 hr (Fig.12, A1) and 5 hr (Fig.12, B1). But MT2 showed increased immunoreactivity in the splenic tissue of 5 hr mice group (Fig.12, B2) than the 1 hr group (Fig.12, A2). MT1 immunoreactivity was also stronger in the spleen of control mice than the 41^{0}C and 43^{0}C exposed mice groups (Fig.13) of the second experimental set. However, less MT1 immunoreactivity was noted in spleen of mice exposed to 43^{0}C than mice exposed to 41^{0}C. In contrary, MT2 showed stronger immunoreactivity in both 41^{0}C and 43^{0}C exposed mice groups than the control one of the second experimental set (Fig.14). However, 43^{0}C exposed mice group showed stronger MT2 immunoreactivity than the 41^{0}C exposed mice group.

Semiquantitative analysis of Immunohistochemistry:

The immunohistochemical studies were interpreted by following the semiquantitative analysis method described by Rezzani *et al.* (2005) with a petite modification. Careful photographic examination of the immunohistochemical figures showed no reactivity in the negative control sections, which was presented by '-'. In a similar way, the experimental groups which showed less immunoreactivity presented by '+', medium immunoreactivity presented by '+ +', high immunoreactivity presented by '+ + +' within the splenic tissue of mice groups. The table no.1 depicts the semiquantitative analysis immunohistochemical figures of the various treatment groups stained with specific antibodies.

Reverse-Transcriptase-PCR of AANAT:

AANAT is the rate limiting enzyme of melatonin biosynthesis and the expression level of AANAT gene in melatonin synthesising cell directly reflects the melatonin biosynthesis process. The sets of enzyme required for melatonin biosynthesis are also present in lymphoid organs and immune cells. Melatonin from extra pineal sources shows localized effects. The reverse-transcriptase-PCR of AANAT mRNA of the spleen tissue of second experimental subset showed increase in expression pattern along with the rise of temperature of thermally stressed groups (Fig.15A). 43^{0}C exposed mice group showed significant (P<0.01) increased expression than the 41^{0}C exposed mice and control mice group (Fig.15C).

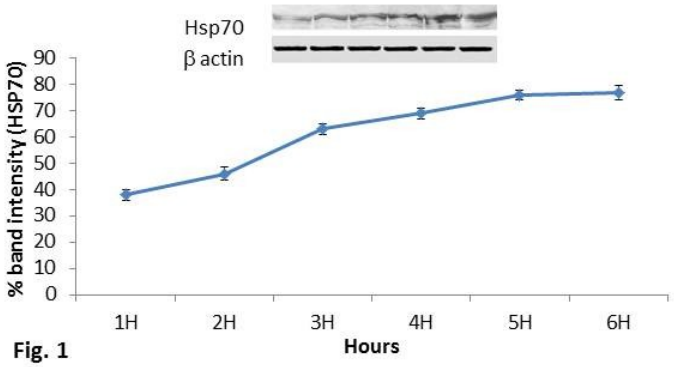

Figure 1. Line graph showing western blot analysis of inducible heat shock protein 70 (Hsp70). β-actin was used as loading control.

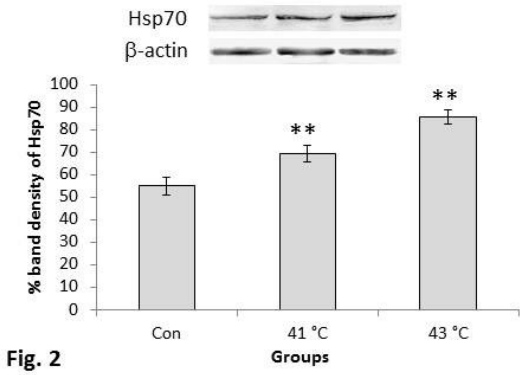

Figure 2. Western blot analysis of inducible heat shock protein 70 (Hsp70). β-actin was used as loading control. Lower panel shows % expression of protein following Scion Image analysis. Histogram represents Mean + SEM. The mean differences were considered significant when $p < 0.05$.

**$p<0.01$: Con vs. Group 41^0C; Con vs. Group 43^0C; Group 41^0C vs Group 43^0C

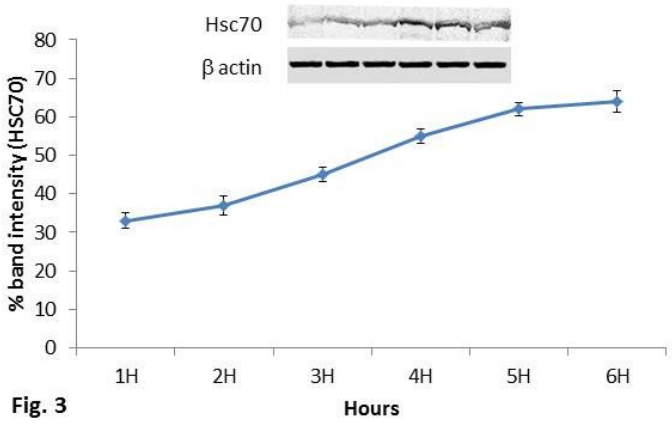

Figure 3. Line graph showing western blot analysis of heat shock cognate protein 70 (Hsc70). β-actin was used as loading control.

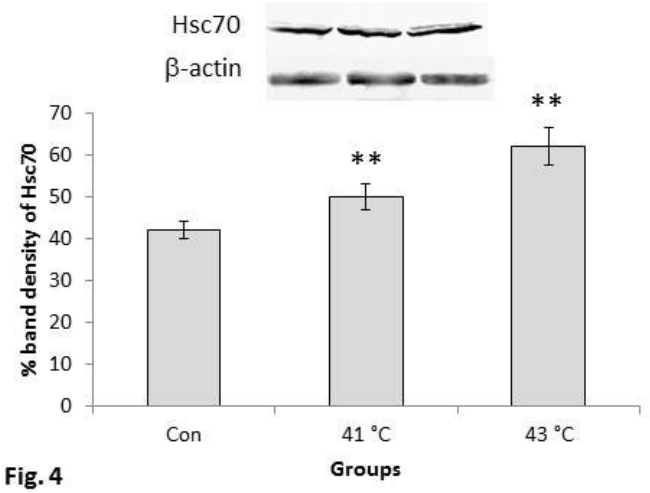

Figure 4. Western blot analysis of heat shock cognate protein 70 (Hsc70). β-actin was used as loading control. Lower panel shows % expression of protein following Scion Image analysis. Histogram represents Mean + SEM. The mean differences were considered significant when p< 0.05.

**p<0.01: Con vs. Group 41^{0}C; Con vs. Group 43^{0}C; Group 41^{0}C vs Group 43^{0}C

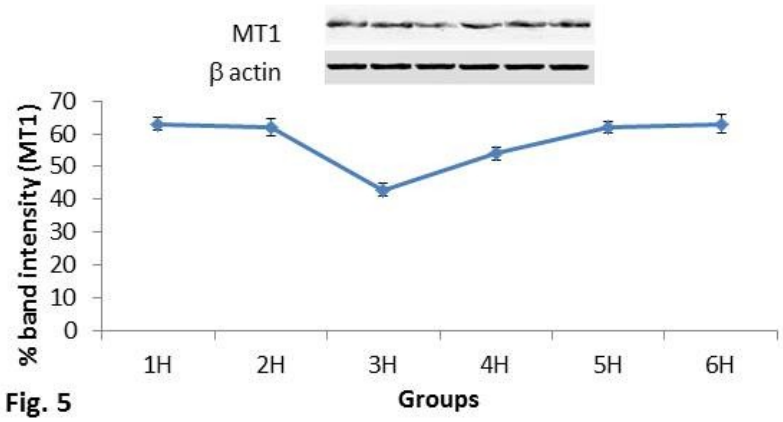

Figure 5. Line graph showing western blot analysis of MT1 melatonin receptor. β-actin was used as loading control.

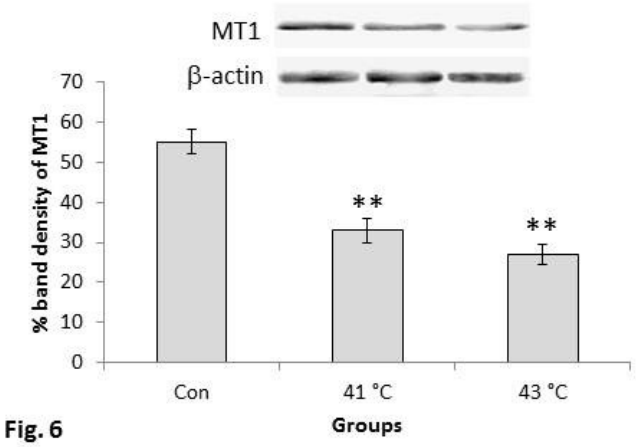

Figure 6. Western blots analysis of MT1 receptor proteins. β-actin was used as loading control. Lower panel shows % expression of protein following Scion Image analysis. Histogram represents Mean + SEM. The mean differences were considered significant when p< 0.05.

**p<0.01: Con vs. Group 41^{0}C; Con vs. Group 43^{0}C; Group 41^{0}C vs Group 43^{0}C

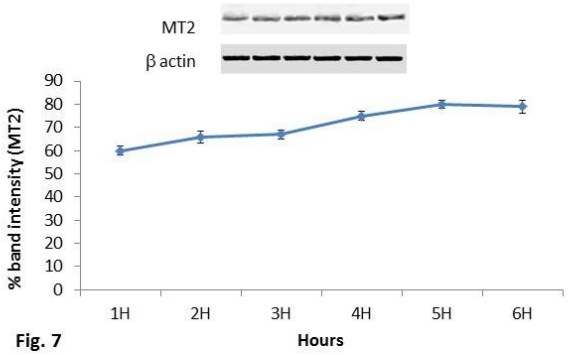

Figure 7. Line graph showing western blot analysis of MT2 melatonin receptor. β-actin was used as loading control.

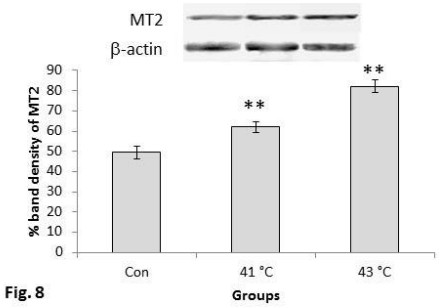

Figure 8. Western blots analysis of MT2 receptor proteins. β-actin was used as loading control. Lower panel shows % expression of protein following Scion Image analysis. Histogram represents Mean + SEM. The mean differences were considered significant when $p < 0.05$.

**$p<0.01$: Con vs. Group 41^0C; Con vs. Group 43^0C; Group 41^0C vs Group 43^0C

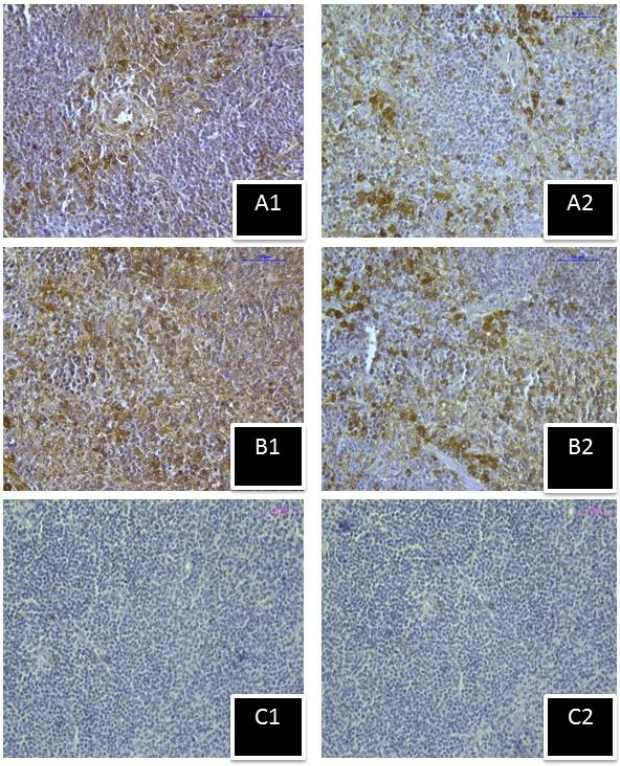

Figure 9. Immunostaining of inducible heat shock protein 70 (Hsp70) in spleen of stress exposed mice group sacrificed after (A1) 1hour, (B1) 5hour respectively, and (C1) negative control section. Immunostaining of heat shock cognate protein 70 (Hsc70) in spleen of stress exposed mice group sacrificed after (A2) 1hour, (B2) 5hour respectively, and (C2) negative control section.(Magnification bars=50µm).

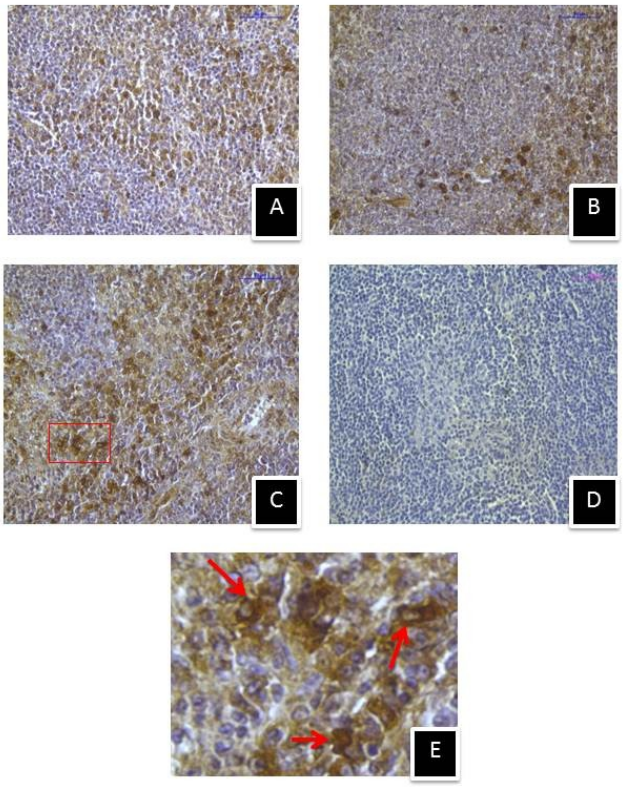

Figure 10. Immunostaining of inducible heat shock protein 70 (Hsp70) in spleen of (A) control, (B) 41^{0}C heat stressed, (C) 43^{0}C heat stressed mice, and (D) negative control section. (E) Enlarged view of selected area showing extracellular and intra cellular Hsp70 immunostaining. (Magnification bars=50µm).

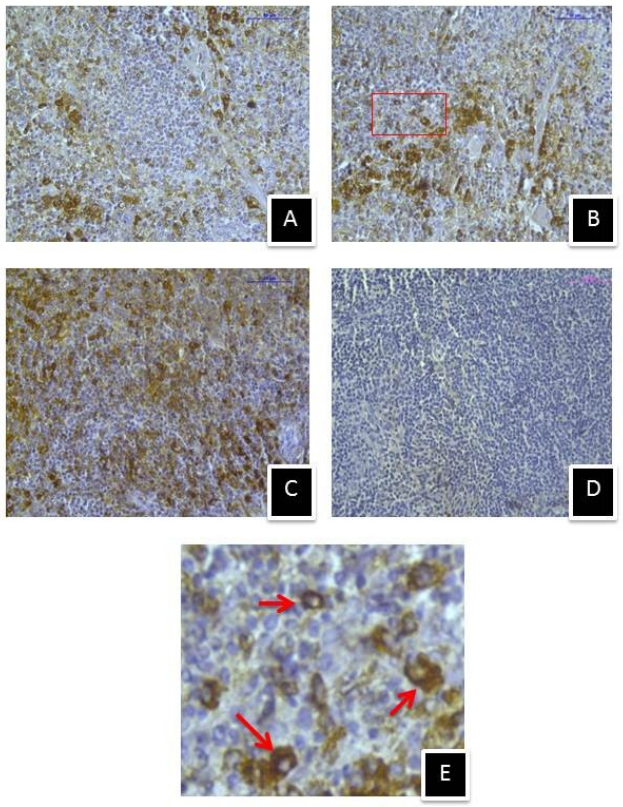

Figure 11. Immunostaining of heat shock cognate protein 70 (Hsc70) in spleen of (A) control, (B) 41^{0}C heat stressed, (C) 43^{0}C heat stressed mice, and (D) negative control section. (E) Enlarged view of selected area showing extracellular and intracellular Hsc70 immunostaining. (Magnification bars=50µm).

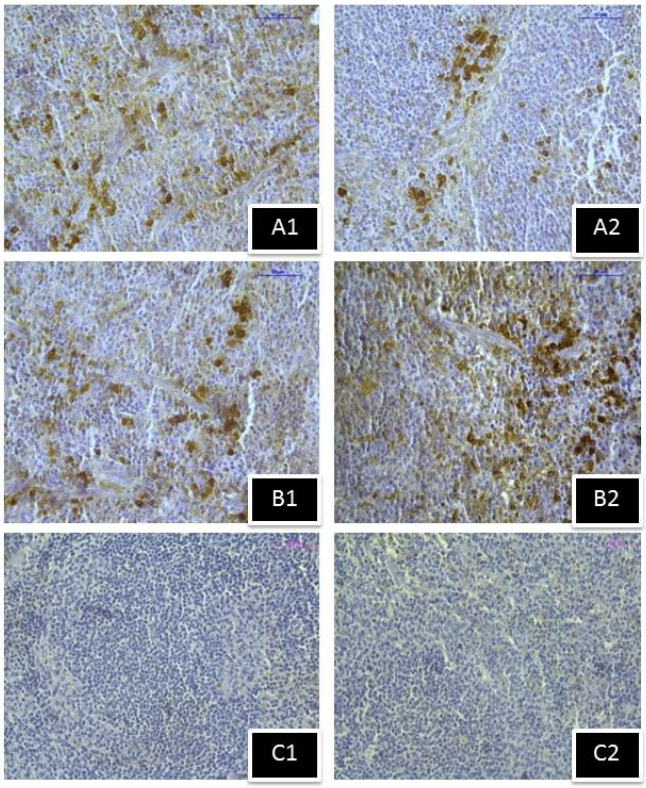

Figure 12. Immunostaining of MT1 melatonin receptor in spleen of stress exposed mice group sacrificed after (A1) 1hour, (B1) 5hour respectively, and (C1) negative control section. Immunostaining of MT2 melatonin receptor in spleen of stress exposed mice group sacrificed after (A2) 1hour, (B2) 5hour respectively, and (C2) negative control section.(Magnification bars=50µm).

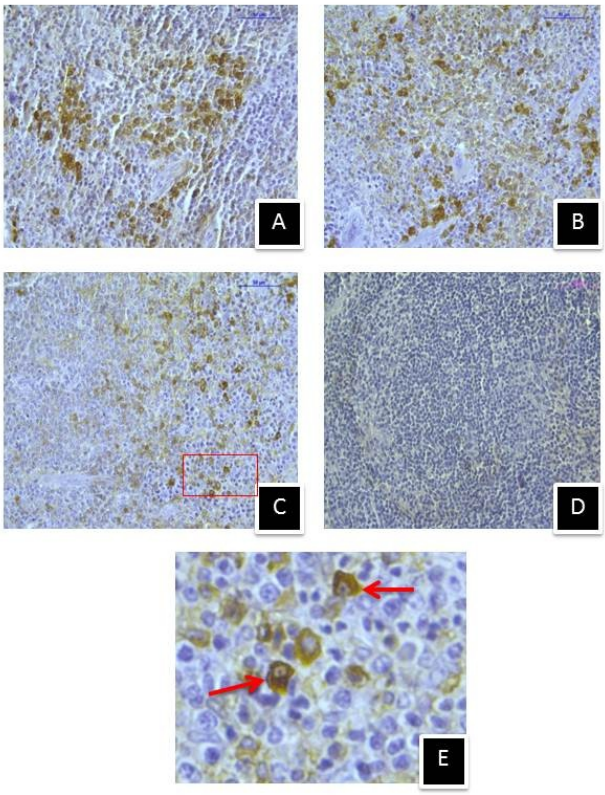

Figure 13. Immunostaining of MT1 melatonin receptor in spleen of (A) control, (B) 41^{0}C heat stressed, (C) 43^{0}C heat stressed mice, and (D) negative control section, (E) Enlarged view of selected area showing membrane specific MT1 immunostaining. (Magnification bars=50µm).

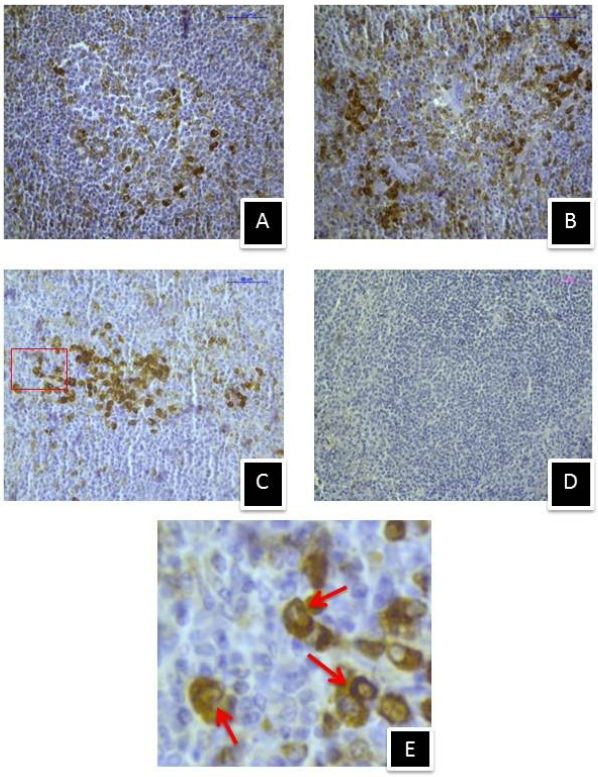

Figure 14. Immunostaining of MT2 melatonin receptor in spleen of (A) control, (B) 41^{0}C heat stressed, (C) 43^{0}C heat stressed mice, and (D) negative control section, (E) Enlarged view of selected area showing membrane specific MT2 immunostaining. (Magnification bars=50µm).

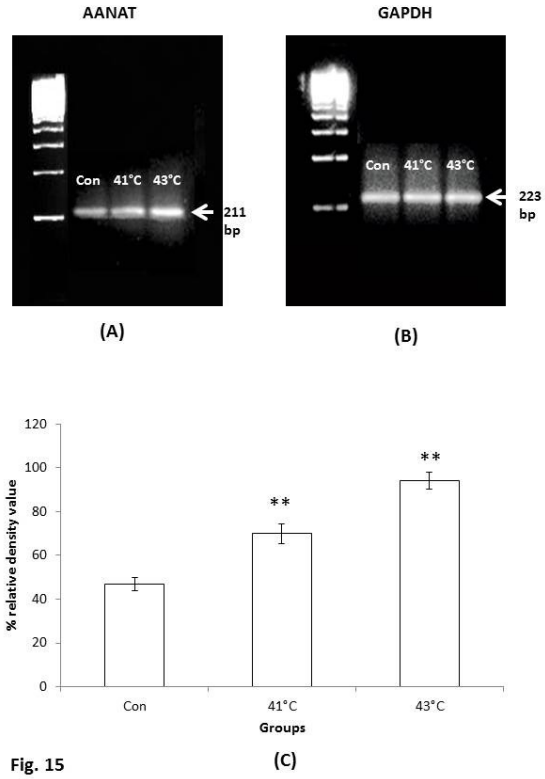

Figure 15. Reverse-transcriptase-PCR of (A) AANAT mRNA and (B) GAPDH mRNA in the spleen of thermally stressed mice groups (41^{0}C and 43^{0}C temperature exposed mice groups). (C) Lower panel shows the histogram after the densitometry analysis. The values were expressed as Mean + SEM. The mean differences were considered significant when $p< 0.05$.

Table 1. Semi quantitative analysis of immunohistochemistry: '+' denotes less immunoreactivity; '+ +' denotes medium immunoreactivity ; '+ + +' denotes high immunoreactivity of the splenic tissue by specific antibodies.

		Temperature dependent study			Time dependent study	
	Treatment Groups	Control group	Thermal stress at 41^0C	Thermal stress at 43^0C	Mice sacrificed after 1 hour thermal exposure	Mice sacrificed after 5 hour thermal exposure
Antibody Immuno-reactivity in immuno–histoche–mistry	Hsp70 immunoreactivity	+	+ +	+ + +	+	+ +
	Hsc70 immunoreactivity	+	+ +	+ + +	+ / + +	+ + /+ + +
	MT1 immunoreactivity	+ + +	+ +	+	+ / + +	+ / + +
	MT2 immunoreactivity	+	+ +/ + + +	+ + / + + +	+	+ + +

DISCUSSION

Thermal stress is an important cue for the production of heat shock protein in relation to cellular protection. Therefore, heat shock protein expression is considered as a sensitive biomarker for identifying challenging conditions of environment (Mukhopadhyay *et al.*, 2003). The thermal sensitivity and tolerance ability in the diverse animal groups could be assessed via the estimation of these heat shock protein expression level (Webster *et al.*, 2013; Lencioni *et al.*, 2013; Sorensen *et al.*, 2013; Tutar *et al.*, 2013; Sharma *et al.*, 2013). Expression and characterization of these proteins were also reported in various vertebrate tissues like intestine, kidney, spleen, gut, gills, thymus and brain (Stolte *et al.*, 2009; Ozacmak *et al.*, 2009; Dang *et al.*, 2010; Lollo *et al.*, 2013). Further, the temperature dependent time delay matter was considered for improvement of accuracy in Arrhenius model of cell death assay (Pearce, 2015). Therefore, differential temperature dependent expression of heat shock protein (Hsp70/Hsc70) is an important aspect, because sometimes extreme hyperthermia might cause degradation of cellular proteins rather than refolding them into their native structure and conformation by the help of heat shock proteins assembly. Recently, a study on pond snail showed that the thermal stress increases Hsp70 and Hsp40 gene expression at transcriptional level in a time dependent manner. The same report also documented that after thermal stress the Hsp40 gene expression increases 40 folds and returns to control level within 8 hours of heat exposure, whereas Hsp70 gene expression rises to 100 folds and not returned to control level within 8 hours (Foster *et al.*, 2015). Therefore, the time dependent expression of heat shock protein after hyperthermic stress might be different in different animal groups and it is also an important query which should be understood in the studied mice strain, which is considered as an important model in the biological research. Therefore, temperature and time dependent expression pattern of heat shock proteins along with melatonin receptors in the concerned experimental mice strain was investigated in the present study. Our immunohistochemical studies showed strong extra and intra cellular Hsp70 and Hsc70 immunoreactivities in the spleen of 41^0C and 43^0C heat stressed mice as well as mice group sacrificed after 5 hours of heat exposure. Earlier reports suggested that cytosolic Hsp70 associates with the antigenic peptide and mediates their translocation and processing (Ishii *et al.*, 1999); whereas extra cellular Hsp70 stimulates dendritic cells through TLR-4 (Chen *et al.*, 2009; Asea *et al.*, 2002). Our western blot analysis showed increased expression of Hsp70 and Hsc70 along with the increase in temperature as well as increase in time after hyperthermic stress in the splenic tissue of experimental groups sacrificed after 1hr, 2hr, 3hr, 4hr, 5hr and 6hr respectively. Semi-quantitative analysis of immunohistochemistry within the splenic tissue of the

concerned groups also supported the expression pattern observed during western blot analysis. Lovell and his co-workers (2007) suggested temperature dependent Hsp70 response during *in vitro* heat shock treatment on peripheral blood mononuclear cells. Another report showed increase in body temperature during exercise induces increased expression of Hsp70 in immune cells (Fehrenbach and Northoff, 2001). The time dependent increasing trend in the expression pattern of Hsp70 and Hsc70 was maximized and became in plateu after 5hr. Similar kind of report was suggested by Kiang and Tsokos (1998) where maximum heat shock proteins expression was noticed within 3-5 hours of thermal exposure. The study of Lovell *et al.*, (2007) also showed that during *in vitro* heat shock treatment on peripheral blood mononuclear cells the time profile of Hsp70 response shows temperature dependent manner, which increases up-to four hour and then again returned to baseline by the sixth hour of post heat shock treatment. Our study showed that *in vivo* heat shock treatment to mice also shows temperature dependent and time dependent increase in expression of the Hsp70 and Hsc70 response in the splenic tissue of mice. In the study, it was also observed that 43^0C temperature was optimum for this mice strain for induction of thermal stress, as above this temperature the mortality of the subject was increased. It could be also resolved from the study that the time gap of 5 hour between the heat stress and animal sacrifice was optimum for expression of heat shock protein. Increased levels of both Hsp70 and Hsc70 in the present study indicate the involvement of heat shock proteins in thermal acclimation process.

Several studies showed that melatonin is involved in nocturnal thermoregulation (Aschoff, 1983; Smolander *et al.*, 1993; Krauchi *et al.*, 1997, 2000). Reports also suggested that melatonin ingestion causes fall in internal temperature (Cagnacci *et al.*, 1998; Gilbert *et al.*, 1999; Harris *et al.*, 2001). Day time exogenous melatonin administration also reduces internal core temperature both under control as well as heat stressed environment (Aoki *et al.*, 2006). Melatonin mediates most of its activities through membrane receptors MT1 and MT2 in mammals (Zlotos *et al.*, 2014). In the present study, thermal stress caused a change in the expression pattern of heat shock protein along with melatonin receptors which indicate their possible involvement in thermoregulation process. The study showed temperature and time dependent decreased and increased pattern of expression of MT1 and MT2 receptors respectively. MT2 receptor is responding in all experimental conditions of temperature dependent as well as time dependent manner corresponding to changes of Hsp70 and Hsc70 expression. Increased AANAT gene expression along with the rise of temperature suggested the induction of melatonin synthesis in the spleen of thermally stressed mice. Earlier reports suggested that heat stress causes increase of melatonin secretion (Abbas *et al.*, 2007; Sejian *et al.*, 2008) and the circulatory increase of melatonin favours the increase in AANAT activity in lymphoid tissues (Gupta *et al.*, 2015). Our study intensely suggested that heat stress generated such melatonin, as a signal molecule might be upregulating the activity of the stress responsive genes in splenic tissue which ultimately results the increase of Hsp70/Hsc70 proteins, whereas the simultaneous increased expression of MT2 receptors shows its possible involvement during such mechanism. Reports on exogenous melatonin mediated upregulation of the gene expression of heat shock proteins like: Hsp60, Hsp70 and Hsp90 in PBMC of thermally stressed goat (Sharma *et al.*, 2013), and in AR42J cells (Bonior *et al.*, 2005) are present. Cabrera and his co-workers (2003) also suggested that melatonin through its MT2 receptors showed anti-apoptotic activity in the heat shocked HL-60 cells, where simultaneously induced expression of Hsp27 was observed. Earlier reports are also suggesting that melatonin regulates differentially its own receptors in different tissues and organs in mammals (Masana *et al.*, 2003) and mediates most of its immunoenhancing activity through MT2 receptors present in immune organs (Guerrero and Reiter, 2002; Dubocovich and Markowska, 2005).

CONCLUSION

Inducible heat shock protein 70 (Hsp70) and heat shock cognate protein 70 (Hsc70) levels showed proportional increase with the rise of temperature as well

as along with the increase in time after exposure to hyperthermic stress. The expression of these heat shock proteins were maximised at 5-6 hours of heat exposure and might protect the splenic cells from hostile effects of stress. In studied mice, 43^{0}C was optimum temperature for induction of thermal stress for conduction of such experiments. The increased expression of heat shock proteins in the splenic tissue after hyperthermic stress might be preparatory phenomenon of spleen cells for the protection against the adverse effects of stress. At the same time, melatonin MT1 and MT2 receptors are expressed differentially on the spleen cells in response to heat stress. MT1 receptors and MT2 receptors showed reduced and increased expression in the splenic tissue respectively along with the increase of temperature and increase of time after thermally stressed condition. MT2 receptor showed similar pattern of increased response along with heat shock protein (Hsp70 and Hsc70) might be responsible for acclimatization under hyperthermic stressed conditions. Further, increased AANAT gene expression suggested the heat induced synthesis of melatonin which might upregulated the heat response genes and resulted in the increased heat shock proteins expression. Moreover, possible extended efforts to evaluate the signalling mechanism involved behind such phenomenon, may bring new information in the melatonin mediated regulation process of stress responses.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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