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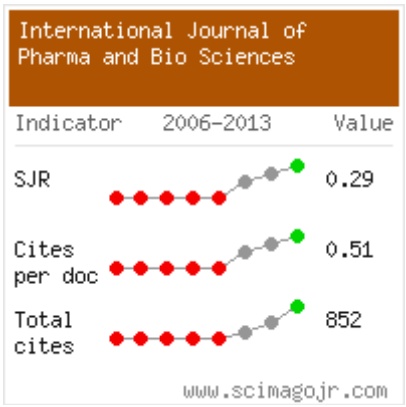

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Research Article **Cell Biology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EXPRESSION OF HEAT SHOCK PROTEINS (HSP70 & HSC70) AND RESPONSIVENESS OF MELATONIN RECEPTORS (MT1 & MT2) IN SPLEEN OF SWISS ALBINO MICE SUBJECTED TO HYPERTHERMIC STRESS CONDITION

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ABSTRACT

The most universal phenomenon in the thermal stress physiology is the expression of heat shock proteins (Hsps) following application of heat stress stimuli. Heat shock proteins are involved in the activation process of the cellular defence mechanism. Melatonin is well known as an anti-stress molecule and protects cells and tissues as an antioxidant. Melatonin also exerts hypothermic effects. Present study is mainly focused on the evaluation of expression pattern of melatonin receptors (Mt1 & Mt2) and heat shock proteins (Hsp70 & Hsc70) in an immunomodulatory organ, spleen of mice at different temperatures. Heat shock treatments against different temperature gradient (41^0C and 43^0C for 45 minutes) brought about a significant increase in the Hsp70/Hsc70 proteins and melatonin receptor Mt2 expression and decrease in Mt1 receptor expression in the spleen of experimental mice groups. Interestingly, Mt2 receptor responded in all experimental conditions corresponding to changes in Hsp70/Hsc70 protein expressions. Therefore, our present study suggests that Mt2 receptor and heat shock proteins (Hsp70 & Hsc70) are mainly responsive and probably involved in the thermoregulation process of thermally stressed mice.

KEY WORDS: **Thermal stress, heat shock proteins, melatonin receptors, spleen, mice.**

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INTRODUCTION

Thermal stress exerts deleterious effects on cellular organisations and hyperthermia activates apoptosis pathway. Cell death via the induction of apoptosis pathway is an anticipated mechanism through which heat stress persuades cellular loss of the physiological system[1,2]. 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. Molecular chaperone belongs to the major protein family members called heat shock proteins (Hsps) that works against the apoptosis process[3,4,1]. Expression of heat shock proteins as a result of thermal shock is a natural phenomenon by which cell protects itself. Stress at higher temperature causes aggregation of heat shock proteins with different polypeptides for their structural refolding trapped in the aggregates. Among the various family members, inducible heat shock protein 70 (Hsp70) plays a vital role in the structural refolding process by actively participating with other chaperones[5]. Another constitutive family member, heat shock cognate 70 (Hsc70) protein is a sensitive biomarker against the various physiological and environmental assaults[6]. Both of these heat shock proteins protect other proteins from unfolding, or refold denatured proteins, or drive them for degradation[7]. Pineal gland product melatonin has multidimensional performance in all organisms ranging from unicellular algae to humans[8,9]. Melatonin heaving a great role in circadian rhythm[10], also influences the cardio vascular system[11,12] and immune system activity[13,14]. It also works as an anti-stress hormone in the physiological system[15,16,17]. Melatonin performs of all its functions either via non-receptor mediated action mechanisms or receptor mediated action mechanisms. As stress generated free radicles are scavenged by melatonin, it is therefore considered as a potential antioxidant working on the cellular system. The cellular protection by scavenging free radicles is an approach in which melatonin non-receptor mediated action mechanism works[18,19,20,10]. At the same time, melatonin also mediates its functions through membrane receptors i.e. Mt1 and Mt2 (previously identified as Mel1a and Mel1b)[21,22,23]. Literatures are available on the involvement of melatonin in thermoregulation process of heat stressed humans[24,25,26]. Heat stressed broilers also shows adverse physiological effects[27], whereas melatonin improves the negative effects of heat stress[28]. But reports are scanty on the mode of their receptor activity in a thermally stressed animal. Therefore, the present aim of our investigation is to find out the mechanism of melatonin receptor expression along with the prominence of heat shock proteins in thermally stressed mice. As the heat shock proteins are the fundamental proteins expressed in stressed conditions for the protection of cells, therefore evaluation of their level of expressions in accordance with the different temperature gradients is also an important aspect of this study, considering spleen as a target organ, which is a vital immune organ working throughout the life of mammals.

MATERIALS AND METHODS

(i) Animal Procurement and Maintenance

Mice were procured from National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad, India. Healthy 10 weeks old (23-25g) adult male Swiss albino mice were acclimatized for 1 month in ambient laboratories (25^0C -27^0C) under normal day-night (12L: 12D) conditions. Mice were kept in groups of six in polycarbonate cages (43cm x 27cm x 14 cm) to avoid the population stress and fed 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.

(ii) Experimental Design

Experimental mice were randomly divided into three groups, each group containing 6 mice. The first mice group was treated as control group (Con) without any kind of stress. The second and third groups of mice were exposed to thermal or heat stress (H) at different temperatures. For heat stress, the second group of mice were exposed to 41^0C and the third group to 43^0C for 45 minutes in

an isolated humidified thermal chamber. To determine maximum heat tolerance level, another mice group were subjected to heat stress at 46^0C for 45 minutes. But their survivability was 50% compared to other mice groups. Therefore, 41^0C and 43^0C exposed mice groups were mainly considered for the present study, as this temperature range did not bring about any mortality under laboratory conditions. The groups of mice subjected to heat stress were sacrificed after 5 hours of heat exposures. The maximum heat shock protein expression was reported after 3-5 hours of thermal exposure and ceases after 8 hours[29]. The mice were sacrificed and spleen was dissected out on ice. Half of the spleen of each mouse of each group was immediately kept in deep freezer at -40^0C for western analysis and the rest fixed in aqueous Bouin's fluid for immunohistochemical studies.

(iii)Immunohistochemical studies

Immunohistochemical studies of control and experimental tissues (spleen) were done following the procedure adopted by Savaskan *et al*[30]. Paraffin sections (6μm) 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 laboratory temperature (25^0C). The sections washed thrice with phosphate buffered saline (PBS: 0.1M Na_2HPO_4, NaH_2PO_4, 0.9% NaCl, pH=7.4) were placed in blocking solution (horse blocking serum, diluted 1:200 in PBS, PK -6200, Vector Laboratories, Burlingame, CA). Then the sections were incubated with primary antibodies [Hsp70; ab79852 and Hsc70; ab1427, rabbit polyclonal, Abcam, USA; Mel 1AR (Mt1); sc13186 and Mel 1BR (Mt2); sc13177, goat polyclonal, Santacruz Biotech, USA, diluted 1:200] overnight at 4^0C. Next day, the 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).The 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 0.1% H_2O_2 and counterstained with Ehrlich's haematoxylin. The sections were dehydrated and mounted with DPX. Microphotographs of the stained sections were taken under 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 4^0C. Next morning the immunohistochemical protocol was followed under the same conditions.

(iv) 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 albino Swiss 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 aprotonin, sodium orthovanadate and phenylmethylsulphonylfluoride (PMSF)] and quantified by Lowry method[31]. Aliquots containing 100μg proteins were resolved by 10% (w/v) SDS polyacrylamide gel electrophoresis followed by electrotransfer 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 β-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 β-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, USA). Values were expressed as ratio of the density of the specific signal to β-actin signal and expressed as the % control value[32]. Each sample

corresponds to tissue from a single animal and at least four gels corresponding to each subunit and experimental conditions were analysed.

(v) Statistical Analysis

Statistical analysis of the data was performed by one way ANOVA followed by Student's Newman-Keul's multiple range tests. The differences were considered significant when $p<0.05$.

RESULTS

Effects of thermal stress (41^0C and 43^0C)

1. Immunohistochemical Localization

Strong immunoreactivity of both Hsp70 and Hsc70 were observed in the intra and extra cellular space of spleenic tissue of the 43^0C exposed mice group compared to the 41^0C exposed and control mice group (Fig:1,2). The Mt1 immunoreactivity in control mice group was stronger than the other experimental groups (Fig:3). Immunoreactivity of Mt2 was noted higher in both 41^0C and 43^0C exposed mice groups than the control one. However, 43^0C exposed mice group showed much stronger Mt2 immunoreactivity than the 41^0C exposed mice group (Fig:4) In negative immunohistochemical control sections no reaction were detected.

2. Western Blot Analysis

Hsp70 and Hsc70 proteins were detected as a single band corresponding to 70KDa. Both melatonin receptors were detected as a single band in between 35–40 KDa, which precisely corresponded to the predicted molecular mass of the receptors[33]. Hsp70 expression increased significantly ($P<0.01$) at both 41^0C and 43^0C exposed mice groups compared to the control. But the mice group exposed to 43^0C had higher expression than the 41^0C groups (Fig:5). Similar pattern of significantly increased Hsc70 expression was observed in 41^0C and 43 ^{0}C experimental groups compare to control. Higher expression of Hsc70 was noted at 43^0C than the 41^0C exposed groups of mice (Fig:6). Significant ($P<0.01$) decrease in Mt1 expression was noted in 41^0C and 43^0C exposed mice groups compared to control group (Fig:7). In contrast, significant ($p<0.01$) increase in Mt2 expression was noted in 41^0C and 43^0 C exposed mice groups compared to control one (Fig:8). Interestingly, the expression of Mt2 showed similar kind of change with the heat shock proteins expression under different experimental conditions.

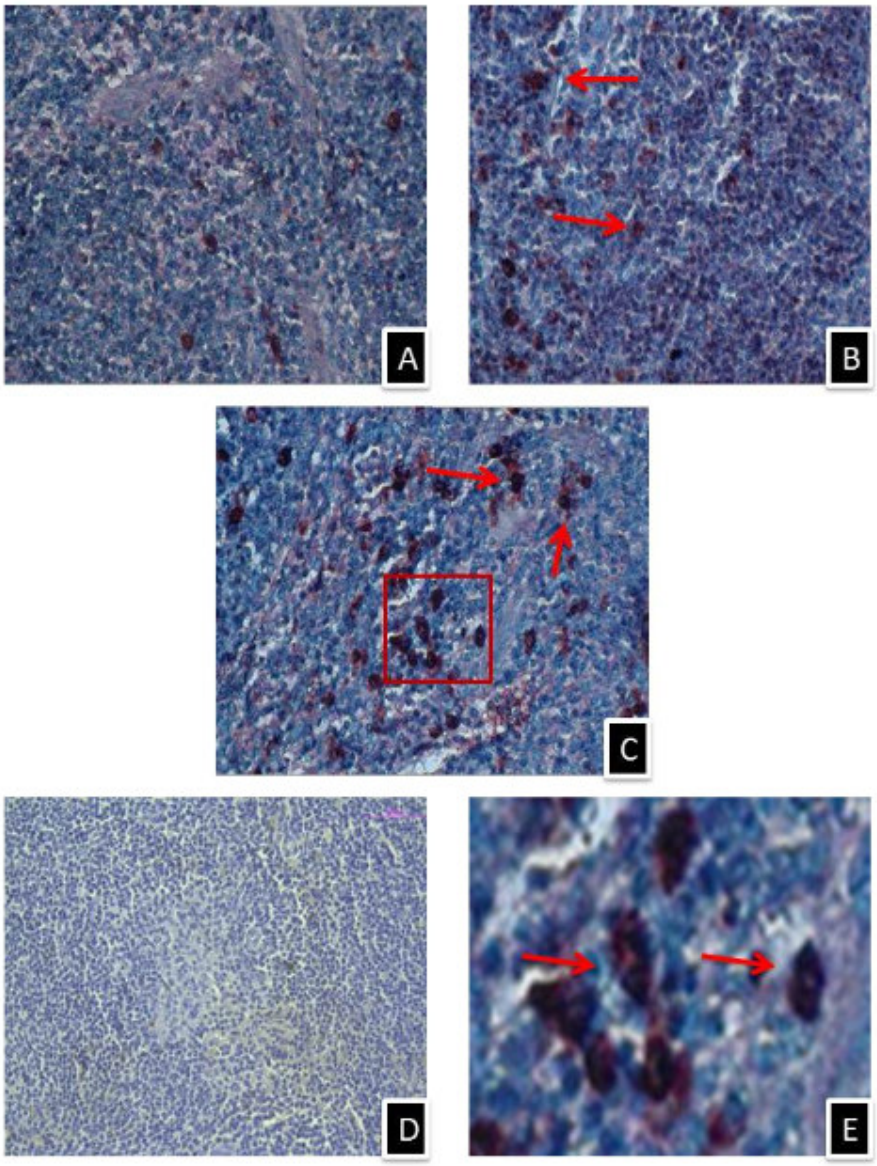

Fig. 1

Figure 1

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, and (D) Negative immunohistochemical control section, (E)Enlarged view of selected area showing extracellular and intra cellular Hsp70 immunostaining.(X40,Magnification bars=50μm)

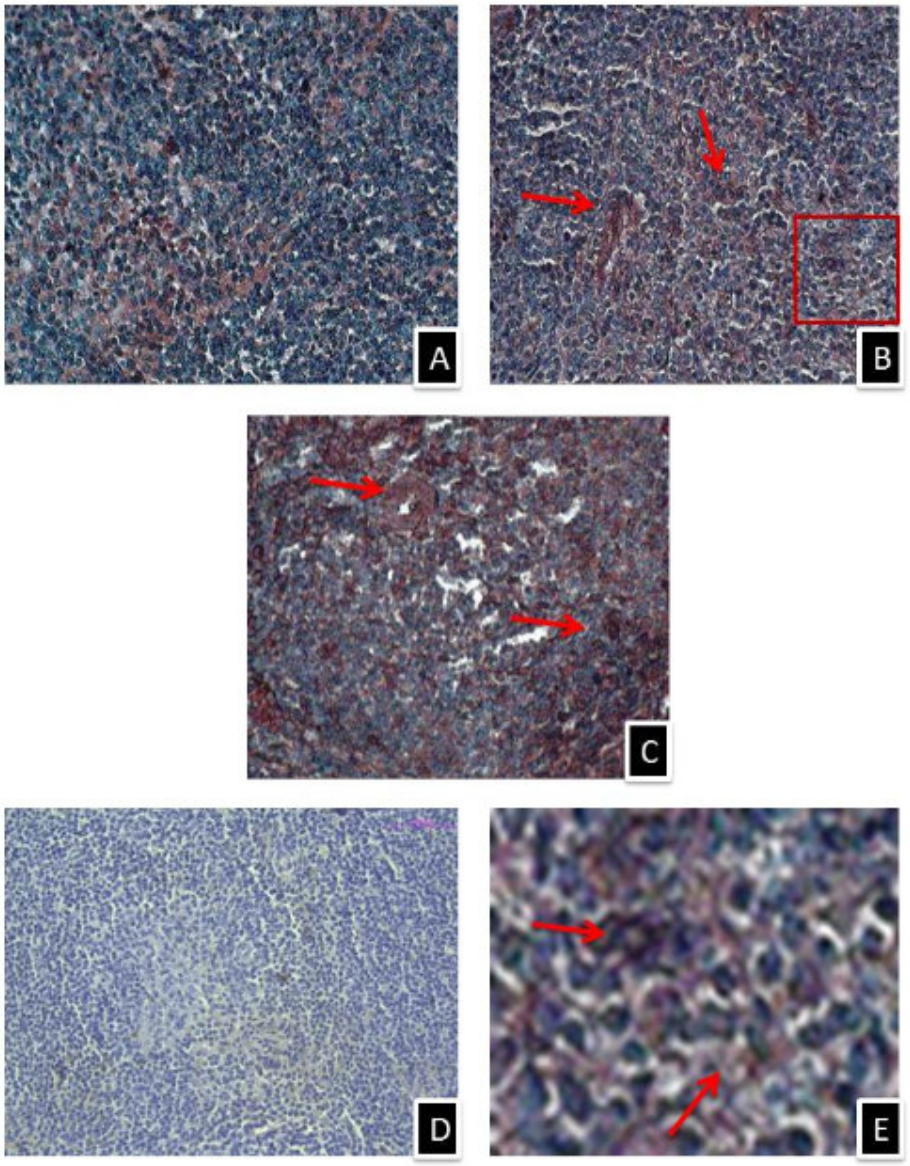

Fig. 2

Figure 2

Immunostaining of heat shock cognate protein 70 (Hsc70) in spleen of (A) control, (B) 41^0C heat stressed, (C) 43^0C heat stressed, and (D) Negative immunohistochemical control section, (E) Enlarged view of selected area showing extracellular and intra cellular Hsc70 immunostaining.(X40, Magnification bars=50μm)

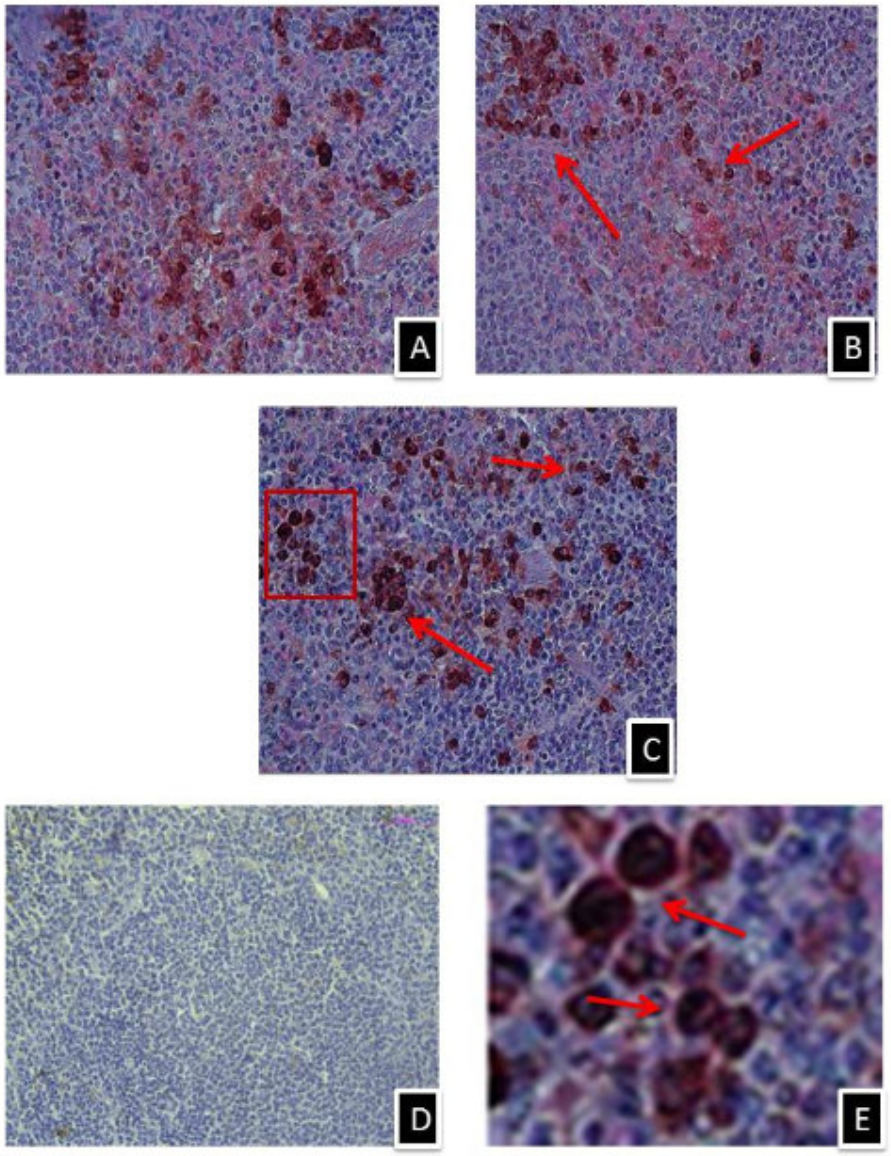

Fig. 3

Figure 3

Immunostaining of Mt1 melatonin receptor in spleen of (A) control, (B) 41^{0}C heat stressed, (C) 43^{0}C heat stressed, and (D) Negative immunohistochemical control section, (E) Enlarged view of selected area showing membrane specific Mt1 immunostaining. (X40, Magnification bars=50μm)

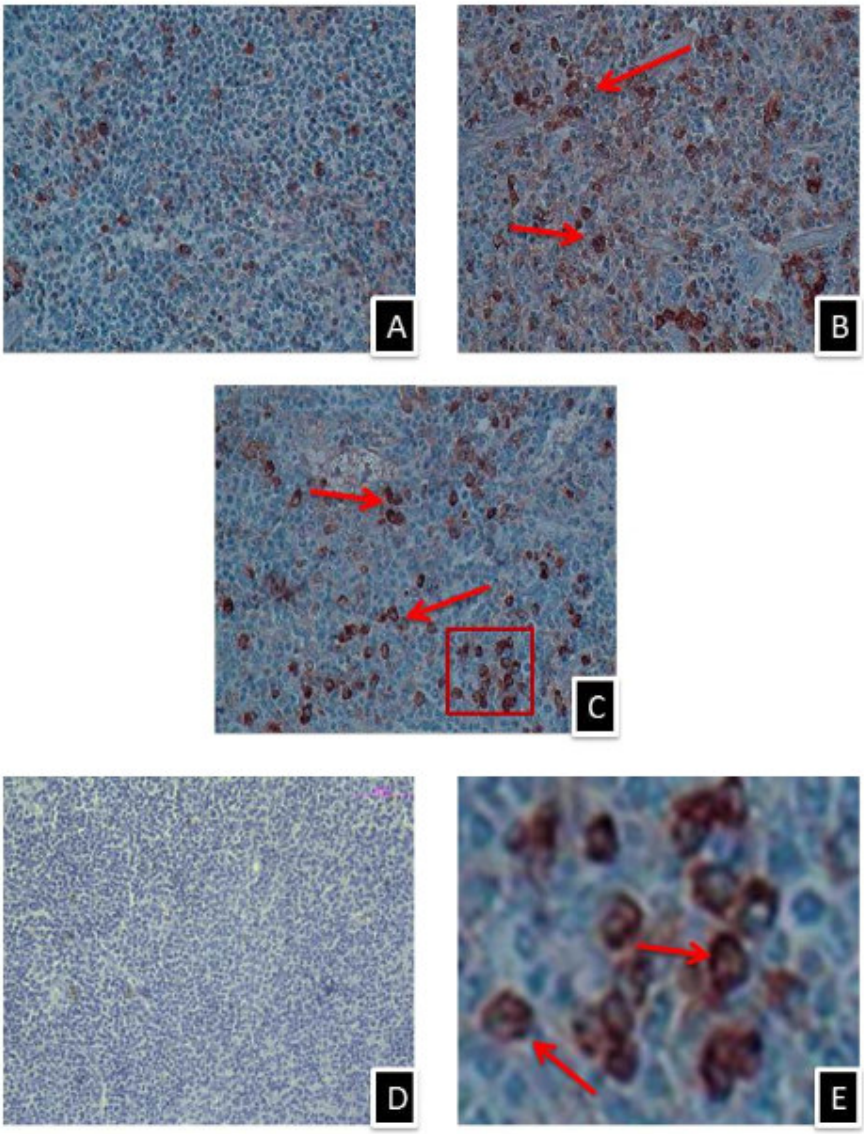

Fig. 4

Figure 4

Immunostaining of Mt2 melatonin receptor in spleen of (A) control, (B) 41^0C heat stressed, (C) 43^0C heat stressed, and (D)Negative immunohistochemical control section, (E) Enlarged view of selected area showing membrane specific Mt2 immunostaining.(X40, Magnification bars=50μm)

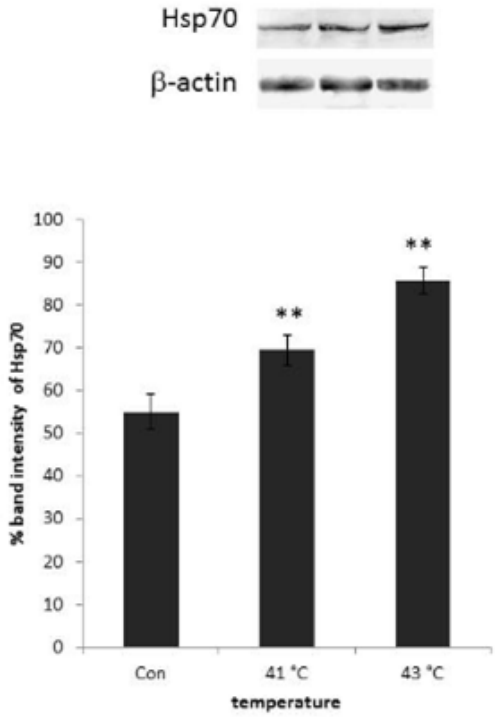

Figure 5

Histogram showing western blot analysis of inducible heat shock protein 70 (Hsp70). β-actin was used as loading control. Lower panel (below western blot) presents percent band intensity of experimental groups. Vertical bars represents Mean ± SEM, n=4. Control vs. Experimental significance of difference **P<0.01.

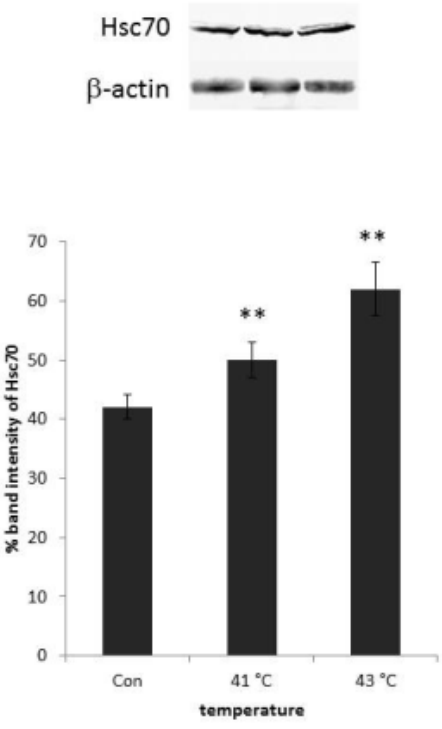

Figure 6

Histogram showing western blot analysis of heat shock cognate protein 70 (Hsc70). β-actin was used as loading control. Lower panel (below western blot) presents percent band intensity of experimental groups. Vertical bars represents Mean ± SEM, n=4. Control vs. Experimental significance of difference **P<0.01.

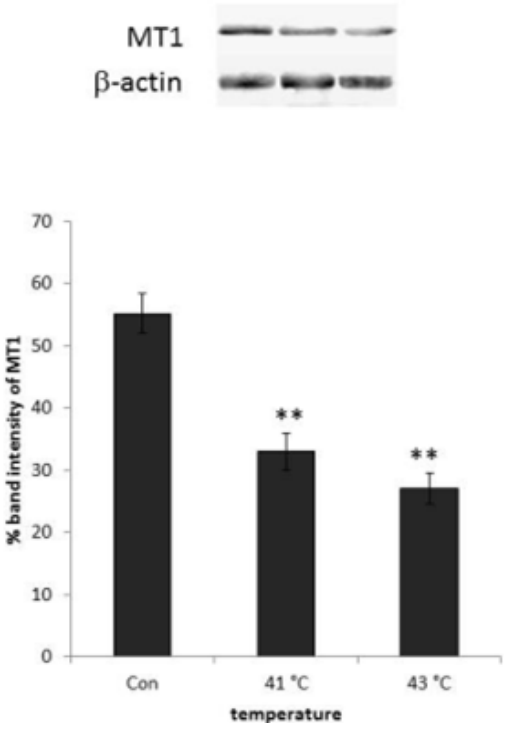

Figure 7

Histogram showing western blot analysis of Mt1 melatonin receptor. β-actin was used as loading control. Lower panel (below western blot) presents percent band intensity of experimental groups. Vertical bars represents Mean ± SEM, n=4. Control vs. Experimental significance of difference **P<0.01.

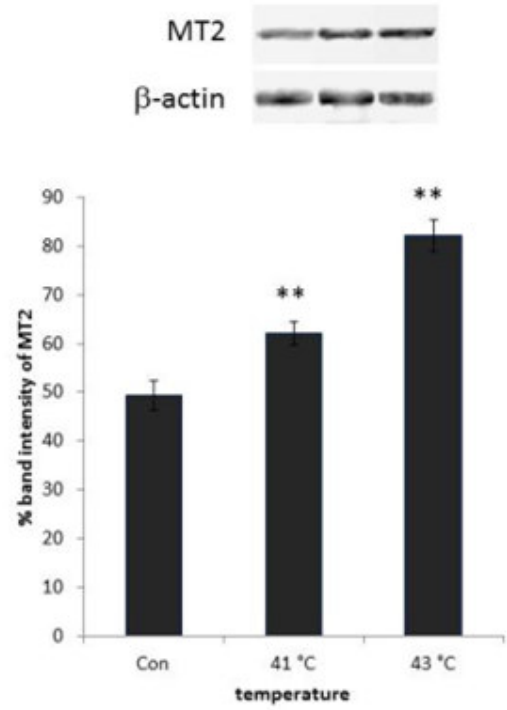

Figure 8

Histogram showing western blot analysis of Mt2 melatonin receptor. β-actin was used as loading control. Lower panel (below western blot) presents percent band intensity of experimental groups. Vertical bars represents Mean ± SEM, n=4. Control vs. Experimental significance of difference **P<0.01.

DISCUSSIONS

Thermal stress is an important cue for the production of heat shock proteins in relation to cellular protection. Therefore, heat shock protein expression is considered as a sensitive biomarker for identifying challenging conditions of environment[6]. The thermal sensitivity and tolerance ability in the diverse animal groups could be accessed via the estimation of these chaperone proteins expression level [34,35,36,37,38,39].Expression and characterization of these proteins were also reported in various vertebrate tissues like intestine, kidney, spleen, gut, gill, thymus and brain[40,41,42,43,44]. But differential temperature dependant localization and expression of heat shock proteins (Hsp70/Hsc70) is also very important aspect which should be understood, as the 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. Our immunohistochemical results showed strong extra and intra cellular Hsp70 and Hsc70 immunoreactivities in the spleen of 41^{0}C and 43 ^{0}C heat stressed mice. Earlier reports suggested that cytosolic Hsp70 associates with antigenic peptide and mediates their translocation and processing[45]; whereas extra cellular Hsp70 stimulates dendritic cells through TLR-4[46,47]. Our present western blot analysis also showed similar kind of increased expression of Hsp70 and Hsc70 in experimental mice groups. Higher expression of Hsp70 and Hsc70 under 43^{0}C exposed mice group corroborates with the findings of Fehrenbach and Northoff [48], who also reported the over expression of heat shock protein (Hsp72) in relation to heat tolerance in leukocytes. Rise in levels of both Hsp70 and Hsc70 in our present studies indicate that both heat shock proteins are involved in thermal acclimation. Several studies presented that melatonin is involved in nocturnal thermoregulation[49,50,51,52]. Available literatures also suggests that melatonin ingestion causes fall in internal temperature[53,54,55]. Day time exogenous melatonin administration also reduces internal core temperature both under control as well as heat stressed environment[26]. Melatonin mediates most of its activities through membrane receptors Mt1 and Mt2 in mammals[56]. In the present study, thermal stress resulted a change in the expression pattern of heat shock proteins along with melatonin receptors which indicate their possible involvement in thermoregulation process. Mt2 receptor is responding in all experimental conditions corresponding to changes of Hsp70/Hsc70 expression. This indicates Mt2 receptor subtype responses higher in the thermally stressed environment. Melatonin might prefer Mt2 receptors for thermoregulation process for the mediation of its anti stress activity. Earlier reports are also in agreement with the fact that melatonin regulates differentially its own receptors in different tissues and organs in mammals[57] and mediates most of its immunoenhancing activity through Mt2 receptors in immune organs[58,14]. Cabrera and his co-workers[59] reported that melatonin mediates anti-apoptosis through Mt2 receptors and also causes the induction of Hsp27 expression in heat shocked HL-60 cells.

CONCLUSION

Levels of heat shock protein 70 (Hsp70), the inducible form as well as heat shock cognate protein 70 (Hsc70), the constitutive form increase proportionally with the rise of temperature. This might be due to a preparatory phenomenon of spleen cells for protection against the adverse effects of stress. Melatonin receptors Mt1 and Mt2 are expressed differentially in response to heat stress. Melatonin receptor Mt2 along with heat shock proteins (Hsp70 and Hsc70) exhibited higher response to acclimate under hyperthermic condition (41^{0}C & 43^{0}C). More in depth investigation are required at mRNA level to understand the mechanism of action of melatonin receptor mediated stress regulation process in an *in vivo* study model at the transcriptional level. This may lead an outcome for the management of heat shock by regulation of circulatory melatonin.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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