



Melatonin Ameliorates Heat Stress Induced Dysregulation of Testicular Function In Wistar Rat By Restoring Tissue Health, Hormone And Antioxidant Status And Modulating Heat Shock Protein Expression

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Abstract: Hyperthermia results in changes in spermatozoa and ultimately causes infertility by inducing heat-stress related oxidative stress. Melatonin is a potent endogenous antioxidant and free radical scavenger. However, the efficacy of melatonin to protect the testis from the deleterious effects of hyperthermia is not clear entirely. This study was intended to look into the protective effect of melatonin against the testicular damage inflicted by thermal stress in adult rats. We investigated the consequences of hyperthermia-induced changes in hormones, antioxidant system, and the status of heat stress proteins in testes of rats and the effectiveness of melatonin to prevent these changes. Heat stress was induced at 43°C for 30 min/day for 15 consecutive days, using a hyperthermia induction chamber. Four groups of rats (n=6 per group) consisting of a control group, heat-stressed group, melatonin group and melatonin treated heat stress group of rats were considered for the study. Analysis of blood and testicular tissue of heat-stressed rats exhibited a significant reduction in serum testosterone, testicular superoxide dismutase, catalase, glutathione and 17β -HSD3; whereas, a significant rise in serum corticosteroid and testicular lipid peroxidation, along with the substantial increase in testicular HSP72 and HSP90 and HSF-1 expressions were also observed. In testicular histology of rats, exposed to heat stress alone, revealed remarkable germ cell degeneration and tubular deformations. Melatonin treatment in heat-stressed rats significantly prevented the aforementioned heat stress-related alterations of physiological parameters. The results indicates that consumption of melatonin may substantially protect testis from heat-stress -induced testicular dysfunctions.

Keywords: Global warming, Heat stress, Hyperthermia, Melatonin, Pineal gland, Testis

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I. INTRODUCTION

The cellular health is governed by the associated microenvironment surrounding the tissue; cumulative health of a population too largely depends upon the environment it resides in. The advancement of modern civilization comes at the price of the emission of deadly greenhouse gases, triggering a steep rise in the global mean temperature. The most affected are the parts of the world with tropical and hot climatic conditions. Hence certain heat stress-related health disorders are found to be prevalent in these areas. Hyperthermia, the resultant effect in individuals residing or working in intense heat, may prompt adverse effects in various organ systems. Therefore, the regulation of body and organ temperature is critical to keep up the ideal organ functions via proper coordination of diverse physiological tools¹. Previous data suggested that elevated body temperature induced death signalling cascade in germ cells of testes involving a common pathway, oxidative toxicity, in almost all of the cases, which is triggered by the generation of reactive oxygen species (ROS)²⁻⁴. It is, therefore, a widely admitted conception that spermatogenesis is greatly abused, subject to the intensification of the testicular or scrotal temperature. Men with scrotal temperatures above the physiological range have been reportedly associated with increased rates of infertility⁵. Moreover, in addition to the demographic data, volunteered individual experiments also supported the same. An individual was reportedly exposed to scrotal hyperthermia via exposure at 43°C water bath for 30 min a day for 10 consecutive days. Consequently, inhibition of spermatogenesis and reduced sperm concentration was observed. The investigations regarding the relationship between stress and testicular dysfunction also revealed that heat exposure resulted in enhanced serum corticosterone (CORT) level⁶ and CORT administration did reduce the serum testosterone (T) level resulting apoptosis of Leydig cells⁷. The heat-stress generates a highly conserved class of peptides termed as heat shock proteins (HSPs), which protect cells providing thermo-tolerance against the adverse effects of heat shock and other stress conditions causing cellular proteins to become partially unfolded⁸⁻¹⁰. Studies have shown that some fruits like red and purple grapes and natural products like polyphenols (catechin, anthocyanidins, resveratrol, etc.) can decrease the caustic effects of oxidative stress¹¹⁻¹³. These foods are rich in natural antioxidant compounds such as polyphenols, carotenoids, and vitamin C, etc. These have shown the ability to protect the cells from damage due to stress¹⁴. Therefore, there is an adequate amount of rationality that propels us to look forward to preventing the heat-induced toxicity employing any sort of antioxidant supplements that lacks too many bad effects either. Melatonin, a well-known antioxidant, made an appropriate choice in our experiments to neutralize the stress-induced toxicity. This is supported by the earlier investigations where melatonin had reportedly ameliorated the harmful effects of ROS, particularly the hydroxyl radical (OH) that attacks cellular macromolecules¹⁵. Several other studies confirmed melatonin to be capable of reducing the amount of hypochlorous acid, peroxy radicals, and singlet oxygen, the causative agents of cellular injury¹⁶⁻¹⁸. All these data from previous studies give enough support to qualify melatonin as a competent stress buster. Effect of melatonin on the male reproductive system as well as testosterone production from Leydig cells had also been reported in the studies on animals. Because melatonin receptors have been

detected in the reproductive system of different species, it also seems reasonable to assume that melatonin exerts its actions through direct interaction with the steroidogenic cells of the reproductive organs¹⁹. Since melatonin has excellent antioxidant properties and shows both direct and indirect mechanisms of antioxidant actions, exogenous melatonin would likely to exert its ameliorative activity on testis against heat stress-induced germ cell damages. The current investigation is intended to scrutinize the effectiveness of melatonin on tissue health and the status of antioxidant and heat shock proteins in the testis of adult rats exposed to hyperthermia.

2. MATERIALS AND METHODS

2.1 Chemicals & Reagents

Sodium Chloride (NaCl), Haematoxylin and Eosin stains, Xylene, Paraffin, EDTA, Tween-20, Phenylmethylsulfonyl fluoride (PMSF) were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India) and 5-5-dithiobis-(2-nitrobenzoic acid) (DTNB) from Sigma-Aldrich (St. Louis, Missouri, USA). For the immunohistochemical study, HSP-90 and HSP-72 antibodies were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). Corticosterone and Testosterone detection kits were purchased from Accubind Elisa Microwells kit system (California, USA).

2.2 Animals

Experiments were carried out using adult male Wistar rats (11-12 weeks old and weighing 150-160g) maintained in a temperature (30±2°C) and humidity (60±5%)-controlled room on a 12hr dark/light cycle, with a balanced diet and water *ad libitum*^{11, 21-22}. All animal experiments were approved (Registration no. 1394/ac/10/CPCSEA, West Bengal State University) by the Institutional Animal Ethics Committee (IAEC) and they were handled as per the guidelines in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3 Grouping and Monitoring of Animals after Induction of Hyperthermia

Animals were allocated into four groups – group I (Control), group II (Heat stress), group III (only Melatonin), and group IV (Melatonin + Heat stress), each consisting of six rats. All groups of rats including the control group were maintained at room temperature of 30±3°C except the period of experimental manipulations for heat stress treatment in Group-II and Group-IV animals. The heat stress was induced in a hyperthermia induction chamber. A thermostatically controlled heater was fitted at the top-lid of the chamber and temperature was continuously monitored by a thermometer throughout the experiment. The core temperature of the animals was monitored by using a rectal thermistor throughout the experiment. Body temperature (rectal) was recorded by a tele-thermometer (Aplab, India) connected through a thermistor probe. At the end of the experiment all the animals were sacrificed, ventricular blood samples were collected in microfuge tubes and used for biochemical assays. The testes were collected, weighed individually, and utilized for necessary biochemical, histological, and immunoblotting studies. The results of each

parameter obtained were compared among the groups. The experimental design was done as per our earlier report¹¹.

2.4 Melatonin Administration

Rats were weighed individually at the start of the experiment and freshly prepared melatonin or water was administered to the respected groups of animals by gavage at a dose of 10mg/kg bodyweight for 15 days²². Melatonin or water was administered before the induction of heat stress every time.

2.5 Epididymal Sperm Counts

The spermatozoa were collected and examined from the caudal epididymis, as previously reported²³. Briefly, the cauda epididymis was cut into small pieces in a 35 mm petri dish and placed in a centrifuge tube containing 3 ml of normal saline to let the sperms swim up for 10 min at 37°C. After incubation, the sperm saline mixture was diluted to 1:100 with a solution containing 5g sodium bicarbonate, 1ml 35% formalin, and 25mg eosin per 100ml of distilled water. Then approximately 10µl of the diluted sperm suspension was transferred to the counting chamber of the haemocytometer and was allowed to stand for 5 min. The cells were then counted under an inverted phase-contrast microscope (Dewinter, Italy) at 200X¹¹.

2.6 Determination of Antioxidant Status in Tissue Level

2.6.1 Superoxide Dismutase Activity

The superoxide dismutase (SOD) activity in testicular tissue was assessed by using the standard protocol, described by Ghosh et al., (2013)²⁴. Superoxide anions are produced by the oxidation of hydroxylamine hydrochloride. The reduction of nitro blue tetrazolium indicator to blue formazan facilitated by superoxide anions was measured at 560 nm under aerobic conditions. Interference by SOD prevents the reduction of nitro blue tetrazolium and therefore, the extent of inhibition to form the blue formazan is taken as a measure of enzyme activity. The activity of SOD was presented as units/mg of protein, in the manuscript.

2.6.2 Reduced Glutathione Assay

Reduced glutathione (GSH) content was measured according to the method of Mukherjee et al., (2012)²⁵. In brief, 0.5ml of the sample or standard solution was mixed with 0.25ml of 1M sodium phosphate buffer (pH 6.8) and 0.5ml of 5-5-dithiobiis-(2-nitrobenzoic acid) (DTNB, 0.8 g/L in phosphate buffer). After 5 minutes, the absorbance was measured at 412 nm using a spectrophotometer (BioSpectrometer, Eppendorf, Germany). The GSH concentration was calculated using the plotted standard curve prepared with different concentrations of standard GSH solutions.

2.6.3 Catalase Assay

Catalase (CAT) activity in testes was estimated by the described by Aebiet et al., (1974)²⁶. In brief, an aliquot of 0.5 mL supernatant was mixed with 2.5 mL of 50 mM phosphate buffer (pH 7.0) and 20 mM H₂O₂. The CAT activity was estimated spectrophotometrically following the decrease in absorbance at 240 nm. The specific activity of CAT was expressed as µM H₂O₂ consumption/min/mg protein as compared with the standard curve.

2.6.4 Lipid Peroxidation

Lipid peroxidation levels in testes were measured by the thiobarbituric acid (TBA) reaction using the method of Mukherjee et al., (2012)²⁵. This method was used to measure spectrophotometrically, the color produced by the reaction of TBA with malondialdehyde (MDA) at 532 nm. Lipid peroxide level was expressed in terms of nM of malondialdehyde.

2.7 Biochemical Assays

After the collection of blood samples from animals, serum was obtained through centrifugation at 4°C. Following the manufacturer's protocol, free serum corticosterone, and testosterone (Accubind Elisa Microwells kit system, USA) Serum SGPT, SGOT were also measured according to the manufacturer's protocol (EBRA assay kits).

2.8 Histological and Immunohistochemical Studies of Testes

Histological studies were performed according to our pre-standardized protocol (Halder et al., 2018)¹¹. Testes were fixed in 10% neutral buffered formalin, routinely processed for embedding in paraffin and used for microtome sectioning. The sections were stained with hematoxylin and eosin and observed under the microscope. For immunohistochemical staining of HSP90 and Caspase-3, the sections were deparaffinized and then rehydrated. In order to unmask the antigen, the sections were placed in 10 mM phosphate buffer saline (pH 7.4) and then heated twice in a microwave oven for 3 min each. Inactivation of endogenous peroxidase was done by incubating with 3% H₂O₂ in methanol (CH₃OH) for 20 min. Nonspecific binding sites were blocked by incubating with blocking solution for 20 min. Sections were overlaid with primary antibody (1:50 dilution) at RT for 30 min and washed thrice using a washing solution. Samples were then treated with a biotinylated anti-mouse secondary antibody for 15 min at room temperature, followed by horseradish peroxidase (HRP) streptavidin for 5 min at room temperature. The slides were counterstained with Mayer's hematoxylin for 30sec and then mounted. An immunohistochemical study was performed as per manufacturer instruction using Chemicon, USA kit. The slides were then observed and photographs were taken using EVOS® XL CORE Cell Imaging System (Life Technologies, USA).

2.9 HSF-1, HSP72 and 17B-HSD3 Expression Study by RT-PCR

Total RNA was isolated from the testicular cells using the RNeasy® Mini Kit (Qiagen, Venlo, Limburg, Netherlands). An equal volume of each sample was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Invitrogen™, CA, USA) following the manufacturer's protocol after the RNA was measured using Qubit® 2.0 Fluorometer (Life Technologies, USA). The cDNAs then were tested with PCR reaction for the expression analysis of genes: HSF-1, HSP72, and 17-HSD3 with respect to GAPDH. PCR amplification of the genes was carried out using AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Life Technologies, USA). The primers, used for the PCR amplification, were the same as used in our previous report¹¹ (Table – 1). The values of the densitometric analysis bands in agarose gels were plotted in a graphical form.

Table – I: Sequence of primers used for RT PCR of expressed mRNAs

Gene	Primer	
	Forward	Reverse
HSP72	5'-ATggCCAAGAAAACAgCg-3'	5'-TAATCCACCTCCTCgATggT-3'
HSF1	5'-ATggATCTggCTgTgggC-3'	5'-CTAggAgACAgTggggTCCTT3'
17β-HSD3	5'-TTCTgCAAggCTTACCAgg-3'	5'-ACAAACTCATCggCggTCTT-3'
GAPDH	5'-CCCTTCATTgACCTCAACTAC3'	5'-gTCCTTCCACgATACCAAAg-3'

2.10 Analysis of Heat Shock Protein Expression by Western Immuno-Blotting Technique

Western blot analysis was performed essentially according to the protocol standardized in our lab²⁷. Briefly, the tissue was dissected out, washed with PBS and homogenized in lysis buffer containing protease inhibitor cocktail (Sigma, USA), 1 mM phenyl-methyl-sulfonyl fluoride, 10 mM NaF, 1 mM Na₃VO₄, and 50 mM glycerophosphate (Sigma, USA). Then the homogenate was centrifuged at 12000 rpm for 20 min, the supernatant was used for Western blot analysis. In all the analyses, the protein concentration determined by Bradford assay was equalized among the samples. Aliquots of tissue lysates containing 50g of proteins were separated by SDS-PAGE. Subsequently, the proteins were electro-trans blotted onto a PVDF membrane and the membrane was blocked with a blocking buffer containing 5% nonfat dried milk at room temperature with gentle rocking for 1 hr. The respective membrane was then incubated with a primary antibody (1:500/1:1000 dilution) followed by respective HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (1:5000 dilution) and finally desired proteins were detected by TMB reactions (Sigma Aldrich, USA).

3. STATISTICAL ANALYLSIS

All data were expressed as Mean \pm Standard deviation (S.D). Data were analyzed all pairwise by one-way ANOVA using Tukey's test with sigma plot version 11 software.

4. RESULTS

4.1 Determination of Body Weight and Organ Weight and Testicular Weight

Animals lost weight during stressful circumstances and administration of melatonin prevented the reduction in body weight by means of their changes in food intake behaviour. The mean body weight of stressed group (Group-II) rats was significantly ($p<0.001$) lower as compared to that of the control group (Group-I) and Group-IV animals, those administered with melatonin along with stress, showed significant ($p<0.05$) increase in body weight as compared to group II animals (Figure-1A). A similar pattern of change and restoration was observed in the case of testicular weight as well (Figure-1B). These findings indicate the reduction of body weight and testicular weight after exposure to heat stress and which was restored by the treatment of melatonin.

4.2 Change in Rectal Temperature

The significant ($p<0.001$) rise of rectal temperature after induction of heat stress in animals of Group-II was observed

while comparing with the control. After treatment with melatonin, the rise in rectal temperature was significantly ($p<0.01$) restored in Group-IV animals (Figure-1 C). This indicates that the restoration of the increased rectal temperature involves physiological modifications owing to the intake of melatonin. There were no significant changes observed in Group-III animals when compared to that of the control group of animals.

4.3 Measurement of Corticosterone, Testosterone & Other Biochemical Parameters

The significant increase of blood corticosterone (CORT) levels in the Group-II animals ($p<0.001$), in comparison to that of control animals, was indicative of the effect of heat stress. However, significantly restored CORT level in Group-IV rats ($p<0.001$) compared to that of Group-II also validated the preventive action of melatonin against the CORT inducing effect of heat stress (Figure-2A). The serum testosterone (T) levels of stressed group (Group-II) rats were found significantly ($p<0.001$) reduced as compared to that of the control group (Group-I). Increased T levels were found in the Group-III and Group-IV animals (all $p<0.05$) as compared to Group II (Figure-2B). These findings showed a reduction of serum testosterone after exposure to heat stress and the stress-busting effect of melatonin, as it was witnessed when administered to the animals exposed to heat stress. The significant increase of SGPT and SGOT levels in the animals of Group-II ($p<0.001$) were found in comparison to the control batch of animals. However, similar to the change in the level of CORT, we found a significant ($p<0.001$) reduction in the level of SGOT and SGPT in Group-IV animals (Figure-2C, 2D). This supports the preventive action of melatonin against the effect of heat stress-induced dysregulation of CORT and T levels.

4.4 Assessment of Testicular Antioxidants

The antioxidant status of the testes was altered markedly by heat stress. Activities of the antioxidant enzymes like SOD, GSH, and CAT were significantly (all $p<0.001$) reduced in heat-stressed group II (Figure-3A,3B, 3C). Moreover, exposure to heat stress generated an elevated level of lipid peroxidation as denoted by a significantly increased level of LPO in Group-II ($p<0.001$) (Figure-3D). Analysis of SOD, CAT, GSH, and LPO in Group-III and Group-IV in comparison to control animals revealed that both of melatonin treated control group (Group-III) and melatonin treated stress group (Group-IV) respectively, which were similar to that in control group indicated the efficacy of melatonin in the restoration of antioxidant status of the testis.

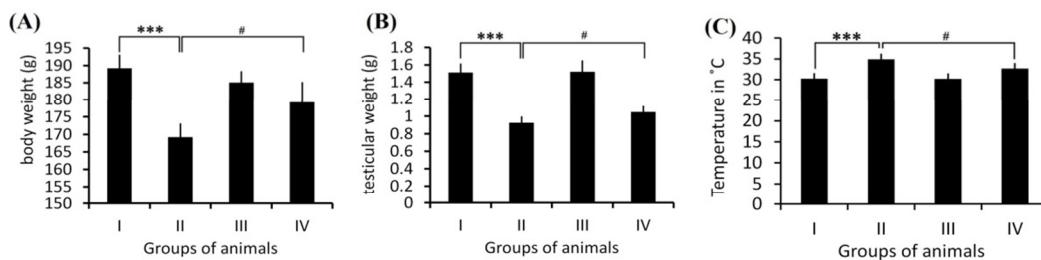


Fig-1: Effects of melatonin on the body, testicular weight and rectal temperature in the presence or absence of heat stress (A) Data (One-way ANOVA; $F=9.694$, DF=3) shows that the bodyweight of animals (Group-II) declined significantly ($p<0.001$) upon induction of heat stress, in comparison to the control group while the same was considerably ($p<0.05$) restored in Group IV animals to which melatonin had been administered along with heat treatment (Group-IV). (B) Similarly, the testicular weight also decreased in the stress group (Group-II) significantly ($p<0.001$) compared to the normal untreated animals only to be reinstated considerably ($p<0.05$) to the optimal testicular weight in Group-IV.(One-way ANOVA; $F=32.555$, DF=3) (C) The figure showed that the rectal temperature, after induction of heat stress in group II, was significantly ($p<0.001$) increased in absence of melatonin as compared to the control group and the same was considerably ($p<0.01$) brought back to near-optimal in group IV.(One-way ANOVA; $F=25.299$, DF=3) This, in fact, points towards the involvement of certain physiological reforms, owing to the intake of melatonin, which triggered the restoration of normal rectal temperature in heat-stressed animals. As depicted in figure, '*' signifies the difference in Group II in comparison with Group I, whereas '#' signifies the difference after restoration of respective parameter in Group IV in comparison with Group II. (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; # = $p<0.05$; ## = $p<0.01$; ### = $p<0.001$)

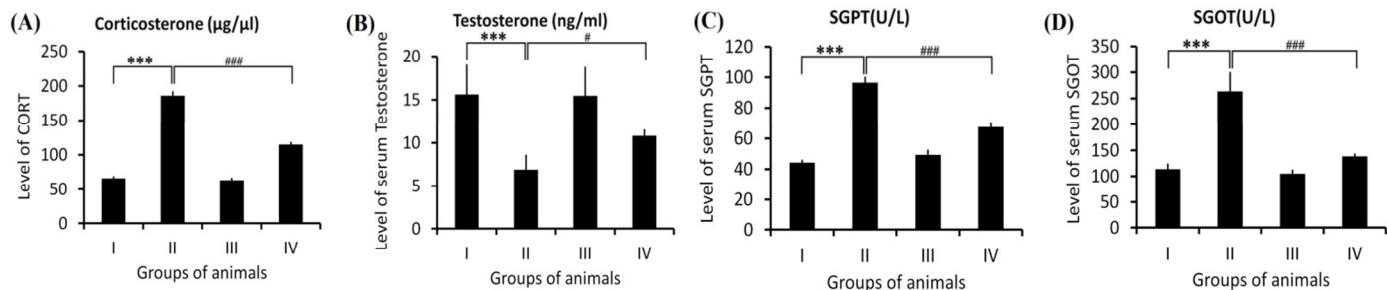


Fig-2: Effects of Melatonin on serum corticosterone and testosterone, SGPT, and SGOT level in the presence or absence of heat stress. (A) Corticosterone, a stress hormone showed its characteristic augmentation in blood serum upon induction of heat stress in animals of group II which reduced after the exogenous administration of melatonin. Our interpretations of data showed the serum CORT level in terms of ng/ml of group II animals increased significantly ($p<0.001$) in comparison to the control group. The same was detected at a significantly ($p<0.001$) restored level in group IV animals to which melatonin had been administered together with heat stress.(One-way ANOVA; $F=1445.957$, DF=3)(B) In line with a strong inverse correlation of corticosterone with the synthesis of testosterone, present data showed that the serum testosterone level (in ng/ml) in the stress group (group II) was significantly ($p<0.001$) decreased in comparison to the control group and upon treatment with the exogenous dosage of melatonin the level of serum testosterone was significantly ($p<0.001$) restored in group IV, as compared to group II. The conclusion also includes the preventive role of melatonin against testicular dysregulation. (One-way ANOVA; $F=16.502$, DF=3) (C – D) Similar to the CORT and T, the serum concentration of SGPT (One-way ANOVA; $F=458.796$, DF=3) and SGOT (One-way ANOVA; $F=89.19$, DF=3) enzymes were estimated to diagnose the effect of heat treatment on tissue (liver) toxicity. In the heat stress group (group II), the level of serum concentration of both enzymes was detected to be significantly ($p<0.001$) higher than that of the control animals (group I), indicating higher tissue toxicity. This was significantly ($p<0.001$) restored after melatonin administration in heat-stressed animals (group IV). As depicted in figure, '*' signifies the difference in Group II in comparison with Group I, whereas '#' signifies the difference after restoration of respective parameter in Group IV in comparison with Group II. (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; # = $p<0.05$; ## = $p<0.01$; ### = $p<0.001$)

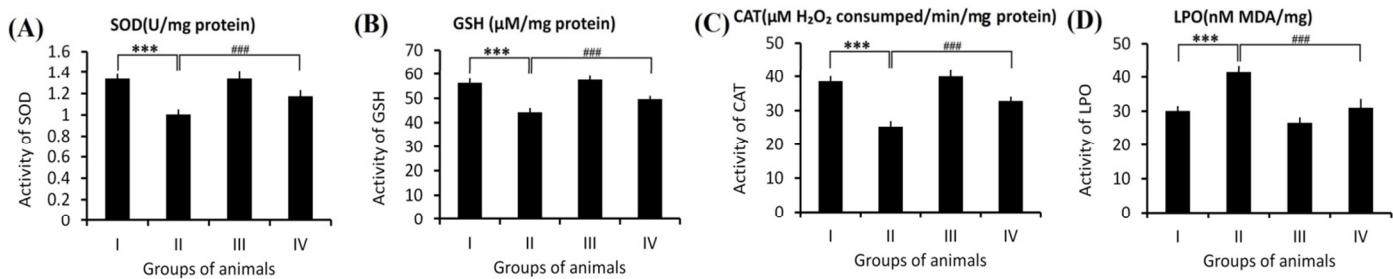


Fig-3: Effects of melatonin on testicular antioxidant profile in the presence or absence of heat stress. (A) Present data explain the status of the activity of SOD. It shows that the activity of dismutase enzyme in the heat stress group was significantly ($p<0.001$) inhibited in comparison to the untreated control group and there was a significant ($p<0.001$) re-establishment of the normal level of the antioxidant status after oral treatment with Melatonin. (One-way ANOVA; $F=62.805$, DF=3) (B) Similar to the SOD activity, GSH activity also diminished with the introduction of heat stress which is indicative of the oxidative stress in the tissue microenvironment. The administration of melatonin did ameliorate the antioxidant status ($p<0.001$). (One-way ANOVA; $F=119.169$, DF=3) (C) Data regarding CAT activity explained a similar change (all $p<0.001$). (One-way ANOVA; $F=124.363$, DF=3) (D) Consistent with SOD, GSH and CAT activity data, regarding cellular stress response, the LPO level in stress group was significantly elevated, as compared to the control group, which was detected to get restored significantly ($p<0.001$) to the normal level in case of the oral administration of melatonin in stressed animals of group IV(One-way ANOVA; $F=76.13$, DF=3). As depicted in figure, '*' signifies the difference in Group II in comparison with Group I, whereas '#' signifies the difference after restoration of

respective parameter in Group IV in comparison with Group II. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; # = $p < 0.05$; ## = $p < 0.01$; ### = $p < 0.001$)

4.5 Histological Study of Testicular Tissue

A gross estimate of overall testicular architecture was undertaken using the sections stained with haematoxylin and eosin. Extensive changes evident by germ cell loss were observed in tests obtained from rats that were exposed to heat stress. Many tubules appeared to lack of spermatocytes, round spermatids, and spermatozoa. Destruction of germinal epithelium, germ cells, spermatocytes, and round spermatids caused a breakdown of seminiferous epithelium (Figure-4B). The testes of rats that received melatonin –both after exposure to heat stress and without heat stress, revealed no significant degenerative changes in the seminiferous tubules, which were rather retained the structural characteristics similar to the controls (Figure-4C, 4D, 4A).

4.6 Immunohistochemical Study of Testicular HSP90 Expression

Immunohistochemical analysis of the testicular tissue showed a positive correlation with the changes in heat shock protein marker. We could observe a marked elevation in the tissue level expression of HSP90 protein in the heat stress group. The administration of melatonin ameliorated the adverse tissue microenvironment by lessening the pro-apoptotic signaling by caspase-3. The significant suppression of caspase 3 expression level in Group-IV animals as compared with Group-II indicated the protective nature of Melatonin against heat stress-induced testicular tissue damage and germ cells apoptosis (Figure-8A, 8B, 8C, 8D).

marker. The significant suppression of HSP90 expression level in Group-IV animals was found as compared with Group-II animals indicating the protective nature of melatonin against heat stress-induced testicular tissue damage (Figure-5A, 5B, 5C, 5D). The melatonin might maintain cellular homeostasis and prevent tissue damage in a way that does not demand further enhancement of HSP90 expression to protect cellular proteins further.

4.7 Immunohistochemical Study of Testicular Caspase-3 Expression

Immunohistochemical analysis of the testicular tissue showed a positive correlation with the changes of the apoptosis marker. We could observe a marked elevation in the tissue level expression of the apoptogenic caspase-3 protein in the heat stress group. The administration of melatonin ameliorated the adverse tissue microenvironment by lessening the pro-apoptotic signaling by caspase-3. The significant suppression of caspase 3 expression level in Group-IV animals as compared with Group-II indicated the protective nature of Melatonin against heat stress-induced testicular tissue damage and germ cells apoptosis (Figure-8A, 8B, 8C, 8D).

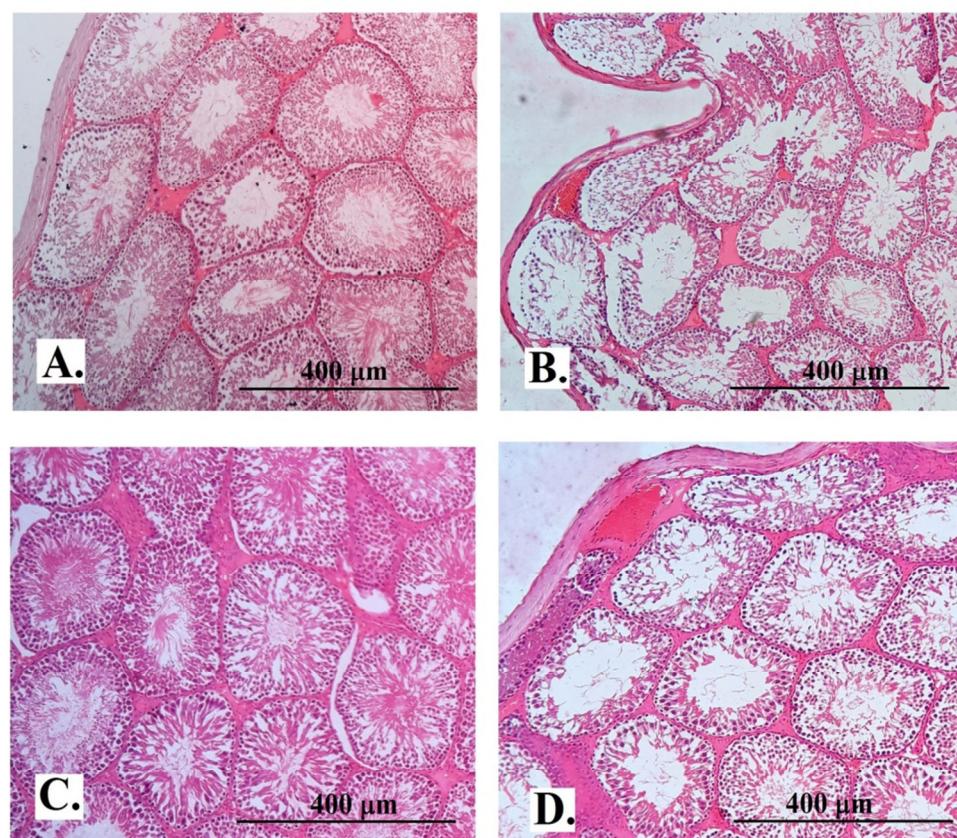


Fig-4: Histological study of testicular tissue in the presence or absence of heat stress with the administration of melatonin. (A) Transverse section of testis of control (Group I) rat showing seminiferous tubules in an organized manner in the panel A. (B) A similar tissue section of testis of sub-chronic heat stress (Group II) rat in the panel B, showing disorganized and distorted Seminiferous tubules and spermatogonia demonstrating the detrimental status of the tissue due to heat stress. (C) Treatment of melatonin alone (Group III) showed no significant change in the tissue architecture of rat testis, showing seminiferous tubules similar to those shown in the control group. (D) The beneficial effects of the melatonin sustained in the case of the heat-stressed animals when treated with melatonin and the tissue structure showed marked protection from that of the only heat-stressed groups. (magnification 200x)

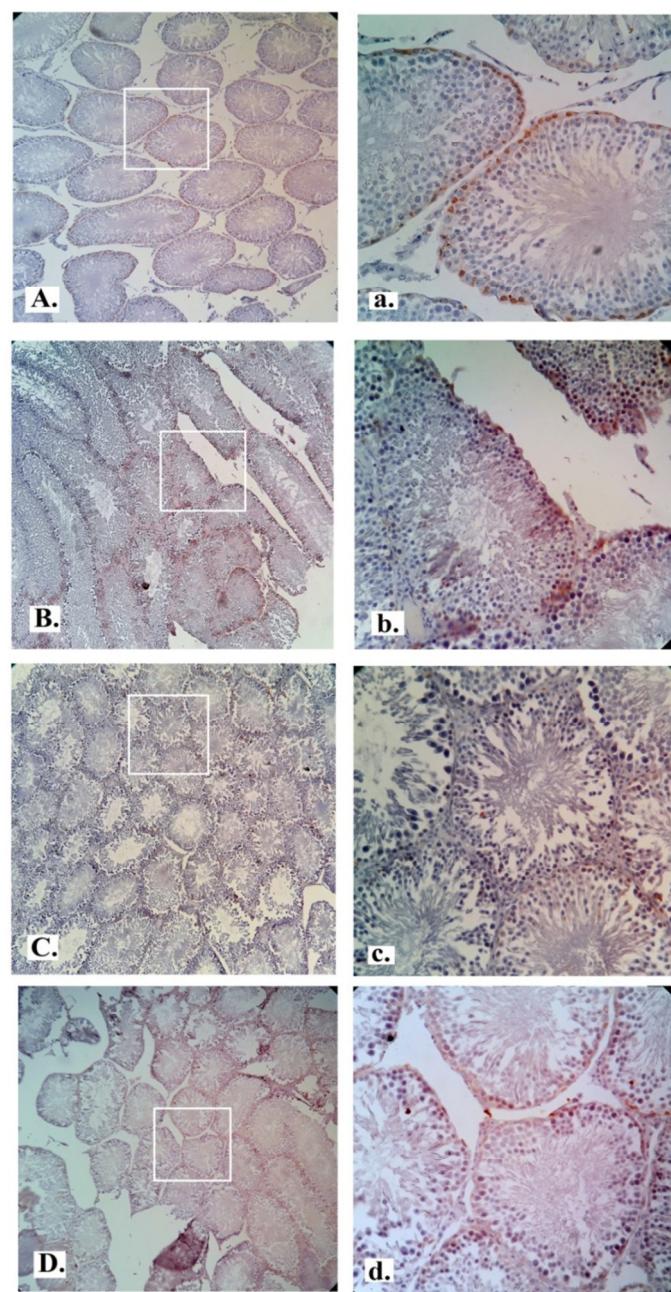


Fig-5: Immunohistochemical study of testicular tissue for expression of HSP90 in the presence or absence of heat stress with the administration of melatonin. Immunohistochemical analysis of the testicular tissue to test heat sensitivity, due to heat stress revealed that heat shock protein was induced by heat stress and got ameliorated with the effect of melatonin. In the figure panel A describes the control group of animals, panel B describes the heat-stressed group, panel C showed the melatonin only treated group whereas, panel D showed the group of animals which were heat-stressed and simultaneously given melatonin treatment (magnification 100x where panel name is in caps, and 1000x where panel name is in small letters).

4.8 HSP72, HSF1 and 17β -HSD3 gene expression

RT-PCR analysis of the heat shock protein genes revealed that in comparison to control (Group-I), the expression of the *HSP72* protein gene markedly increased in heat-stressed (Group-II) animals but this increment occurred at a much lesser extent in melatonin fed stressed animals (Group-IV). In other words, in comparison with Group-II, *HSP72* expression restored significantly in Group-IV rats. Along with the *HSP72*, parallel changes were noticed in *HSF1* expression (Figure-6) as well. This result showed a compensatory effect of melatonin on the heat stress-induced overexpression of *HSP72* and *HSF1*. 17 -hydroxysteroid dehydrogenase-3 (17β -HSD3), the predominant rate-limiting enzyme subtype-specific for male steroidogenic testosterone production has been taken into consideration to see the

expressional correlation of this enzyme with the serum level of testosterone. It was found that in heat-stressed (Group-II) rats, 17β -HSD3 expression reduced significantly ($p<0.001$) and drastically compared to control group, whereas that was restored considerably ($p<0.001$) in Group-IV rats indicating the protective effect of melatonin against heat stress-induced reduction of testicular testosterone production (Figure-6).

4.9 Determination of Heat Shock Protein Expression by Western Immuno-Blotting

The densitometric analysis of the Western blots has been represented by the bar diagrams Figure 7. Panel A and B, showing similar kinds of expression patterns for each of *HSP72* and *HSP90*. The level of expression of all heat stress-related proteins increased in the stress group as compared

to the control group which was reduced after administration of melatonin in heat exposed animals of group IV. No significant change in protein expression levels could be observed in melatonin treated groups (Group-III), as compared to the control group (Group-I) (Figure-7A and 7B). It also indicated that melatonin itself does not alter the

basal level of expression of stress-related proteins. These results also indicated that melatonin prevents tissue damage in a way that does not demand further enhancement of heat shock protein expression to protect other cellular proteins from damage.

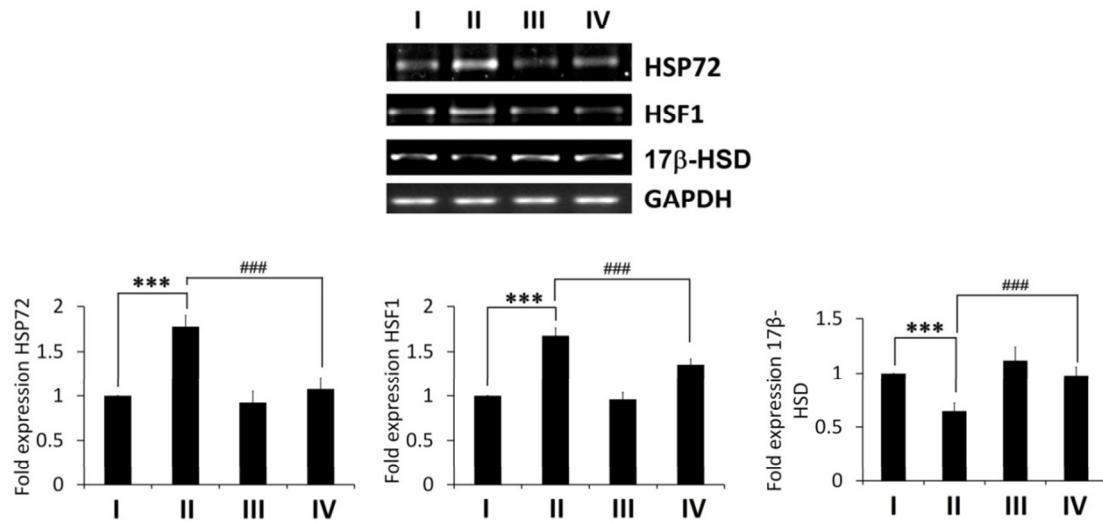


Fig-6: Effects of melatonin on testicular HSP72, HSF1, and 17 β -HSD3 gene expression in the presence or absence of heat stress. In this figure mRNA expression of HSP72 remained unchanged with the treatment of melatonin only but increased significantly in the case of heat-stressed animals devoid of melatonin administration as compared with the control. The HSP72 showed a significant reduction when the heat-stressed animals were given melatonin (One-way ANOVA; $F=1490.107$, $DF=3$). The same was found true for the HSF1 mRNA expression (One-way ANOVA; $F=155.931$, $DF=3$). In heat-stressed (Group-II) rats, 17 β -HSD3 expression reduced significantly ($p<0.001$) and drastically compared to the control group, whereas the 17 β -HSD3 expression was significantly ($p<0.001$) restored in Group-IV rats (One-way ANOVA; $F=464.576$, $DF=3$) indicating the protective effect of melatonin against heat stress-induced reduction of testicular testosterone production. As depicted in figure, “*” signifies the difference in Group II in comparison with Group I, whereas “#” signifies the difference after restoration of respective parameter in Group IV in comparison with Group II. (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; # = $p<0.05$; ## = $p<0.01$; ### = $p<0.001$)

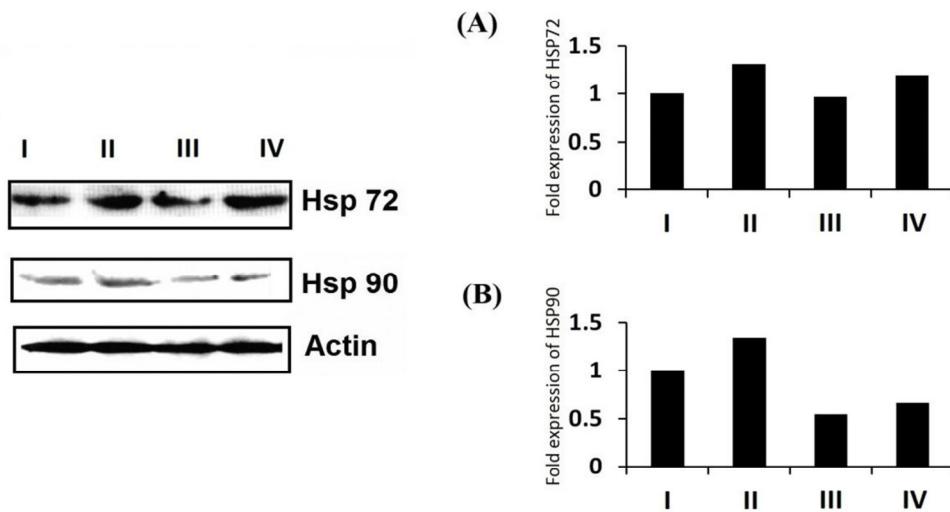


Fig-7: Effect of melatonin on heat shock protein expression. The densitometric analysis of the middle panel was given by the bar diagrams. Panel A and B showed a similar kind of expression of Hsp72 and Hsp90 respectively. The expression of heat shock proteins increased in Heat stressed animals as compared to the control animal which was restored on the administration of melatonin in group IV.

4.10 Assessment of Epididymal Sperm Count, Motility And Viability

Epididymal sperm count study showed a protective effect of

melatonin against heat stress-induced reduction of testicular sperm production. The number of epididymal sperms in the heat stress group was significantly ($p<0.001$) decreased in comparison to the control group and it was significantly

($p<0.001$) restored in Group-IV (heat stress + melatonin) as compared with the heat-stressed group (Group-II) (Figure-8E). The changes in sperm count levels were also correlated

with the serum level of testosterone in different groups (Figure-2B) and sperm motility and viability (Table-2) (Figure-8E, 8F, 8G).

Table-2: Effect of melatonin on sperm viability and motility in heat stress

	I	II	III	IV
Sperm motility(%)	85.215 \pm 0.89	56.582 \pm 1.106**	84.553 \pm 1.096 NS	70.528 \pm 2.755**
Sperm Viability(%)	89.005 \pm 3.560	58.00 \pm 3.742**	91.072 \pm 2.323 NS	74.401 \pm 3.455**

Values are expressed as mean \pm standard deviation. * $p<0.05$; significant when compared with control Group ** $p<0.001$; significant, NS=not significant. One Way Anova was performed in both cases out of percentages of Sperm motility ($F=272.45$; $DF=3$) and Sperm Viability ($F=85.34$; $DF=3$ followed by Tukey's all pairwise test.

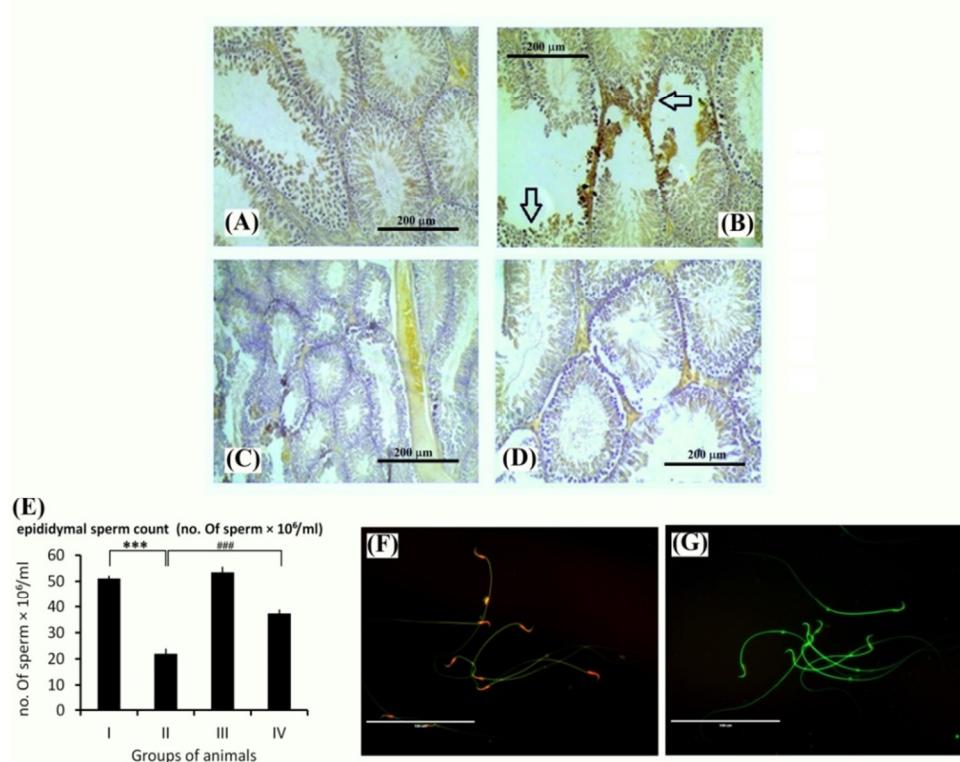


Fig-8: (A-D) Immunohistochemical study of testicular tissue for expression of caspase-3 in the presence or absence of heat stress with the administration of melatonin. Immunohistochemical analysis of the testicular tissue to test for apoptogenic activities due to heat stress revealed that apoptosis was induced by heat stress and got ameliorated with the administration of melatonin. In the figure panel A describes the control group of animals, panel B describes the heat-stressed group, panel C showed the melatonin only treated group whereas, panel D showed the group of animals which were heat-stressed and simultaneously given melatonin treatment (magnification 300x). (E) Effects of melatonin on epididymal sperm count in the presence and absence of heat stress. This figure explained that the number of epididymal sperms in heat stress group (Group-II) were significantly ($p<0.001$) decreased in comparison to the control group (Group-I) and it was significantly ($p<0.001$) increased in Group-IV (heat Stress + Mel) as compared with the heat stress group (Group-II). (One-way ANOVA; All Pairwise Multiple Comparison Procedures (Tukey Test); $F=85.335$, $DF=3$) (F-G) Fluorescence microscopic pictures of epididymal sperm. After fixing the sperm suspension with Carnoy's fixative stain with AO-EtBr and was observed under a fluorescent microscope in 400x magnification. (F) Live sperm (G) Dead sperm. As depicted in figure, '*' signifies the difference in Group II in comparison with Group I, whereas '#' signifies the difference after restoration of respective parameter in Group IV in comparison with Group II. (* = $p<0.05$; ** = $p<0.01$; * = $p<0.001$; # = $p<0.05$; ## = $p<0.01$; ### = $p<0.001$)**

5. DISCUSSION

In this study, we investigated the changes in different heat stress associated physiological parameters in testis upon induction of hyperthermia and the efficacy of melatonin treatment in the restoration of these parameters. In heat-stressed animals (Group II), the reduction of serum T was possibly the result of the rise in circulating CORT and direct testicular tissue damage due to heat stress. A number of earlier studies have revealed that glucocorticoid directly inhibits testosterone production by Leydig cells ^{29,30}. In our previous study, we reported protective antioxidant activity of red grape juice against detrimental effects of heat stress ¹¹

and on a similar ground; we chose to investigate the usability of melatonin to combat heat stress. In this study, reduction of expression of 17β -HSD3, an enzyme essential for testosterone synthesis in Leydig cell, may be the consequence of this inhibitory action. We observed remarkable testicular weight loss and germ cell destruction parallel to the reduction of a prominent antioxidant defense system consisting of SOD, GSH, CAT along with the elevation of lipid peroxidation after exposure to hyperthermia. SGPT and SGOT levels also elevated by exposure to hyperthermia. Oxidative stress is brought about during exposure to high temperatures that causes uncontrolled transcription, RNA processing, translation,

oxidative metabolism which leads to the disruption of structure and physiology of cells³¹. Transient mild testicular hyperthermia may cause an imbalance in the oxidative capacity as well as antioxidant capacity³². High levels of free radicals and reactive oxygen species (ROS), superoxide anion and hydrogen peroxide (H_2O_2) make human spermatozoa highly vulnerable to oxidative stress³³⁻³⁴. In support of this, we studied the gene expression pattern of 17β -HSD3, an enzyme that is predominantly expressed in testis and is responsible for the conversion of androstenedione to testosterone. Daily administration of melatonin prior to induction of hyperthermia could significantly prevent the pathophysiological changes in Group-IV rodents than that of heat-stressed groups (Group II). Previous reports revealed that ROS was involved in the process of induction of apoptosis in testicular germ cells³⁵ and caspase 3 played roles in the process of apoptosis as one of the downstream effectors³⁶. Expression of caspase 3 in heat-stressed rats, which we observed here, indicated the involvement of the apoptotic process in testicular germ cell destruction. Recently, Chen et.al (2019) reported that melatonin treatment successfully reduced the caspase 3 activity and apoptosis in granulosa cell³⁷. In the present investigation, we observed the similar role of melatonin in attenuating testicular caspase 3 activity, which was possibly the cause of protection of the germ cells from destructive actions induced by hyperthermia. Heat shock proteins (HSPs) do come into play as soon as the heat stress is introduced to the experimental animals. Highly conserved heat shock protein family members perform cellular protection in stressful situations. Heat shock proteins perform their activities via regulating conformational changes, translocation, assembly and degradation of targeted cellular proteins. Plumier J-CL et.al. (1996) reported the accumulation of RNA for HSPs during conditions known to produce ROS³⁸. Madamanchiet.al.(2001) investigated whether H_2O_2 caused accumulation of these proteins in rat Vascular Smooth Muscle Cell (VSMCs) and observed that H_2O_2 stimulated expression of both HSP70 and HSP90³⁹. Therefore, heat shock protein expression in various tissues is considered as a sensitive biomarker for identifying challenging conditions of the environment. So, the heat-stress generated expression pattern of heat shock proteins in the reproductive organ, the testis, is considered an important objective in our study. Among the heat shock proteins, the HSP72 that belongs to the heat shock protein 70 (HSP70) family, have a role in attenuation of oxidative cell damage. They also protect against ER-stress induced apoptosis⁴⁰. In this study, the HSP72 mRNA and protein expressions were significantly increased in the stressed group of animals (Group-II) as compared to the control group. In both cases, melatonin restored the expression of HSP72 in melatonin treated heat stressed group, proving the stress-buster character of melatonin. 90 kDa heat shock proteins (the Hsp90 family) are chaperons and during stress, these chaperons hold their targets in a folding competent state until the whole cell recovers. Once recovery is accomplished, these proteins provide the energy for the refolding process to maintain the functionality of the macromolecules⁴¹. In eukaryotic organisms, the expression of heat shock factor I (HSF-I) plays a major role in heat shock response. In resting cells, HSF-I remains in complex with several heat shock proteins viz. HSP70 or HSP90. In the stressed state, damaged proteins become abundant and liberate the heat shock factor from

their HSP70/HSP90 complexes. This process sets the stage for the trimerization, nuclear translocation and phosphorylation of HSF-I⁴². HSP90 plays a special role as "signalling-chaperones". It binds to steroid receptors and to several serine and tyrosine kinases⁴³⁻⁴⁷. HSP90 helps these kinases proteins to reach their fully signalling competent form, as well as regulating their association with other proteins and membranes. When the steroid hormone binds to its receptor, the receptor dissociates from HSP90 and translocates to the nucleus resulting in the dimerization of the receptor-hormone complex to the steroid response element DNA-region. Chaperones like HSP90 are also believed to play a role in the termination of the steroid response promoting the recycling of the receptor. In our experiment, we observed an elevated HSF-I level with an increased HSP90 level after heat stress, which supports the previous reports. On melatonin administration, HSF-I and HSP72 and HSP90 level restored to the basal level. This is also likely due to the stress buster mechanism of melatonin which minimizes the demand for expression of HSPs to compensate for the protein damage due to heat stress, hence no further elevation of HSPs is required in melatonin treated heat-stressed rats (Group-IV). The maintenance of structural proteins may also be a key to HSP associated stress tolerance. In this regard, HSP27, a protein homologous with a-crystalline lens protein, prevents actin microfilament disruption under stress conditions. This effect on the cytoskeleton may be important not only in individual cell tolerance to stress through cytoskeletal stabilization but may also be essential to the safety of the entire organism through the maintenance of endothelial and epithelial barrier functions. The mechanism of cellular protein management is through the chaperone function across the cell membranes. HSP70 and HSP60 perform a unique relay in the movement of cellular proteins through the mitochondrial membrane. HSP70 transporting the protein to the outer mitochondrial membrane and participating in the protein's unfolding. In our study, we found that the expression of HSP72 and HSP90 is amplified after exogenously induced heat stress. This overexpression of HSPs has been successfully ameliorated by melatonin as discussed earlier. The present work has been extensively presented and discussed in the Ph.D. thesis awarded to the present author⁴⁸. Earlier reports showed that melatonin treatment also reduces Hsp70 family protein expression induced by chronic cerebral hypoperfusion in rats⁴⁹. The pineal gland hormone melatonin is involved in nocturnal thermoregulation of internal body temperature. Ahn and Thiele (2003) reported that heat and hydrogen peroxide exerted redox-dependent activation of HSF1 establishing a common mechanism in the stress activation of HSP gene expression by mammalian HSF1⁵⁰. We observed the treatment of melatonin prior to induction of hyperthermia prevented heat stress-induced expression of HSF1 and HSPs. Here, melatonin, possibly by virtue of its ROS scavenging activity, repelled the stimulatory action of ROS on HSF-1 and HSPs expressions.

6. CONCLUSION

Human testes are more vulnerable to reproductive toxicants to that of laboratory animals including rat. The infertility in human male caused by different environmental stressors is more common than the experimental laboratory animals⁵¹. So, current findings in rat model is justifiable to

understand the applicability towards more sensitive human testis. Our experimental data suggest that melatonin effectively prevents the hyperthermia-induced testicular dysfunction and germ cell degeneration exerting its antioxidant action by its ROS scavenging activity. This study showed the protective effects of melatonin which may be useful to prevent environmental and occupational heat stress-related disorders of testicular function that may cause infertility. There is also a large scope to investigate further details regarding this beneficial effect of melatonin in this field.

6.1 Limitation or lacunae of this study

Melatonin is a natural nontoxic antioxidant, that mainly synchronizes biological rhythm. It is evident from different scientific researches that, melatonin as a competent stress buster. In this study we have examined effects of melatonin against hyperthermia induced germ cell damage in rodent model. This study showed the protective effects of melatonin to prevent heat stress-induced dysregulation of testicular function that may cause infertility. Further detailed investigations on signalling molecules and their signalling pathways and their cross talks underlying this protective effect is yet to be studied. In addition to that, there are huge scopes of research on the effectiveness of melatonin against physiological stress in human.

10. REFERENCES

1. Setchell BP. Spermatogenesis and spermatozoa in Austin C.R and R.V short (Eds). *Germ Cell and Fertilization*. Cambridge: Cambridge University Press. 1993; 63-102.
2. Jung A, Schill WB, Schuppe HC. Improvement of semen quality by nocturnal scrotal cooling in oligozoospermic men with a history of testicular maldevelopment. *Int J Androl*. 2005;28(2):93-8. doi: 10.1111/j.1365-2605.2004.00517.x, PMID 15811070.
3. Foldesy RG, Bedford JM. Biology of the scrotum. I. Temperature and androgen as determinants of the sperm storage capacity of the rat cauda epididymidis. *Biol Reprod*. 1982;26(4):673-82. doi: 10.1095/biolreprod26.4.673, PMID 7082731.
4. Mieusset R, Bujan L, Mondinat C, Mansat A, Pontonnier F, Grandjean H. Association of scrotal hyperthermia with impaired spermatogenesis in infertile men. *Fertil Steril*. 1987;48(6):1006-11. doi: 10.1016/s0015-0282(16)59600-9, PMID 3678498.
5. Rao M, Zhao XL, Yang J, Hu SF, Lei H, Xia W, Zhu CH. Effect of transient scrotal hyperthermia on sperm parameters, seminal plasma biochemical markers, and oxidative stress in men. *Asian J Androl*. 2015;17(4):668-75. doi: 10.4103/1008-682X.146967, PMID 25652627.
6. Gong S, Miao YL, Jiao GZ, Sun MJ, Li H, Lin J, Luo MJ, Tan JH. Dynamics and correlation of serum cortisol and corticosterone under different physiological or stressful conditions in mice. *PLOS ONE*. 2015;10(2):e0117503, PMID 25699675. doi: 10.1371/journal.pone.0117503, PMID 25699675.
7. Gao HB, Tong MH, Hu YQ, Guo QS, Ge R, Hardy MP. Glucocorticoid induces apoptosis in rat Leydig cells. *Endocrinology*. 2002;143(1):130-38. doi: 10.1210/endo.143.1.8604, PMID 11751601.
8. Becker J, Craig EA. Heat-shock proteins as molecular chaperones. *Eur J Biochem*. 1994;219(1-2):11-23. doi: 10.1007/978-3-642-79502-2_2, PMID 8306977.
9. Chaki SP, Misro MM, Ghosh D, Gautam DK, Srinivas M. Apoptosis and cell removal in the cryptorchid rat testis. *Apoptosis*. 2005;10(2):395-405. doi: 10.1007/s10495-005-0813-7, PMID 15843900.
10. Reiter RJ, Tan DX, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress. A review. *J Biomed Sci*. 2000;7(6):444-58. doi: 10.1007/BF02253360, PMID 11060493.
11. Halder S, Sarkar M, Dey S, Kumar Bhunia SK, Ranjan Koley A, Giri B. Protective effects of red grape (*Vitis vinifera*) juice through restoration of antioxidant defense, endocrine swing and Hsfl, Hsp72 levels in heat stress induced testicular dysregulation of Wister rat. *J Therm Biol*. 2018;71:32-40. doi: 10.1016/j.therbio.2017.10.011, PMID 29301698.
12. Dani C, Oliboni LS, Pasquali MA, Oliveira MR, Umezu FM, Salvador M, Moreira JC, Henriques JA. Intake of purple grape juice as a hepatoprotective agent in Wistar rats. *J Med Food*. 2008;11(1):127-32. doi: 10.1089/jmf.2007.558, PMID 18361748.
13. Dani C, Oliboni LS, Vanderlinde R, Bonatto D, Salvador M, Henriques JA. Phenolic content and antioxidant activities of white and purple juices manufactured with organically or conventionally produced grapes. *Food Chem Toxicol*. 2007;45(12):2574-80. doi: 10.1016/j.fct.2007.06.022, PMID 17683842.

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8. AUTHORS CONTRIBUTION STATEMENT

Soma Halder developed the HS model, carried out the experiments, calculated the results; Mrinmoy Sarkar, Sananda Dey, Prasanta Ghosh, Sujay Kumar Bhunia carried out some experiments; Soma Halder, Mrinmoy Sarkar, Sananda Dey wrote the manuscript; Debasish Bandyopadhyay helped to carry out antioxidant experiment and analysis; Biplab Giri initiated the hypothesis, helped supervise the project, analysed the supplied data, helped in writing the manuscript

9. CONFLICT OF INTEREST

Conflict of interest declared none.

14. Ferguson LR. Role of plant polyphenols in genomic stability. *Mutat Res.* 2001;475(1-2):89-111. doi: 10.1016/s0027-5107(01)00073-2, PMID 11295156.
15. Reiter R, Tang L, Garcia JJ, Muñoz-Hoyos A. Pharmacological actions of melatonin in oxygen radical pathophysiology. *Life Sci.* 1997;60(25):2255-71. doi: 10.1016/s0024-3205(97)00030-1, PMID 9194681.
16. Cagnoli CM, Atabay C, Kharlamova E, Manev H. Melatonin protects neurons from singlet oxygen-induced apoptosis. *J Pineal Res.* 1995;18(4):222-26. doi: 10.1111/j.1600-079x.1995.tb00163.x, PMID 8531053.
17. Marshall KA, Reiter RJ, Poeggeler B, Aruoma OI, Halliwell B. Evaluation of the antioxidant activity of melatonin in vitro. *Free Radic Biol Med.* 1996;21(3):307-15. doi: 10.1016/0891-5849(96)00046-9, PMID 8855441.
18. Pieri C, Marra M, Moroni F, Recchioni R, Marcheselli F. Melatonin: a peroxyl radical scavenger more effective than vitamin E. *Life Sci.* 1994;55(15), PL271-PL276. doi: 10.1016/0024-3205(94)00666-0, PMID 7934611.
19. Öner-İyidogan Y, Gürdöl F, Öner P. The effects of acute melatonin and ethanol treatment on antioxidant enzyme activities in rat testes. *Pharmacol Res.* 2001;44(2):89-93. doi: 10.1006/phrs.2001.0828, PMID 11516256.
20. Eng JVJL, Reed CB, Kokolus KM, Repasky EA. Housing temperature influences the pattern of heat shock protein induction in mice following mild whole body hyperthermia. *Int J Hypertherm.* 2014;30(8):540-46. doi: 10.3109/02656736.2014.981300, PMID 25430986.
21. Gordon CJ. Thermal physiology of laboratory mice: defining thermoneutrality. *J Therm Biol.* 2012;37(8):654-85. doi: 10.1016/j.jtherbio.2012.08.004.
22. Belyarova G, Tancheva S, Hristova M. The effects of melatonin on burn-induced inflammatory responses and coagulation disorders in rats. *Methods Find Exp Clin Pharmacol.* 2010;32(5):299-303. doi: 10.1358/mf.2010.32.5.1437717, PMID 20664819.
23. Yokoi K, Uthus EO, Nielsen FH. Nickel deficiency diminishes sperm quantity and movement in rats. *Biol Trace Elem Res.* 2003;93(1-3):141-54. doi: 10.1385/BTER:93:1-3:141, PMID 12835498.
24. Ghosh D, Firdaus SB, Mitra E, Dey M et al. Hepatoprotective activity of aqueous leaf extract of *Murrayakoenigii* against lead-induced hepatotoxicity in male Wistar rat. *Int J Pharm Pharm Sci.* 2013;5(1):285-95.
25. Mukherjee D, Ghosh AK, Bandyopadhyay A, Basu A, Datta S, Pattari SK, Reiter RJ, Bandyopadhyay D. Melatonin protects against isoproterenol-induced alterations in cardiac mitochondrial energy-metabolizing enzymes, apoptotic proteins, and assists in complete recovery from myocardial injury in rats. *J Pineal Res.* 2012;53(2):166-79. doi: 10.1111/j.1600-079x.2012.00984.x, PMID 23050266.
26. Aebi H, Wyss SR, Scherz B, Skvaril F. Heterogeneity of erythrocyte catalase II. Isolation and characterization of normal and variant erythrocyte catalase and their subunits. *Eur J Biochem.* 1974;48(1):137-45. doi: 10.1111/j.1432-1033.1974.tb03751.x, PMID 4141308.
27. Kole L, Giri B, Manna SK, Pal B, Ghosh S. Biochanin-A, an isoflavon, showed anti-proliferative and anti-inflammatory activities through the inhibition of iNOS expression, p38-MAPK and ATF-2 phosphorylation and blocking NF κ B nuclear translocation. *Eur J Pharmacol.* 2011;653(1-3):8-15. doi: 10.1016/j.ejphar.2010.11.026, PMID 21147093.
28. Mukherjee D, Ghosh AK, Dutta M, Mitra E, Mallick S, Saha B, Reiter RJ, Bandyopadhyay D. Mechanisms of isoproterenol-induced cardiac mitochondrial damage: protective actions of melatonin. *J Pineal Res.* 2015;58(3):275-90. doi: 10.1111/jpi.12213, PMID 25652673.
29. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. *Endocr Rev.* 1984;5(1):25-44. doi: 10.1210/edrv-5-1-25, PMID 6368214.
30. Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev.* 2000;21(1):55-89. doi: 10.1210/edrv.21.1.0389, PMID 10696570.
31. Miura M, Sasagawa I, Suzuki Y, Nakada T, Fujii J. Apoptosis and expression of apoptosis-related genes in the mouse testis following heat exposure. *Fertil Steril.* 2002;77(4):787-93. doi: 10.1016/s0015-0282(01)03255-1, PMID 11937135.
32. Pino JA, Osses N, Oyarzún D, Fariñas JG, Moreno RD, Reyes JG. Differential effects of temperature on reactive oxygen/nitrogen species production in rat pachytene spermatocytes and round spermatids. *Reproduction.* 2013;145(2):203-12. doi: 10.1530/REP-12-0330, PMID 23241345.
33. Tremellen K. Oxidative stress and male infertility—a clinical perspective. *Hum Reprod Update.* 2008;14(3):243-58. doi: 10.1093/humupd/dmn004, PMID 18281241.
34. Gavella M, Lipovac V. Protective effects of exogenous gangliosides on ROS-induced changes in human spermatozoa. *Asian J Androl.* 2013;15(3):375-81. doi: 10.1038/aja.2012.144, PMID 23503425.
35. Maheshwari A, Misro MM, Aggarwal A, Sharma RK, Nandan D. Pathways involved in testicular germ cell apoptosis induced by H₂O₂ in vitro. *FEBS Journal.* 2009;276(3):870-81. doi: 10.1111/j.1742-4658.2008.06831.x, PMID 19143845.
36. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim Biophys Acta.* 2016;1863(12):2977-92. doi: 10.1016/j.bbamcr.2016.09.012, PMID 27646922.
37. Chen Z, Lei L, Wen D, Yang L. Melatonin attenuates palmitic acid-induced mouse granulosa cells apoptosis via endoplasmic reticulum stress. *J Ovarian Res.* 2019;12(1):43. doi: 10.1186/s13048-019-0519-z, PMID 31077207.
38. Plumier JC, Robertson HA, Currie RW. Differential accumulation of mRNA for immediate early genes and heat shock genes in heart after ischaemic injury. *J Mol Cell Cardiol.* 1996;28(6):1251-60. doi: 10.1006/jmcc.1996.0115. PMID 8782066.

39. Madamanchi NR, Li S, Patterson C, Runge MS. Thrombin regulates vascular smooth muscle cell growth and heat shock proteins via the JAK-STAT pathway. *J Biol Chem.* 2001;276(22):18915-24. doi: 10.1074/jbc.M008802200, PMID 11278437.

40. Gupta S, Deepti A, Deegan S, Lisbona F, Hetz C, Samali A. HSP72 protects cells from ER stress-induced apoptosis via enhancement of IRE1α-XBPI signaling through a physical interaction. *PLoS Biol.* 2010;8(7):e1000410. doi: 10.1371/journal.pbio.1000410, PMID 20625543.

41. Merin AB, Yaglom JA, Gabai VL, Zon L, Ganiatsas S, Mosser DD, Zon L, Sherman MY. Protein-damaging stresses activate c-Jun N-terminal kinase via inhibition of its dephosphorylation: a novel pathway controlled by HSP72. *Mol Cell Biol.* 1999;19(4):2547-55. doi: 10.1128/mcb.19.4.2547, PMID 10082520.

42. Morimoto RI. Cells in stress: transcriptional activation of heat shock genes. *Science.* 1993;259(5100):1409-10. doi: 10.1126/science.8451637, PMID 8451637.

43. Morimoto RI, Santoro MG. Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nat Biotechnol.* 1998;16(9):833-8. doi: 10.1038/nbt0998-833, PMID 9743115.

44. Morimoto RI, Tissières A, Georgopoulos C. Stress proteins in biology and medicine. *Cold Spring Harb.* 1990.

45. Morimoto RI, Tissières A. The biology of heat shock proteins and molecular chaperones (No. 577.112 BIO). Vol. 1994; 1994.

46. Buzzard KA, Giaccia AJ, Killender M, Anderson RL. Heat shock protein 72 modulates pathways of stress-induced apoptosis. *J Biol Chem.* 1998;273(27):17147-53. doi: 10.1074/jbc.273.27.17147, PMID 9642282.

47. Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev.* 1997;18(3):306-60. doi: 10.1210/edrv.18.3.0303, PMID 9183567.

48. Halder S. Molecular response and role of heat shock protein during heat stress induced dysregulation of testicular function and its management by natural [products Ph.D thesis]. West Bengal State University; 2019.

49. Ozacmak VH, Barut F, Ozacmak HS. Melatonin provides neuroprotection by reducing oxidative stress and HSP70 expression during chronic cerebral hypoperfusion in ovariectomized rats. *J Pineal Res.* 2009;47(2):156-63. doi: 10.1111/j.1600-079X.2009.00695.x. PMID 19552637.

50. Ahn SG, Thiele DJ. Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. *Genes Dev.* 2003;17(4):516-28. doi: 10.1101/gad.1044503. PMID 12600944.

51. Working PK. Male reproductive toxicology: comparison of the human to animal models. *Environ Health Perspect.* 1988;77:37-44. doi: 10.1289/ehp.887737, PMID 3289906.