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Pharmacological and biochemical studies on the protective effects of melatonin during stress-induced behavioral and immunological changes in relation to oxidative stress in rats

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Abstract

Stress is known to precipitate neuropsychiatric diseases and depending upon its nature and intensity it can also influence functioning of the immune system. Melatonin (N-acetyl-5-methoxy tryptamine) a pineal gland hormone and potent antioxidant is known to protect many diseases. Effect of melatonin in stress-induced neuro-immunomodulation is not well elucidated. Therefore in the present study protective effects of melatonin were evaluated in restraint stress (RS)-induced behavioral and immunological changes in rats. RS for 1hr. significantly reduces a) % open-arm entries b) % time spent on open-arm in elevated plus maze (EPM) test parameters ($p < 0.01$) and significant increase in MDA levels in brain homogenate when compared to no RS control groups ($p < 0.05$). In immunological studies both humoral and cell-mediated immune responses to antigen were significantly suppressed by RS for 1 hr. for 5 consecutive days as evidenced by significant reduction in (a) anti-SRBC antibody titre (b) PFC counts (c) % change in paw volume and (d) Th₁ (IFN- γ) and Th₂ (IL-4) cytokine levels ($p < 0.001$ in all parameters). These RS-induced immunological changes were associated with significantly increased lipid peroxidation (MDA) levels in serum and significantly decreased activity of (a) SOD (b) CAT and (c) GSH levels in RS (X5)-exposed group ($p < 0.02$). Pretreatment with melatonin (10, 50 and 100 mg/kg) significantly reversed these RS-induced changes in EPM test parameters and humoral & cell-mediated immunological parameters as well as oxidative stress markers in a dose dependent manner by differential degrees ($p < 0.001$). Results are strongly suggestive of the involvement of free radicals during stress-induced neurobehavioral and immunological changes. These changes were significantly restored by melatonin pretreatment. We can conclude that melatonin may have protective role during such stress-induced neuro-immunomodulation.

Key words: Restraint stress, Free radicals, Oxidants, Melatonin, Behavior, Cytokine, Immunity

Introduction

There are various factors which are known to affect physiological homeostasis of the organisms (Chrousos and Gold 1992). Several interactive changes are induced by different types of known stressors. They also elicit a complex repertoire of behavioral and physiological responses (Davis et al. 2005). These processes reflect activation of specific central neuro-circuit and constantly modulated by psychological or environmental factors (Fogaca et al. 2014; Kochanek et al. 2013; Stojkov et al. 2014).

It is known that stress suppresses both humoral and cell-mediated immune responses to antigen. Cell-mediated immune regulation can be influenced through Th₁ and Th₂ cytokine network. (Dantzer and Kelley 1989). The involvements of catecholamine and glucocorticoids are also having been reported to modulate stress-induced immune regulation (De Felice and Porreca 2009; George et al. 2013).

Various studies have indicated the role of reactive oxygen species (ROS) in the regulation of stress-induced neurobehavioral changes (Dias et al. 2013; Emerit et al. 2004; Schieber and Chandel 2014). Involvement of free radicals in neurobehavioral changes induced by stress and these changes were reversed by antioxidants (Tseng et al. 2013). In a 2011 study we have shown the implication of free radicals during stress-induced immunomodulation (Pal et al. 2011).

Melatonin (n-acetyl-5-methoxytryptamine), a pineal gland neuro-hormone regulates circadian rhythm of the body. Several studies have been done to prove the antioxidant potential and protective role of melatonin in various diseases including neurological and immunological disorders (Carrillo-Vico et al. 2013; Zaitone et al. 2013). It acts as a direct free radical scavenger and indirect antioxidant and have protective role in various diseases (Hardeland et al. 1993; Kucuktulu 2012; Nakagawa et al. 2004; Shirazi et al. 2011). It has been shown that melatonin stimulate gene expression for antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx) in brain and other tissues. (Turgut et al. 2013). Additionally, it directly neutralizes ROS like hydroxyl radical (OH[•]), superoxide radical (O₂^{•-}), peroxy radical (LOO[•]), singlet oxygen (¹O₂) and hydrogen peroxide (H₂O₂) (Ma et al. 2014; Osborne et al. 1998).

The emotional stress is known to precipitates various neuropsychiatry diseases and anxiety like disorders. It is also known to suppress immune defense mechanism of the body. Involvement of increased free radicals and decreased antioxidant defense system is known to cause precipitation of various pathological and non-pathological disorders. Restraint stress is a well known animal model to induce emotional stress in rodents.

The protective effect of melatonin during RS-induced behavioral and immunological changes is not well elucidated.

Therefore, present study was aim and designed to evaluate possible protective role of melatonin during restraint stress-induced neuro-immunomodulation in rats.

Materials and methods

Experimental animals

Inbred male Wistar rats of (200-250 gm.) and $n=7$ per group were used in this study. All the animals were housed in a standard laboratory conditions with food and water available *ad libitum*. Necessary approval from the Institutional Animal Ethics Committee (IAEC) was obtained before starting the study.

Stress exposure

The restraint stress (RS) procedure is widely used and most accepted experimental model to induce emotional stress in rodents (Maghsoudi et al. 2014). Stress was applied by immobilizing/restraining the animals in Plexiglas restrainers (INCO, Ambala). One hr for 1 day restraint stress was applied to the animals for behavioral and 5 days for immunological studies. Animals were restraint after spontaneous entry in Plexiglas restrainer chamber and allowed to stay in this position for 1 hr. To study the effect of melatonin on behavioral changes, rats were pretreated with melatonin (10, 50 and 100 mg/kg, body weight) for five consecutive days and on day 5 they were exposed to 1hr restraint stress (RSX1). Immediately after stress procedure rats were exposed to EPM test. For immunological studies restraint stress (RS) and melatonin were given for five consecutive days (RSX5).

Drugs and chemicals

All chemicals and melatonin used in the study were procured from Sigma-Aldrich Chemical Co. USA. Melatonin was dissolved in 20 μ L of absolute ethanol and volume was makeup to 2 ml by using distill water. The total volume of ethanol in the injected

dose was not more than 1%. Melatonin in a dose of 10 mg/kg, 50 mg/kg and 100 mg/kg were administered intraperitoneally (i.p) between 10:00-10:30 am for every experimental group.

Neurobehavioral studies

Elevated plus maze test

Elevated plus maze test was performed according to the method described by Pellow *et al.* (Pellow et al. 1985). It consists of two opposite arms of 40X40 cm, crossed with two similar closed arms with wall of 40 cm height. The arms were connected so that the maze had a plus sign look. The entire maze was elevated 50 cm above the ground level and placed in a quite dimly lit room. Naïve or pretreated rats were placed individually in the centre of the maze facing the closed arms and observed for 5 minutes. The parameters measured were a) No. of open-arms entries b) No. of closed arms entries c) time spent on open-arm and d) time spent on closed arms. Subsequently the percentage of open-arms entries and time spent on open-arms were calculated from open-arm entries and time spent on open arms, divided by the total number of entries in both open and closed arms and time spent on open-arms exploration divided by total time spent in both open and closed arms respectively. After the EPM test rats were sacrificed using high dose of anesthesia (pentobarbitone 50 mg/ kg) and were decapitated. Their brain was taken out and brain homogenate was prepared for biochemical analysis.

Immunological studies

Test for Humoral Immunity (Haemagglutination assay)

Haemagglutination assay was performed according to the method described by Clarke and Casals (Clarke and Casals 1958). To perform this assay fresh blood of the healthy sheep was collected by venipuncture and was centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and sheep red blood cells (SRBC) washed three times with 0.9% normal saline. The SRBCs were diluted to 0.5×10^9 cells/ml. The cells were counted on the Neubaur's chamber under the microscope. All the animal groups were immunized with the i.p. injection of 0.5×10^9 cells on day 0. The treatment with different antioxidants was done for five consecutive days, while control group received only vehicle. The stress (RS) exposure was done for one hour daily from the day of immunization to five consecutive days. On day 6th they were bled from the supraorbital-

plexus by micro capillary technique under light ether anesthesia. The serum of each rat was collected in the micro centrifuge tubes. The serum samples were kept at -80°C until analyzed for anti-SRBC antibody titer by indirect haemagglutination (IHA) technique. For secondary antibody response, animals were given a booster dose of 0.1×10^9 cells and again exposed to RS. After 48 hours blood was collected for secondary anti-SRBC antibody response. The serum samples were assayed for primary and secondary anti-SRBC antibody titre using 96 well titre plates (Tarson). The serum samples were diluted 2 folds with phosphate buffer saline (150 Mm/L, pH 7.4). To each well 0.025 mL of 1% v/v SRBC (fresh) in phosphate buffer was added. The plates were incubated at 37°C for 1 hr. and then observed for haemagglutination. The highest dilution showing visible haemagglutination was taken as the antibody titre. The antibody titre was expressed in a graded manner, the minimum dilution (1/2) being ranked as one (1), and the mean ranks for different groups assayed for statistical significance.

Splenic plaque forming cell (PFC) assay

PFC assay was done by the method described by Li *et al.* (Li et al. 2010). This assay is an in-vitro method for identifying antibody forming cells by the action of the freshly synthesized antibodies against SRBC around antibody forming cells. The spleen was dissected out and rinsed in 2 ml of RPMI-1640 (pH 7.2) and dissociated with the help of sterile forceps on the small wire-mesh under aseptic conditions to recover lymphoid cells. 1 ml of histopaque (1.077 g/ml density) and 2 ml cell suspension was taken and centrifuged at 1000 rpm for 20 minutes at 4°C . The whitish interface of the lymphocyte between button red pellet and uppermost layer was taken and adjusted to a volume of 1 million lymphocytes/ml, i.e. 1×10^6 cells/ml. SRBC were washed with phosphate buffer saline (PBS) 150 Mm/L, pH 7.4 and the cell counts were adjusted to 800×10^6 cells /ml. guinea pig serum was taken as complement and diluted 5 times in PBS. For assay, 1 ml of SRBC suspension, 1 million lymphocytes and 0.5 ml of Guinea pig serum were taken in the microtube. From this mixture, a known volume was loaded into a Cunningham's chambers, sealed with vacuum grease, and incubated for 1 hr at 37°C . The plaques were counted under light microscope and results were expressed as plaques/ 10^6 spleen cells.

Test for cell-mediated Immunity (DTH response)

The delayed type hypersensitivity response was measured by the method of Farhath *et al.* (Farhath *et al.* 2013). After immunization with SRBC as for antibody titre on day 0 and on day 5 all the animal groups were challenged with 0.2 mL of 2% v/v fresh SRBCs in 0.9% normal saline into the right hind paw, while left paw received normal saline. After 24 hrs the footpad oedema was measured and difference in the footpad volume (Rt-Lt) was measured by a dial caliper (Mututio Japan) and expressed as percentage difference in DTH response.

Cytokine assay

The cytokine assays were carried out by using commercially available cytokine assays kits (Diclone, France). Briefly, the Th₁ interferon- γ (IFN- γ) and Th₂ interleukin-4 (IL-4) cytokines assays were performed by using solid phase sandwich enzyme linked immunosorbant assay (ELISA) technique. A monoclonal antibody specific to IL-4 or IFN- γ was coated on the well of the microtitre strips provided. Antigen and biotinylated polyclonal antibody specific for rIL-4 and rIFN- γ were simultaneously incubated at 37 °C for 1 hr. The revelation steps included streptavidin horse-radish peroxidase (HRP) and 3, 3', 5, 5'-Tetramethylbenzidine (TMB) as chromogen. The readings were taken on Microscan-5405A ELISA reader (ECIL) and results were expressed in pg/ml.

Biochemical studies

Lipid peroxidation markers

Thiobarbituric acid reactive substance (TBARS) estimation in brain

Malondialdehyde (MDA), a measure of lipid peroxidation was determined as describe by Lowry *et al.* and Garcia *et al.* (Garcia *et al.* 2005; Lowry *et al.* 1951). Briefly, the reaction mixture is consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20 % acetic acid (pH 3.5) and 1.5 ml of 0.8 % aqueous solution of thiobarbituric acid (TBA) was added to 0.2 ml of processed brain homogenate. The mixture was made up to 4.0 ml with distilled water and heated at 95 °C for 60 min. after cooling with tap water, 5 ml of n-butanol and pyridine (15:1 v/v) and 1 ml of distilled water was added and centrifuged. The organic layer was separated out and its absorbance was measured at 532 nm using a UV-Visible spectrophotometer (UV 5740 SS, ECIL). Estimation of protein content in the brain

homogenate was done by using standard method of Lowry *et al* (Lowry *et al.* 1951). MDA content was expressed as nM/mg protein.

Thiobarbituric acid reactive substance (TBARS) estimation in blood

The serum samples were analyzed for the lipid peroxidation by the method of Lowry *et al.* and Garcia *et al.* (Garcia *et al.* 2005; Lowry *et al.* 1951). In this method, 0.5 ml of the serum or brain homogenate samples and 2.5 ml TCA (20%), taken in the test tubes and centrifuged at 3500 rpm for 10 min. The supernatant was decanted and precipitate was washed once with 2.0 ml of 0.05M H₂SO₄. Washed precipitate was dissolved in 2.0 ml of the H₂SO₄ and 3.0 ml of thiobarbituric acid (0.68% TBA in Na₂SO₄) was added and vortexed. The tubes were incubated in boiling water bath for 30 minutes. The tubes were taken out and kept under tap water. To this chromogen 4.0 ml of n-butanol was added with vigorous shaking. Centrifugation was done at 3000 rpm for 10 min. The upper organic layer was separated and absorbance was taken at 530 nm on UV-spectrophotometer (UV 5740 SS, ECIL). The calculation was carried out using extinction coefficient and results were expressed in nM/ml.

Red cell superoxide dismutase (SOD) activity

The red cell SOD activity was measured by the method of Marklund *et al.* (Marklund *et al.* 1981). In this method red blood cells (RBC) were washed with the normal saline and haemolysate prepared by adding 3ml of cold distilled water. This was used for estimation of SOD. The amount of hemoglobin (Hb) in RBCs haemolysate was determined by cyanomethemoglobin method using Drabkin's reagents. 0.5 ml of RBCs haemolysate mixed with 3.0 ml of ice cold H₂O, 1.0 ml of ethanol and 0.6 ml of chloroform (CHCl₃), haemolysate mixed properly after each addition and finally shaken for 10 minutes, and centrifuge at 3000 rpm for 30 minutes. The supernatant used for SOD estimation by taking different aliquots. Add 100 µl pyrogallol in cuvette containing 2.7 ml tris HCl buffer, 100 µl ethylene diamine tetra acetic acid (EDTA) and 100 µl haemolysate. The increased in the absorbance at 420 nm is recorded on UV-Spectrophotometer (UV 5740 SS, ECIL) from 30 sec to 3 minutes, the lag of 30 sec/min allowed for steady state or auto-oxidation of pyrogallol to be attained. The allowance of this lag period is important for reproducibility of results. The concentration of pyrogallol was so adjusted that the rate of change of absorbance was approximately 0.020 to 0.023 per minutes. The increase in

the absorbance at 420 nm after addition of pyrogallol was inhibited by presence of SOD. One unit of SOD is defined as the amount of the enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 ml of assay mixture and results were expressed in U/gm Hb.

Red cell catalase (CAT) activity

Activity of this enzyme was carried out in red cell haemolysate using hydrogen peroxide as a substrate by the method of Marklund *et al.* (Marklund *et al.* 1981). A stock haemolysate was prepared by addition of four parts of ice cold triple distilled water to one part of washed red cells and frozen at -20 °C. A 1:500 of this concentrated haemolysate was prepared with potassium phosphate buffer (50 mM/L, pH 7.0) and immediately assay was performed. The assay was carried out by taking 2 mL of haemolysate in 3 mL cuvette and the reaction was started by addition of 1mL of 30 mM /L H₂O₂. Decrease in the absorbance was recorded at 240 nM for 30 seconds. The results were expressed as U/gm Hb.

Statistical analysis

The data were expressed as Mean $\pm$ SEM. For statistical significance data were analyzed by using one-way analysis of variance (ANOVA) followed by Newman-Keul post-hoc tests for multiple group comparison. A *p* value of at least 0.05 was considered as the level of significance in all statistical tests.

Results

Effect of melatonin in restraint stress (RS)-induced changes in elevated plus maze (EPM) test parameters and lipid peroxidation marker in brain

Acute restraint stress (RS) suppresses neurobehavioral activity, which has been confirmed by the results of EPM test. It is the most acceptable test for the neurobehavioral study for anxiety like syndrome. Rats subjected to RS for 1hr, show significantly reduced % number of open-arms entries and time spent on open arms as compared to vehicle treated non stressed control group (*p*<0.01). RS-induced effects were significantly reversed by pretreatment with melatonin (10 mg/kg) for five consecutive days as compared to vehicle + RS. Higher dose of melatonin (50 and 100 mg/kg) further reversed these RS-induced effects.

Results obtained by thiobarbituric acid reactive substances (TBARS) assay suggest that RS for 1 hr significantly elevates lipid peroxidation marker (MDA) levels in brain homogenate. The significant reduction in the % time spent on open arm entries and time spent in open arms in the EPM test parameters may be associated with free radical damaged induced by restraint stress which is confirmed by significant increase in MDA levels in brain of the stressed rats as compared with no stress group. Melatonin pretreatment with a dose of 10 mg/kg significantly reversed RS-induced elevated levels of MDA in brain homogenate. At higher doses of melatonin (50 and 100 mg/kg) clearly shows a significant reversal ($p < 0.001$) of RS-induced suppression of both % open arm entries and time spent on open arm in elevated plus maze (EPM) test parameters and brain MDA levels (Figure 1).

Effect of melatonin in RS induced changes in humoral and cell-mediated immunological changes in rats

Humoral and cell-mediated both types of immune responses to antigen are suppressed by Restraint stress (RS). In the present study, it was found that RS for 1hr daily for five consecutive days significantly reduces humoral immune response as evidenced by significantly suppressed anti-SRBC antibody titre in immunized animals as compared to non stress control group ($p < 0.01$). Significant reduction in secondary anti-SRBC antibody titre was also observed after RS (X5) but change in secondary antibody titre was not significant as compared to vehicle (no RS) treated group (data not shown). Further splenic plaque forming cell assay shows that RS (X 5) significantly suppressed antibody forming cells (PFC) counts (Table 1). In the cell-mediated immune responses RS (X5) significantly suppressed % change in paw edema as evidenced by the significant reduction in the paw volume in right hind foot with antigen challenge to normal saline challenged left hind foot group as compared to non stressed RS group (Figure-2). Further RS (X5) significantly suppressed the levels of Th₁ (IFN- γ) and Th₂ (IL-4) cytokine levels in the serum of the RS-exposed animals group as compared to controls (no RS) group (Figure-2). Melatonin (10 mg/kg) pretreatment for five consecutive days significantly reversed RS (X5)-induced reduction in (a) anti-SRBC antibody titre and (b) PFC counts (c) % change in footpad thickness (d) IFN- γ and IL-4 cytokine levels when compared with vehicle treated control animals groups. These RS (X5) induced changes in humoral

and cell mediated immune parameters were further significantly reversed with the higher doses (50 and 100 mg/kg) of melatonin pretreatment as compared with Vehicle + RS exposed group. At higher dose of melatonin (100 mg/kg) these RS-induced immunological changes were completely abolished. Data is represented in Table 1 and Figure 2.

Effect of melatonin on restraint stress (RS)-induced changes in oxidative stress markers in blood

Oxidative stress has been established as the one of the major reasons for various diseases. Restraint stress (RS) exposure for five consecutive days causes significant changes in oxidative stress markers in blood. RSX5 significantly increases lipid peroxidation product (MDA) as evidenced by increased TBARS activity in the serum ($p<0.01$). Significant decrease in the erythrocytes endogenous antioxidant enzymes SOD and CAT in stress exposed animal group when compared with vehicle (no RS) treated group was also observed. Pretreatment for five consecutive days with melatonin (10 mg/kg) significantly reverses these RS-induced changes in the oxidative stress markers as compared to Vehicle + RS treated group. Similarly pretreatment with higher doses of Melatonin (50 and 100 mg/kg) further reverses these RS-induced changes in oxidative stress markers. Pretreatment with higher doses of melatonin (50 and 100 mg/kg) significantly decrease MDA levels ($p<0.001$) as well as significantly reversed suppressed activities of antioxidant SOD and CAT. Melatonin pretreatment in a graded and dose dependent manner significantly reverses ($p<0.001$) decrease in levels of reduced glutathione (GSH) in RS-exposed group (Table 2).

Discussion

Restraint stress (RS) is widely used and most acceptable model to induced emotional stress in rodents. Results of our study shows that exploratory behavioral activity in elevated plus maze (EPM) test parameters are significantly suppressed by RS for 1 hr. EPM test is the widely used and most accepted model to study anxiety like behavior (Pellow et al. 1985). RS (X1) induced significant changes in % open arm entries as well % time spent in open arm and were significantly decreased as compared to vehicle treated no RS-group. The significant reduction in the EPM test parameters were associated with the significant increase in the brain lipid peroxidation marker as evidenced by significant

increased in the TBARS (MDA) levels in RS-exposed group which is in agreements with our earlier study (Pal et al. 2006). It might be possible that RS-significantly damage the brain cells due to rich polyunsaturated fatty acids (PUFA) and cause significant increase in lipid peroxidation damage during stress due to poor-antioxidant defense. Results of our study suggest involvement of the free radicals during restraint stress (RS)-induced changes in the neurobehavioral profile.

Melatonin significantly reversed such RS-induced changes in EPM test parameters. Melatonin (10 mg/kg) shows marginal protective effects in behavioral tests parameters in EPM model. Higher dose of melatonin (50 and 100 mg/kg), significantly reversed these RS-induced neurobehavioral changes. Various studies have reported that melatonin significantly reverses stress induced neurobehavioral changes in rats due to its free radical scavenging and anti-oxidant property (Pekarkova et al. 2001). Therefore, protective effect of melatonin in EPM test may not be due to its pharmacological property mediated through MT-2 receptor, but due to its free radical scavenging and antioxidant enzyme modulation properties (Ahmed et al. 2013; Allagui et al. 2014). The results of these studies are in agreement with our previous study in which we have demonstrated that treatment with L-Ascorbic acid significantly reduces stress induced changes in EPM test. Reversal of RS-induced changes in EPM parameters by ascorbic acid pretreatment suggests that free radical generation in central nervous system (CNS) during RS may be responsible for these stress induced changes (Pal et al. 2006). Further, to evaluate the role of free radical damage caused by restraint stress in the brain, the brain homogenate was assessed for lipid peroxidation parameters by TBARS method. It was found that RS for 1 hr. significantly increases MDA levels in brain. The lipid peroxidation marker (MDA) levels in brain was reversed to great extent by melatonin (10, 50 and 100 mg/kg) pretreatment in a dose dependent manner. RS-induced effects in EPM test parameters and oxidative stress markers in brain were totally abolished by the melatonin pretreatment by differential degrees in a dose dependent manner. These results thus obtained suggest that RS-induced changes may be associated with RS-induced oxidative stress in brain which may be associated with neurobehavioral suppression and melatonin has protective role in such stress induced neuromodulation.

RS (X5) exposure significantly suppress humoral immune responses, which is supported by decreased anti-SRBC antibody titre. It may be due to marked suppression of the antibody forming cells during stress. RS-significantly suppressed the splenic plaque forming cell (PFC) counts in Cunningham's chambers against SRBC antigen, which suggests that RS-may act at bone marrow levels and suppress lymphocyte formation. The markers of cell-mediated immune responses, DTH reaction and Th₁ and Th₂ cytokine levels were significantly suppressed in RS (X5) exposed group. RS-significantly suppressed percentage change in paw volume (footpad thickness). It may be due to the suppressed cell trafficking and inflammatory cytokines to the site of antigen challenge. Pretreatment with melatonin (10, 50 and 100 mg/kg) significantly reversed these RS-induced immunological changes in dose dependent manner. In humoral immune responses RS (X5) decreases anti-SRBC antibody titre and splenic plaque forming cells (PFC) counts. It may be due to the suppression of the lymphocytes formation in bone marrow mediated through cytokine regulation. Further RS (X5) significant suppresses cell-mediated immune markers as evidenced by significant decreased in % change in paw volume as well as Th₁ (IFN- γ) and Th₂ (IL-4) cytokine levels. Due to increase in pro-inflammatory cytokines predominantly of Th₁ subtypes, the regulatory (T_{reg}) cells secrete anti-inflammatory cytokines, such as IL-10 (Wu et al. 2012), resulting in the resolution of the inflammatory responses (Motilva et al. 2011). This secretion of IL-10 reduces the ability of antigen presenting cells to efficiently present antigen to T lymphocytes which in turn may inhibit induction of Th₁ mediated response thus maintaining the balance between Th₁/Th₂ subsets that are of decisive importance for immune responses. It was found that the Th₁ cytokine suppression was 24 % more as compared to Th₂. These RS-induced immunological changes may be associated with increased in the oxidative stress or decreased antioxidant levels during stress. Treatment with melatonin not only prevents suppression of immunological markers such as anti body titre and PFC counts it also restored them towards normalcy. The results of the immunological studies are in agreements with our previous study which indicates that role of free radical exists in the restraint stress (RS)-induced immunomodulation in rats (Pal et al. 2011).

The level of lipid peroxidation product malondialdehyde (MDA) significantly increased during stress and significant decrease in the endogenous antioxidant enzymes SOD and

CAT in the red blood cells and GSH in serum of the RS-exposed group. Pretreatment with melatonin (10, 50 and 100 mg/kg) significantly attenuated/reversed these RS-induced changes in oxidative stress markers in blood and lead to marked increase in the activity of superoxide radical ($O_2^{\cdot-}$) scavenging enzyme SOD and CAT as well as GSH levels. This supports that RS-induced immunosuppression may be associated with excessive generation of free radical and decreased antioxidant defense mechanisms during stress and such RS-induced immunosuppression not only prevented but also restored toward normalcy by melatonin pretreatment.

Conclusions: Results of the present study suggests that RS-induced neurobehavioral and immunological changes may be mediated through excessive free radicals and/or decreased antioxidant defense mechanism. The antioxidant property of the melatonin was utilized to protect stress-induced such neuro-immunomodulatory changes. Melatonin significantly restored both stress-induced suppressed behavioral and immune defense mechanisms. Results of our study suggest protective role of melatonin during stress-induced neuro-immunomodulation. Further studies are needed to understand its protective molecular mechanism in stress to establish its clinical uses in stress related disorders.

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Table 1
 Effect of melatonin in restraint stress (RS)-induced modulation of humoral and cell-mediated immunological markers in rats

Treatment (mg/kg, i.p)	Antibody titre (-log ₂)	PFC (x10 ⁶)	Change in paw volume (%)
Vehicle (no RS)	5.6±0.13	432±37.89	10.3±0.50
Vehicle+RS	3.4±0.42 ^a	280±29.8 ^a	5.1±0.64 ^a
MEL(10+RS)	4.3±0.14 ^b	325±28.2 ^b	8.3±0.87 ^b
MEL(50+RS)	5.7±0.10 ^b	375±51.4 ^b	12.1±0.88 ^b
MEL(100+RS)	6.3±0.32 ^c	425±50.9 ^c	14.3±1.07 ^b

All data are Mean±SE (n= 7 per group)
 RS: Restraint stress MEL: Melatonin
 a. *p*<0.001 (Compared to Vehicle)
 b. *p*<0.05 (Compared to Vehicle+RS)
 c. *p*<0.001 (Compared to Vehicle+RS)

Table 2

Effects of melatonin in restraint stress (RS)-induced modulation of oxidative stress markers of blood in rats.

Treatment (mg/kg i.p)	MDA (nM/L)	SOD (U/gm Hb)	CAT (U/gm Hb)	GSH (μ M/L)
Vehicle (no RS)	2.61 $\pm$ 0.13	846 $\pm$ 7.82	3690 $\pm$ 50.8	16.0 $\pm$ 0.62
Vehicle+RS	5.16 $\pm$ 0.22 ^a	450 $\pm$ 6.53 ^a	2425 $\pm$ 29.7 ^a	7.5 $\pm$ 0.49 ^a
MEL(10+RS)	3.12 $\pm$ 0.87 ^c	630 $\pm$ 8.14 ^b	3054 $\pm$ 35.8 ^c	12.6 $\pm$ 0.89 ^c
MEL(50+RS)	1.50 $\pm$ 0.18 ^b	912 $\pm$ 24.4 ^b	3296 $\pm$ 115 ^c	14.2 $\pm$ 1.02 ^c
MEL(100+RS)	0.90 $\pm$ 0.12 ^b	1014 $\pm$ 77.1 ^b	3710 $\pm$ 172 ^b	16.9 $\pm$ 3.95 ^b

All data are Mean $\pm$ SE ($n=7$ per group)

RS: Restraint stress MEL: Melatonin

a. $p<0.02$ (Compared to Vehicle)

b. $p<0.001$ (Compared to Vehicle+RS)

c. $p<0.05$ (Compared to Vehicle+RS)

Figure 1

Effect of melatonin on RS-induced neurobehavioral changes in EPM test and lipid peroxidation marker in brain

All data are Mean $\pm$ SE ($n=7$ per group)

RS: Restraint stress

MEL: Melatonin

a. $p<0.01$ (Compared to Vehicle)

b. $p<0.001$ (Compared to Vehicle+RS)

c. $p<0.05$ (Compared to Vehicle+RS)

Figure 2

Effect of melatonin on RS-induced changes in IFN- γ and IL-4 levels in blood

All data are Mean $\pm$ SE ($n=7$ per group)

RS: Restraint stress

MEL: Melatonin

a. $p<0.001$ (Compared to Vehicle)

b. $p<0.01$ (Compared to Vehicle+RS)

c. $p<0.05$ (Compared to Vehicle+RS)

Effect of melatonin on RS-induced neurobehavioral changes in EPM test and lipid peroxidation marker in brain

189x106mm (300 x 300 DPI)

Effect of melatonin on RS-induced changes in IFN-γ and IL-4 levels in blood

642x402mm (96 x 96 DPI)