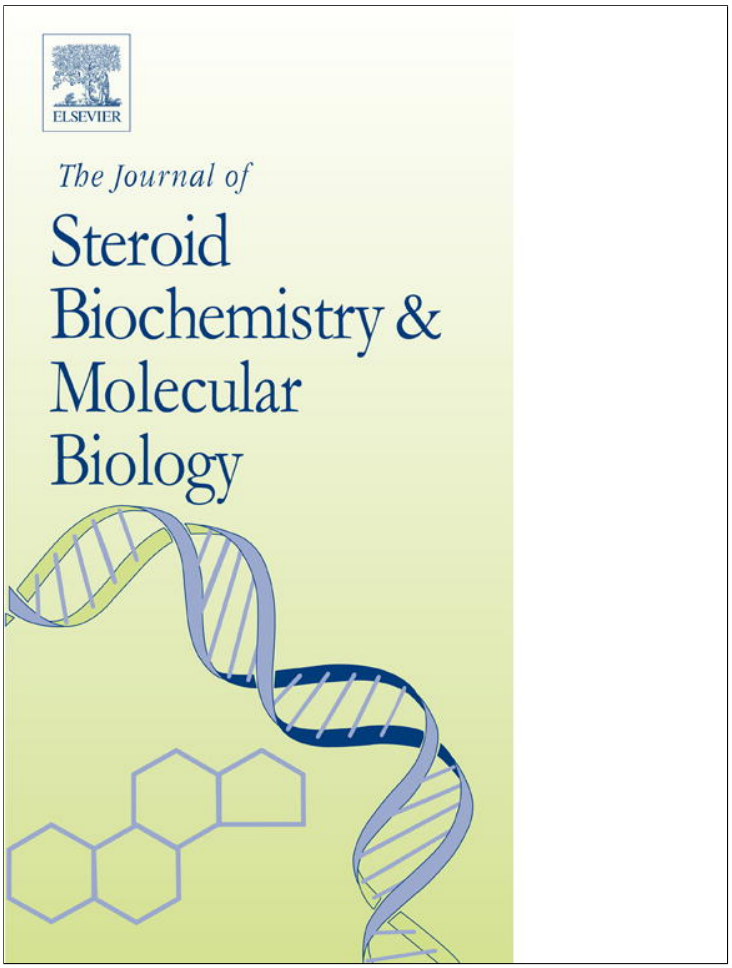

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Physiological crosstalk between melatonin and glucocorticoid receptor modulates T-cell mediated immune responses in a wild tropical rodent, *Funambulus pennanti*

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ABSTRACT

Immunoenhancing attributes of melatonin (Mel) on the immunocompromised state induced by glucocorticoid is well known, but the involvement of their receptors in the modulation of immunity has never been studied in any rodent. The present study explores the role of Mel and its receptors (MT1 and MT2) in amelioration of immunocompromised state induced by a synthetic glucocorticoid, dexamethasone (Dex) in a tropical rodent *Funambulus pennanti*. Immune parameters *viz*. DTH response, Lymphocyte proliferation, cytokine (IL-2) and antibody production were assessed following pretreatment of Mel and Dex alone or in combination. Mel enhanced the IL-2 production, thymic and splenic lymphocyte proliferation thereby increasing T helper cell associated immune responses and anti-KLH-IgG production. MT1 and MT2 receptor expression was downregulated following Dex treatment while glucocorticoid receptors (GR) expression was downregulated in Mel treated groups suggesting that the immunomodulatory effects of glucocorticoids and Mel are mediated *via* their receptors. To gain further insights on the role of Mel receptors, we used nonselective melatonin receptor antagonist luzindole which resulted in reversal of most of the immunomodulatory actions of Mel. Therefore, it may be suggested that a physiological cross talk exist between Mel and GR which is of high adaptive significance in wild animals for balancing the immunity during ecologically stressful conditions.

1. Introduction

Chronobiotic neurohormone melatonin plays a pivotal role in modulation of seasonal reproduction and immunity in mammals [1–4]. Being active at cellular level interacting with various functional proteins and scavenging free radicals, melatonin also acts *via* high affinity G protein coupled receptors (GPCRs). Till date two high affinity melatonin membrane receptors (MT1 and MT2) have been cloned and characterized in mammals [5–7]. These are ubiquitously located on immunocompetent cells and lymphoid tissues like thymus and spleen [8–10].

Glucocorticoid, especially corticosterone is a hallmark of stress in rodents which evokes adaptive physiological strategies and helps animal to cope up with stressful environmental conditions. Glucocorticoids exhibit their biological actions *via* two receptors, belonging to the nuclear hormone receptor superfamily, *viz.* glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). GR is low affinity glucocorticoid receptor and have two isomers/splicing variants. It is mainly active during the periods of abundant glucocorticoid secretion such as circadian peak or systemic inflammation [11,12]. In immune system glucocorticoid effects are primarily mediated by GR [13,14].

Melatonin and glucocorticoid acts in concert to integrate the seasonal changes at the level of immune system that are equipped with the melatonin and glucocorticoid receptors. In general glucocorticoids are known for immunoinhibitory effect on T cell and NK cell function there by hampering the immune status [14] while melatonin is well known for its immunoenhancing attributes [15]. Interaction between glucocorticoids and the immune functions have been explored mostly in relation to stress [16], and glucocorticoids are considered to mediate the immunosuppressive effects of stress. Therefore, the present study has been planned in wild seasonal breeder as they experience more stress in terms of ecofactors (extreme conditions of temperature, scarcity of food), social pressure and avoiding being killed by predators. Although, glucocorticoids are necessary for the maintenance of overall metabolism which ensures survival during periods of stress, their inhibitory and functionally suppressive effects on the immune system is considerably less understood.

Glucocorticoid studies are clinically relevant because synthetic glucocorticoids are used as potent anti-inflammatory and

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immunosuppressive agents for numerous functions associated with host defense [17]. Dexamethasone is one of the commercially available synthetic glucocorticoid which has a wide range of use in clinical conditions like chronic asthma [18], rheumatoid arthritis [19], and tissue/organ transplantation [20], which is based on empirical evidences of its efficacy, while surprisingly little is understood concerning the mechanisms by which glucocorticoids suppress immune function.

Earlier reports suggest that dexamethasone treatment *in vivo* altered lymphoid tissue mass, morphology and resulted in decreased circulating immune cell number in tropical squirrel *Funambulus pennanti* [21]. However, till date the receptor mediated modulation of immune function by melatonin and adrenal steroids has never been studied in any wild rodent. The present study explores the role of melatonin and its membrane receptors (MT1/MT2) in regulation of GR induced immunosuppression in a wild, tropical, seasonally breeding rodent *F. pennanti*. Furthermore, in order to delineate whether the antagonizing effect of melatonin on glucocorticoid induced stress on immune status is mediated by membrane bound melatonin receptor or is independently regulated by melatonin, we treated the splenocytes *in vitro* with luzindole, a nonselective melatonin receptor MT1/MT2 antagonist.

2. Materials and methods

All the experiments were conducted in accordance with Institutional practice and with in the framework of experimental animals (Scientific Procedure) Act 2007, of the Committee for the Purpose of Supervision and Control on Experiments on Animals (CPSCEA), Government of India, on animal welfare.

2.1. Animals and maintenance

The experiment was performed on healthy young adult male squirrels *F. pennanti* approximately of same age (as judged by their cranium diameter, incisor length and body weight (120 ± 5 g) as reported earlier) [22]. The experiment started in the last week of August and was continued till October. During this period the environmental day length in Varanasi (latitude 25°18′N and longitude 83°1′E) is approximately 12 h light and 12 h dark (Temp. minimum 28 °C – maximum 37 °C) and biotic and abiotic stress are minimum in nature. The squirrels were collected from the vicinity of Varanasi during the last week of August. The squirrels were weighed and kept in wire net cages ($25'' \times 25'' \times 30''$ in size) in a room having photoperiod of 12 light:12 dark and temperature 25 ± 2 °C for brief period of acclimatization for 10 days prior to treatment with dexamethasone. All the experimental protocols were continued for 45 days. After completion of treatments the animals were sacrificed during third week of October. During experiment the animals were fed with soaked gram seeds (*Cicer arietinum*), seasonal fruits, nuts and water *ad libitum*. The experimental groups were divided as follows:

2.2. Experimental plan I: in vivo

For *in vivo* experiment the squirrels were randomly divided into four groups each containing 15 animals ($n = 15$/group) and treatment given was as follows:

Group I: Vehicle (normal saline) treated (Con)
Group II: Melatonin treated (Mel); 25 μg/100 g body wt./day
Group III: Dexamethasone treated (Dex); 60 μg/100 g body wt./day
Group IV: Melatonin + Dexamethasone treated (Mel + Dex)

2.2.1. Drugs and treatments

Melatonin, dexamethasone, mitogen Concanavalin A (Con A), luzindole, oxazolone and keyhole limpet hemocyanin (KLH; antigen) were all purchased from Sigma–Aldrich Chemicals, St. Louis, MO, USA). Melatonin and dexamethasone were first dissolved in few drops of ethanol and then diluted with normal saline up to desired concentration and injected. Melatonin was injected subcutaneously during evening hours (between 17:30–18:00 h IST; approximately 1–1.5 h before sunset), dexamethasone was injected intramuscularly; the control animals were injected with the vehicle, normal saline in the same amount. All the injections/doses were selected as reported earlier [21,23], and were continued for 45 days. Experiment was performed in three sets. Second set of squirrels ($n = 5$) was subjected for delayed type hypersensitivity (DTH) response study while the third set ($n = 5$) was used for the study of humoral immune response in terms of anti-KLH-IgG production.

2.2.2. Sample collection

After 24 h of the last injection the squirrels were weighed and sacrificed under deep ether anesthesia inhalation. All the sacrifices were made during the night time between (20:00–22:00 h, IST). The blood was collected by cardiac puncture in heparinized tubes and centrifuged at 3000 rpm for 20 min at 4 °C. Plasma was kept at −20 °C till the hormonal and other humoral estimations were performed. Spleen and thymus were dissected out on ice, weighed and processed for the assay of blastogenic response in terms of lymphocyte proliferation and also a part of it was kept for receptor assay and other biochemical estimations.

2.2.3. Isolation and culture of Peripheral Blood Mononuclear Cells (PBMC)

The PBMC were isolated from heparinized blood collected by cardiac puncture ($n = 5$), by density gradient centrifugation, an adapted method of isolating mononuclear cells by Boyum [24]. Lymphocyte separation media was used according to manufacturer's instruction (HiSep™ LSM 1084, HiMedia, Mumbai, India). Briefly, the white band at the plasma-ficoll (Hisep, Himedia) interphase was collected and washed twice with PBS and finally suspended in RPMI 1640 (Himedia, India) supplemented with 10% fetal calf serum and 100 units of penicillin and streptomycin (Sigma Aldrich, USA). The cell concentration was adjusted to 1×10^7 viable cells/ml with RPMI 1640. MTT assay was performed as mentioned in the following section.

2.2.3.1. MTT assay for thymocyte, splenocyte and peripheral blood mononuclear cells (PBMC) proliferation. Cell-mediated immune function was assessed by measuring splenocyte, thymocyte and PBMC proliferation in response to the T-cell specific mitogen, Concanavalin A (Con A), using a colorimetric assay based on the reduction of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [25]. Spleen and thymus of squirrels were removed in sterile condition and a single-cell suspension was prepared by mincing and grinding them between sterile frosted glass slides. Erythrocytes were lysed by hypotonic shock using equal volume of cold ammonium chloride tris buffer (tris hydroxymethylene aminomethane, SRL, Mumbai, India); 0.5% tris buffer and 0.84% NH_4Cl mixed in 1:10 ratio; pH 7.2). This single cell suspension along with ice cold culture medium (RPMI-1640 supplemented with 1% penicillin (5000 U/ml) streptomycin (100 μg/ml), 1% L-glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5×10^{-2} M/ml), and 10% heat-inactivated fetal calf serum). The cell suspension was washed thrice. The cells were counted using a hemacytometer and viability was determined by trypan blue exclusion method. Viable cells (which exceeded 95%) were adjusted to 1×10^7 cells/ml in culture medium, and 100 μl aliquots of each cell

suspension were added to the wells of sterile flat-bottom 96-well culture plates. 50 μl/well Concanavalin A (Con A, Sigma–Aldrich, St. Louis, USA) was added to the culture medium at the concentration of 10 μg/ml to yield a final volume of 150 μl/well (each in duplicate). Finally 50 μl of complete medium was added to make the final volume of 200 μl/well (each in duplicate). Plates were incubated at 37 °C with 5% CO_2 for 69 h prior to addition of 10 μl of MTT (SRL, Bombay, India; 5 mg/ml in phosphate buffered saline) per well followed by an additional incubation for 4 h. At 72 h, 150 μl of acidified propanol (0.04 mol/l HCL in isopropanol) was added to each culture and the optical density (OD) of each well was determined with a microplate reader (ELx-800, Biotek Instruments, Winooski VT, USA) equipped with a 570 nm wavelength filter. Mean OD values for each set of duplicates were used in subsequent statistical analyses. Response was calculated as percent stimulation ratio (%SR) representing the ratio of absorbance of mitogen stimulated cultures to control cultures [23].

2.2.4. Western blot analysis for MT1, MT2 and GR

The western blot analysis was performed according to method published elsewhere [10]. Briefly, the spleen and thymus were dissected out in chilled PBS. PBMC were separated from the blood of the squirrels as described in previous section. Tissue/cells were homogenized and lysed in RIPA buffer containing aprotinin, sodium orthovanadate and phenyl methylsulphonyl fluoride (PMSF). The protein content of the lysates were quantified using the Bradford method [26]. The aliquots containing 70 μg of protein for lymphoid organs and 40 μg protein for PBMC was resolved on 12% SDS-polyacryl-amide gel electrophoresis (PAGE) for melatonin membrane receptor MT1 and MT2 and 10% SDS-PAGE for GR followed by electrotransfer (Biometra, Germany) on nitrocellulose (Boiscience, Keene, NH, USA) for 1 h. Nitrocellulose membranes were blocked for 60 min in Tris-buffered saline (TBS; Tris 50 mM, pH 7.5, NaCl 150 mM) containing 5% fat free dry milk and were incubated with melatonin receptor MT1 and MT2 and glucocorticoid receptor (GR) antibodies (Mel1aR, R-18, Mel1bR, T-18, and GR, M-20, sc 1004, Santa Cruz Biotechnology, Santa Cruz, CA, USA). All the antibodies *i.e.* MT1, MT2 and GR were used at a dilution of 1:200. The membranes were then washed thrice with TBS. The immunodetection was carried out using horseradish peroxidase (HRP) conjugated secondary antibody (donkey antigoat IgG-HRP for melatonin receptors and donkey antirabbit IgG-HRP for GR; diluted 1:1000). Finally, the blots were washed thrice with TBS and developed with Super Signal West Pico Chemiluminescent substrate (34080, Thermo Scientific, USA). Further, the nitrocellulose membranes were then stripped with stripping buffer (10% sodium azide) and were immunostained with β-actin antibodies in 1:1000 dilutions (A-2228; Sigma–Aldrich, USA) as internal loading control. Immunodetection of β-actin was performed with anti-mouse IgG-HRP (1:1000). Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation). Values were expressed as the ratio of the density of the specific signal to the β-actin signal [10]. The ratio of density was calculated with respect to β-actin (house-keeping gene) and expressed as % band intensity of MT1, MT2 and GR.

2.2.5. Radioimmunoassay (RIA) of melatonin

The radioimmunoassay (RIA) of melatonin was performed following the method of Rollag and Niswender [27] using Guildhey antisera (Guildhey, Surrey, UK). Details of the method and validation have been published elsewhere [28]. The intra- and inter-assay variation for melatonin was 9% and 15%, respectively. The sensitivity for melatonin RIA was 18–20 pg/ml. The recovery of melatonin RIA was 92%.

2.2.6. Corticosterone ELISA

Corticosterone was measured using an ELISA kit (kind gift from Prof. T. G. Srivastava, National Institute for Health and Family Welfare, New Delhi, India) according to the manufacturer's instructions. Plasma samples were thawed, and applied to a microplate. Following incubation, the plates were read on a microplate reader at 405 nm, and values were determined by extrapolation from a standard curve. The recovery, accuracy, and sensitivity of the corticosterone assay were 95%, 99.1%, and 0.27 μg/dl, respectively. Inter- and intra-assay variation was 3.38–5.56% and 5.69–7.84%, respectively. All samples were assayed in a single lot and samples, standards, and replicates were assayed in duplicate.

2.2.7. ELISA for IL-2

Sandwich ELISA was performed to quantify plasma level of IL-2 in all the four groups according to manufacturers instruction (Immunotech, France) Intra assay variation was between 3.3 and 7.2% and inter assay variation was between 6.2 and 8.2%; sensitivity; 5 pg/ml and recovery was between 80 and 132%.

2.2.8. Delayed-type hypersensitivity (DTH) response

After 45 days of treatment, DTH was induced by application of antigen oxazolone (Sigma, St Louis, MO, USA) to the ear pinna of the squirrel. Following the initial immunization by applying 100 μl of Oxazolone [5% (w/v) in 4:1, acetone:olive oil] for two consecutive days to shaved dorsal region. On day 6 the baseline ear thickness was measured using vernier calliper and oxazolone challenge was induced by applying 50 μl of oxazolone [0.5% (w/v) in 4:1, acetone:olive oil] to the skin of the dorsal surface of right ear pinna. Left ear pinna was treated with the vehicle solution alone. Ear swellings were measured for three consecutive days to record the maximum DTH response which eventually was observed on the 2nd day. The DTH response was expressed as change in percent thickness and was measured by comparing differences between oxazolone treated and vehicle treated ear pinna [3,29].

2.2.9. Anti-KLH-IgG estimation for humoral immune status

The anti-KLH-IgG estimation assay was performed following the method of [30], with some modifications which has been published elsewhere [29]. Briefly, 24 h after the last injection the third experimental set of the squirrels received single subcutaneous injection of 150 μg of KLH (Sigma–Aldrich) in 0.1 ml of sterile normal saline (0 day). The squirrels were sacrificed and trunk blood was collected on the days 5 and 10 (post immunization). These periods were chosen to capture basal (5 days) and peak (10 days) levels of IgG. The blood was allowed to clot; after 1 h, the clots were removed and samples were centrifuged (at 4 °C) for 30 min at 700 × *g*; serum aspirated and aliquots were stored at −20 °C until assayed for anti-KLH-IgG.

2.2.9.1. ELISA for anti-KLH-IgG. The humoral immune status was assessed by measuring serum anti-KLH-IgG concentrations using ELISA [29,30]. In brief, the microtiter plates were coated with KLH antigen (0.5 mg/ml in sodium bicarbonate buffer) by overnight incubation at 4 °C. Next day the plates were washed with phosphate buffered saline containing 0.05% Tween 20 (PBST), blocked with 0.5% non-fat dry milk in PBST overnight at 4 °C and washed again with PBST. The serum samples were diluted 1:50, 1:100, 1:200, 1:400 and 1:800 in PBST, and finally 150 μl of each serum dilution was loaded in duplicate in antigen coated microtiter plate. Similarly the positive control (squirrels those have prior been exposed to KLH showing robust immune reaction) and negative control samples (squirrels never exposed to KLH) were diluted and loaded in wells in duplicate. The plates were sealed and incubated at 37 °C for 3 h followed by PBST wash. Secondary antibody (alkaline phosphatase conjugated, anti-mouse IgG; SC-2320, Santa Cruz Biotec, USA) was added to each well in a dilution of 1:500; and plates were

incubated again at 37 °C for 1 h followed by washing with PBST. 150 μl of *p*-nitrophenyl phosphate (enzyme substrate) 1 mg/ml in diethanolamine buffer (Sigma–Aldrich) was added to each well and the plates were kept in dark, the enzyme-substrate reaction was terminated by adding 50 μl of 1.5 M NaOH to each well. The optical density (OD) of each well was determined using a microplate reader equipped with a 405 nm filter (ELx-800, Biotek Instruments, Winooski, VT, USA) and average OD was calculated for each sample in duplicate. The mean for each sample was calculated and expressed as a percentage of the positive control mean (% plate positive).

2.3. In vitro study

For *in vitro* study five squirrels were sacrificed ($n = 5$), splenocyte (and thymocyte) culture was performed in two experimental groups with four and five sets respectively each with quadruplicate. The groups were as follows.

2.3.1. Experimental set I: splenocytes and thymocytes culture

Group I – Control (Con)
Group II – Melatonin supplemented (Mel; 500 pg/ml)
Group III – Dexamethasone supplemented (Dex; 2 μM)
Group IV – Melatonin + Dexamethasone co supplementation (Mel; 500 pg/ml + Dex; 2 μM)

2.3.2. Experimental set II: splenocytes culture

In order to find out the role of melatonin *via* melatonin receptors if any in alleviation of glucocorticoid induced immunocompromised state splenocytes were cultured under five different groups.

Group I – Control (Con)
Group II – Dexamethasone supplemented (Dex); 2 μM
Group III – Dexamethasone + Luzindole (Dex; 2 μM + Luz; 5 μM)
Group IV – Mealtonin + Dexamethasone co supplementation (Mel; 500 pg/ml + Dex; 2 μM)
Group V – Melatonin + Dexamethasone + Luzindole (Mel + Dex + Luz)

Splenocyte and thymocytes proliferation was assessed using MTT assay method [21]. Further, immunoblot analysis of the cultured splenocytes and thymocytes of experimental set I for melatonin receptor and GR was carried out and culture supernatants were also assessed for IL-2 production in all the groups. For experimental set II only %SR and GR expression were monitored in splenocytes following treatment.

2.3.3. MTT assay for splenocyte and thymocyte proliferation

The cells were isolated as explained in the previous section. The viable cells (which exceeded 95%) were adjusted to 1×10^7 cells/ml with culture medium and 50 μl of cell suspension was added to the wells of sterile flat bottom, 96 well culture plate. Con A (Sigma Aldrich) was added to the culture medium at a concentration of 10 μg/ml and finally 50 μl of the mitogen was added to yield a final volume of 100 μl/well each in quadruplicate. Melatonin, dexamethasone and luzindole were all dissolved in absolute ethanol and then diluted with RPMI-1640 to standard stock solution to be used. One hundred microliters of dexamethasone (2 μM) or melatonin (500 pg/ml) or 50 μl of each dexamethasone, melatonin and luzindole at a concentration of 5 μM was added to each well ultimately to yield a final volume of 200 μl/well (each in quadruplicate). The control cultures comprised of the same sets of treatment except for Con A. Melatonin, dexamethasone, Con A and luzindole were added to the plates prior to the addition of cells. Plates were incubated in humidified CO_2 incubator at 37 °C. Harvesting of cultures for having final OD were processed as described in the earlier *in vivo* section of the manuscript.

2.3.4. Western blot analysis of splenocytes and thymocytes

The splenocytes (and thymocytes) were collected under sterile conditions using routine method as explained in previous sections. The cells were suspended in RPMI-1640 at a concentration of 1×10^7 cells/ml. Then, 100 μl of cell suspension, 100 μl of melatonin (500 pg/ml), dexamethasone (2 μM) or both and RPMI-1640 medium were added to each well of a 24-well culture plate to make a final volume of 1 ml. For antagonist part luzindole (5 μM), melatonin, dexamethasone or all the components along with RPMI-1640 were added to make up the final volume to 1 ml. After 48 h of incubation at 37 °C with 5% CO_2 the cells were collected and centrifuged at 2500 rpm for 10 min. The cell pallet was washed twice with PBS (0.1 M, pH 7.4) and finally the homogenate was made using lysis buffer and protein content in the cell lysates was quantified. Aliquots containing 30 μg of protein were resolved on 12% SDS-PAGE for MT1 and MT2 and 40 μg aliquots were resolved on 10% SDS-PAGE for GR. Further details regarding western blot has already been given in the previous section of the manuscript.

2.3.5. IL-2 production and analysis in culture supernatant

IL-2 level was estimated from culture supernatant of activated splenocytes and thymocytes. Splenocytes and thymocytes were collected by a routine method under sterile condition as explained above. The cells were suspended in RPMI-1640 medium at a concentration of 1×10^7 cells/ml. Then, 100 μl of cell suspension, 100 μl of melatonin (500 pg/ml), 100 μl of dexamethasone (2 μM) or both, luzindole (5 μM; [19]), 100 μl of Con A with a final concentration of 10 μg/ml and RPMI-1640 medium were added to each well of a 24-well culture plate to make a final volume of 1 ml in each well. The cultures were centrifuged (500 × *g*, 10 min) after incubation at 37 °C with 5% CO_2 for 48 h. Then, the supernatants were collected and preserved at −20 °C for IL-2 estimation. Sandwich ELISA was performed to quantify the level of IL-2 in culture supernatants according to the manufacturer's instructions (Immunotech, France).

3. Statistical analyses

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

4. Results

4.1. In vivo study

4.1.1. Lymphocyte proliferation index of spleen, thymus and PBMC

Percent stimulation ratio (%SR) representing the change in cell mediated immune status (T-helper cells) in response to a T cell specific mitogen Concanavalin A challenge, presented a significant ($p < 0.01$) increase in splenocyte, thymocyte and PBMC proliferation following melatonin treatment, when compared with saline treated control groups. A significant decrease ($p < 0.01$) was observed in splenic and thymic lymphocyte proliferation along with PBMC following dexamethasone treatment. Melatonin treatment along with dexamethasone however, significantly ($p < 0.01$) increased splenocytes, thymocytes and PBMC proliferation compared to dexamethasone treated group (Fig. 1A).

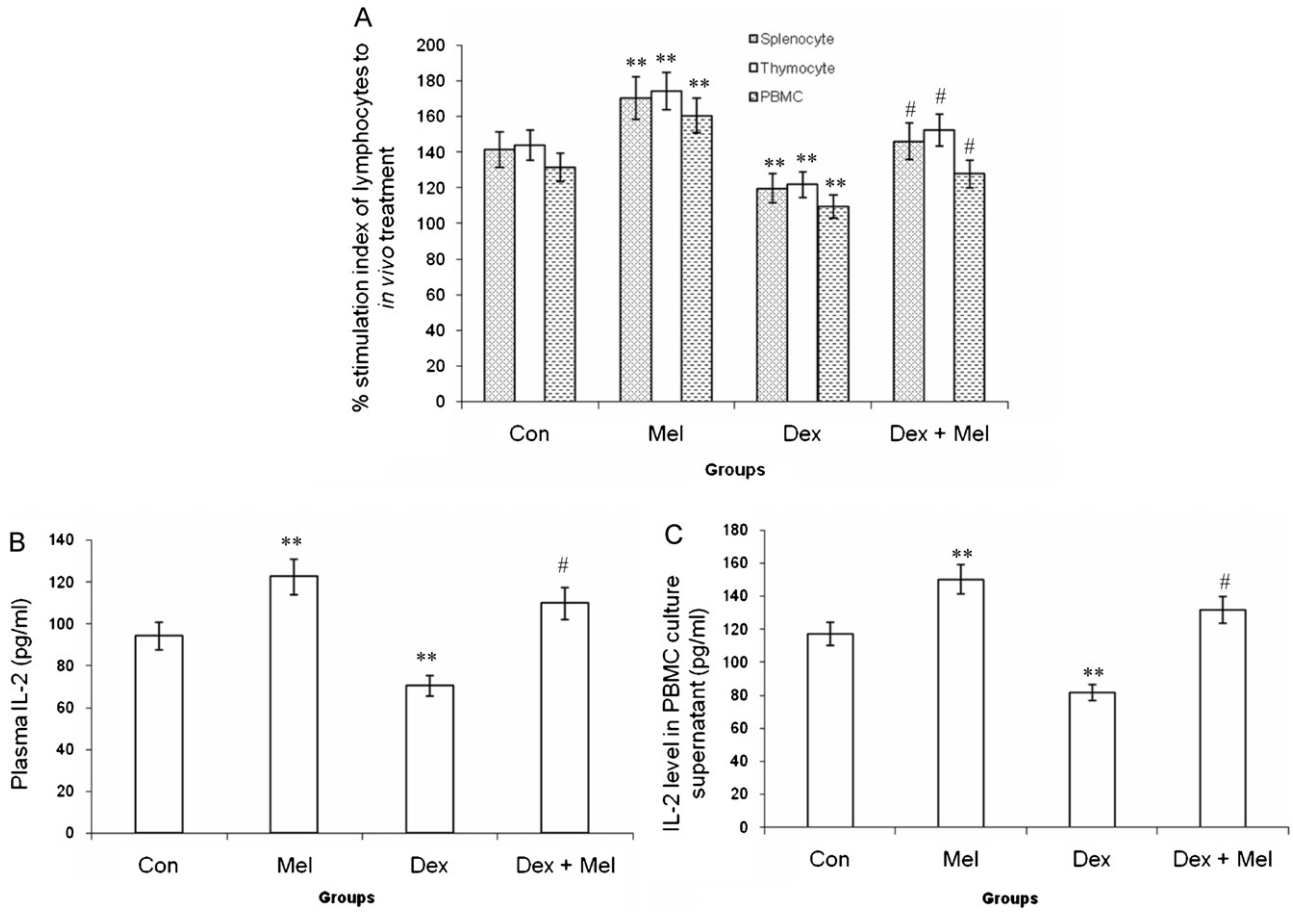

Fig. 1. Effect of melatonin and dexamethasone treatment on proliferation of lymphocyte and mononuclear cells stimulated with Con A. (A) Blastogenic response (%SR) of splenocytes, thymocytes and PBMC following *in vivo* melatonin and dexamethasone administration. IL-2 levels in plasma (B) and in culture supernatant of PBMC (C) of Con, Mel, Dex and Mel + Dex treated *F. pennanti*. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* Mel + Dex.

4.1.2. Western blot analysis

To check the expression of melatonin receptor subtypes (MT1 and MT2) and GR in lymphoid tissues *i.e.* spleen and thymus of *F. pennanti* at translational level, Western blot analysis was performed. Melatonin receptor protein was detected as a single band between 35 and 40 kDa corresponding to the reported molecular mass of the receptor protein [10,23,31]. Single band for GR protein was detected between 80–100 kDa in the lymphoid tissues.

Following the *in vivo* administration of melatonin and dexamethasone injections, both the melatonin membrane receptors (MT1 and MT2) presented similar expression pattern in spleen and thymus. A significant ($p < 0.01$) increase was observed in MT1 and MT2 receptor expression following melatonin injection in both spleen and thymus when compared to vehicle treated control group. Dexamethasone treatment, on the other hand significantly ($p < 0.01$) decreased the melatonin membrane receptor MT1 and MT2 expression in spleen and thymus. Melatonin treatment along

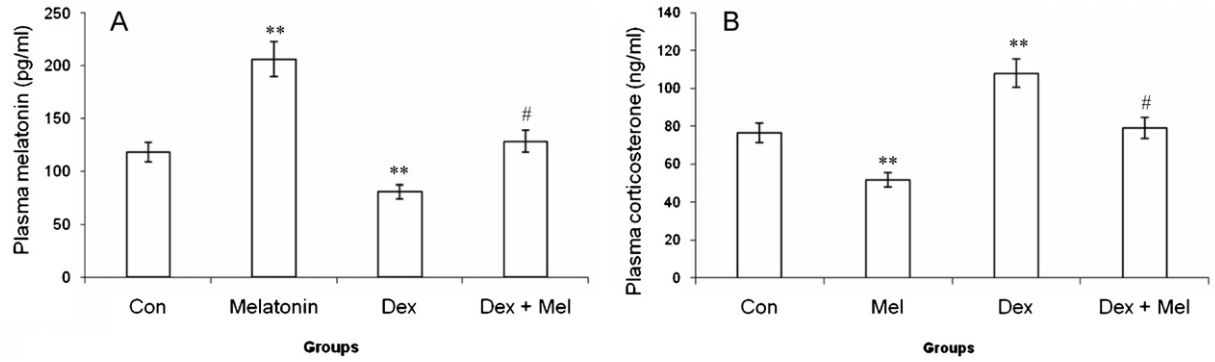

Fig. 2. Effect of melatonin and dexamethasone treatment on plasma melatonin and corticosterone concentration. (A) Plasma melatonin (pg/ml) and (B) corticosterone level (ng/ml) following *in vivo* treatment of Mel, Dex and Mel + Dex in *F. pennanti*. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* Mel + Dex.

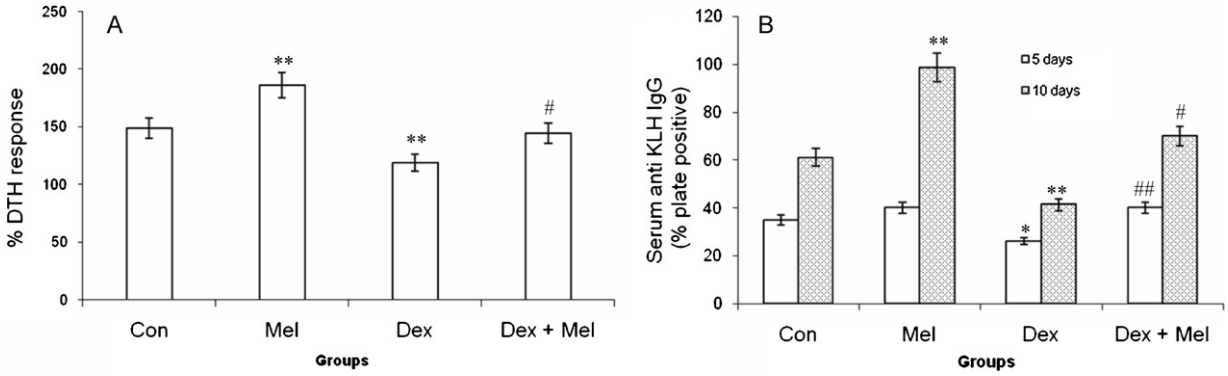

Fig. 3. Effect of melatonin and dexamethasone treatment on T cell mediated immune responses. (A) %DTH response to oxazolone application and (B) anti-KLH-IgG titer (as % plate positive) in Con, Mel, Dex, and Mel + Dex treated groups of *F. pennanti*. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{*}p < 0.05$, (5 days) Con *vs.* all experimental groups; $^{\#\#}p < 0.05$, (5 days) Dex *vs.* Mel + Dex; $^{**}p < 0.01$, (10 days) Con *vs.* all experimental groups and $^{\#}p < 0.01$, (10 days) Dex *vs.* Mel + Dex.

with dexamethasone significantly ($p < 0.01$) restored the melatonin membrane receptor (MT1 and MT2) expression in both spleen and thymus when compared to dexamethasone treated squirrels (Fig. 4A–D).

The PBMC isolated from the blood of the squirrels treated with melatonin and dexamethasone injections were also subjected to Western blot analysis. The melatonin membrane receptor MT1 and MT2 presented significantly ($p < 0.01$) higher expression in PBMC isolated from the melatonin treated group; while significant ($p < 0.01$) decrease in expression of MT1 and MT2 was observed in dexamethasone treated group. Combined treatment of both melatonin and dexamethasone caused significant ($p < 0.01$) increase in MT1 and MT2 expression when compared to dexamethasone group suggesting that melatonin significantly restored the melatonin receptor expression when given in combination with dexamethasone (Fig. 4E and F).

Glucocorticoid receptor (GR) expression was significantly ($p < 0.01$) decreased in lymphoid organs *i.e.* spleen and thymus of melatonin treated groups while dexamethasone treatment significantly ($p < 0.01$) increased the GR expression when compared with vehicle treated control group. Melatonin in combination with dexamethasone treatment significantly ($p < 0.01$) decreased the GR expression in spleen and thymus of *F. pennanti* compared to dexamethasone treated squirrels (Fig. 5A–C).

Melatonin alone and in combination with dexamethasone significantly ($p < 0.01$) decreased GR expression in the PBMC isolated from the treated groups compared to control and dexamethasone treated group respectively. Squirrels treated with dexamethasone showed significant ($p < 0.01$) increase in GR expression in the PBMC when compared with the vehicle treated control group (Fig. 5D and E).

4.1.3. Radioimmunoassay (RIA) of melatonin

The plasma melatonin levels were found to be significantly ($p < 0.01$) elevated in melatonin treated group when compared to vehicle treated control group. Dexamethasone treatment resulted in significant ($p < 0.01$) decrease in the plasma melatonin levels when compared with control. Melatonin administration along with dexamethasone however significantly ($p < 0.01$) increased the plasma melatonin level when compared to dexamethasone treatment alone (Fig. 2A).

4.1.4. Corticosterone ELISA

A significant ($p < 0.01$) decline was observed in plasma corticosterone levels in melatonin treated group of squirrels when compared to control. Dexamethasone, on the other hand significantly ($p < 0.01$) increased the corticosterone levels while melatonin in concert with dexamethasone significantly ($p < 0.01$) decreased plasma corticosterone levels when compared with dexamethasone treated group (Fig. 2B).

4.1.5. ELISA for IL-2

Plasma IL-2 level was found to be significantly increased in melatonin treated group when compared with control group while dexamethasone administration significantly ($p < 0.01$) decreased the IL-2 level. Melatonin in combination with dexamethasone significantly ($p < 0.01$) increased plasma IL-2 level when compared with dexamethasone treated group (Fig. 1B).

The estimation of IL-2 production from PBMC isolated from pretreated groups presented almost a parallel profile as plasma level of IL-2. PBMC isolated and cultured from melatonin treated squirrels showed significantly ($p < 0.01$) high IL-2 level in the culture supernatant when compared with vehicle treated control group while dexamethasone treatment resulted in significantly ($p < 0.01$) less production of IL-2. Melatonin in combination with dexamethasone significantly ($p < 0.01$) increased IL-2 production when compared to dexamethasone treatment alone.

4.1.6. Delayed-type hypersensitivity (DTH) response

Significant increase ($p < 0.01$) in % DTH response was observed in squirrels pretreated with melatonin when compared with the vehicle treated control group squirrels. Dexamethasone treatment on the other hand, resulted in significant ($p < 0.01$) suppression of the DTH response compared with control group. Melatonin pretreatment along with dexamethasone significantly ($p < 0.01$) increased the DTH response when compared with the dexamethasone pretreated groups (Fig. 3A).

4.1.7. Anti-KLH-IgG estimation for humoral immune status

Serum anti-KLH-IgG was measured twice during the post immunization phase *i.e.* after 5 and 10 post immunization days. Anti-KLH-IgG titer was found to be significantly ($p < 0.01$) increased at 10 days compared to 5 days. Bleed after 5 post immunization days, a significant ($p < 0.05$) decrease was observed in dexamethasone treated group when compared with the control. Melatonin and dexamethasone co-treatment resulted in significantly ($p < 0.05$) high antibody titer when compared with dexamethasone treatment alone. Measured after 10 post immunization days, melatonin treatment significantly ($p < 0.01$) increased the serum anti-KLH-IgG while dexamethasone administration significantly decreased the serum anti-KLH-IgG titers compared to vehicle treated squirrels. Melatonin along with dexamethasone significantly ($p < 0.01$) increased the anti-KLH-IgG titer compared with the dexamethasone treated squirrels (Fig. 3B).

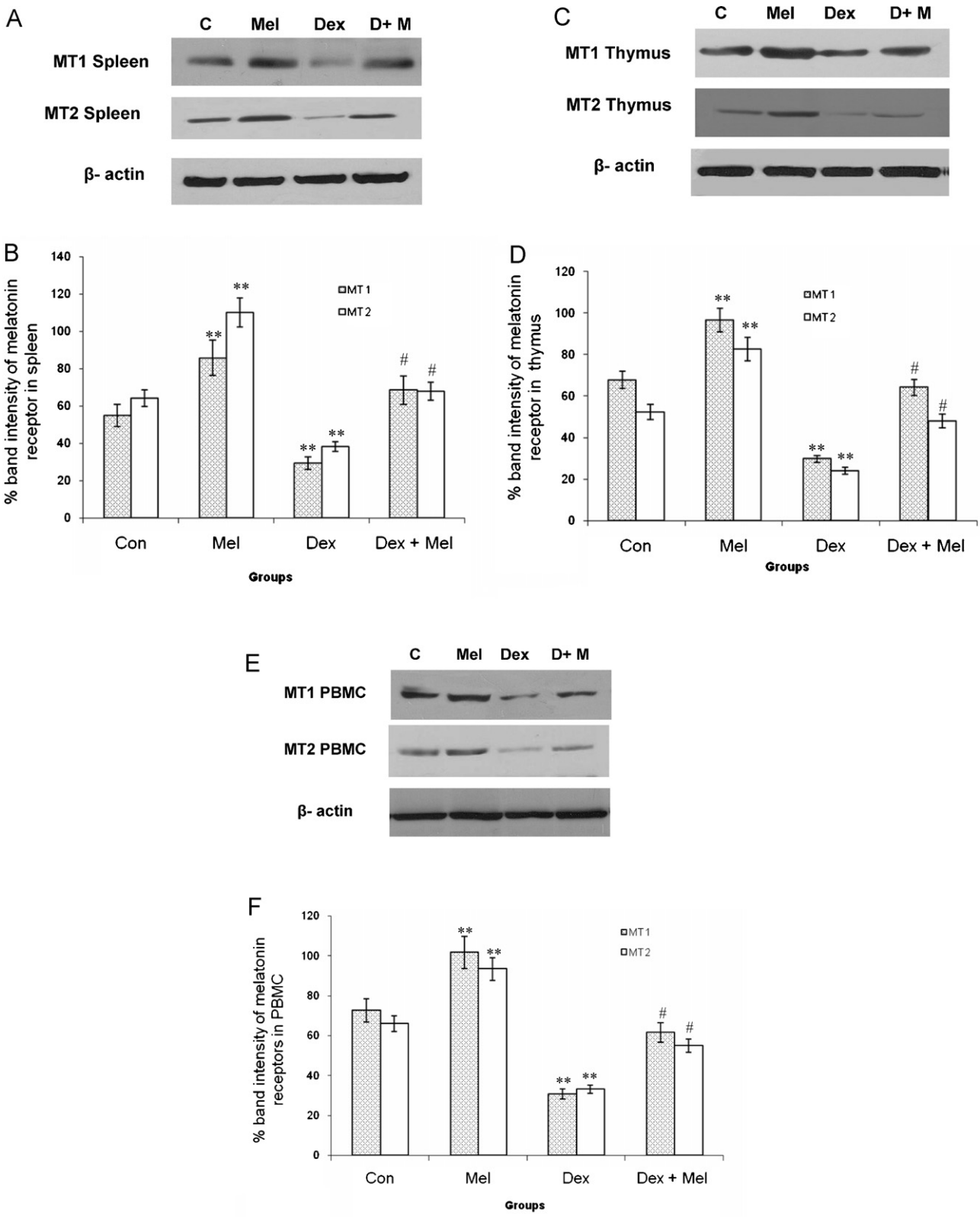

Fig. 4. Effect of melatonin and dexamethasone treatment on melatonin membrane receptor MT1 and MT2 expression. Western blot analysis of MT1 and MT2 in (A) spleen (C) thymus and (E) PBMC. The data are expressed as % band intensity of receptor expression in (B) spleen (D) thymus and (F) PBMC in Con, Mel, Dex, and Mel + Dex treated groups of *F. pennanti*. β-Actin expression was used as loading control. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* Mel + Dex.

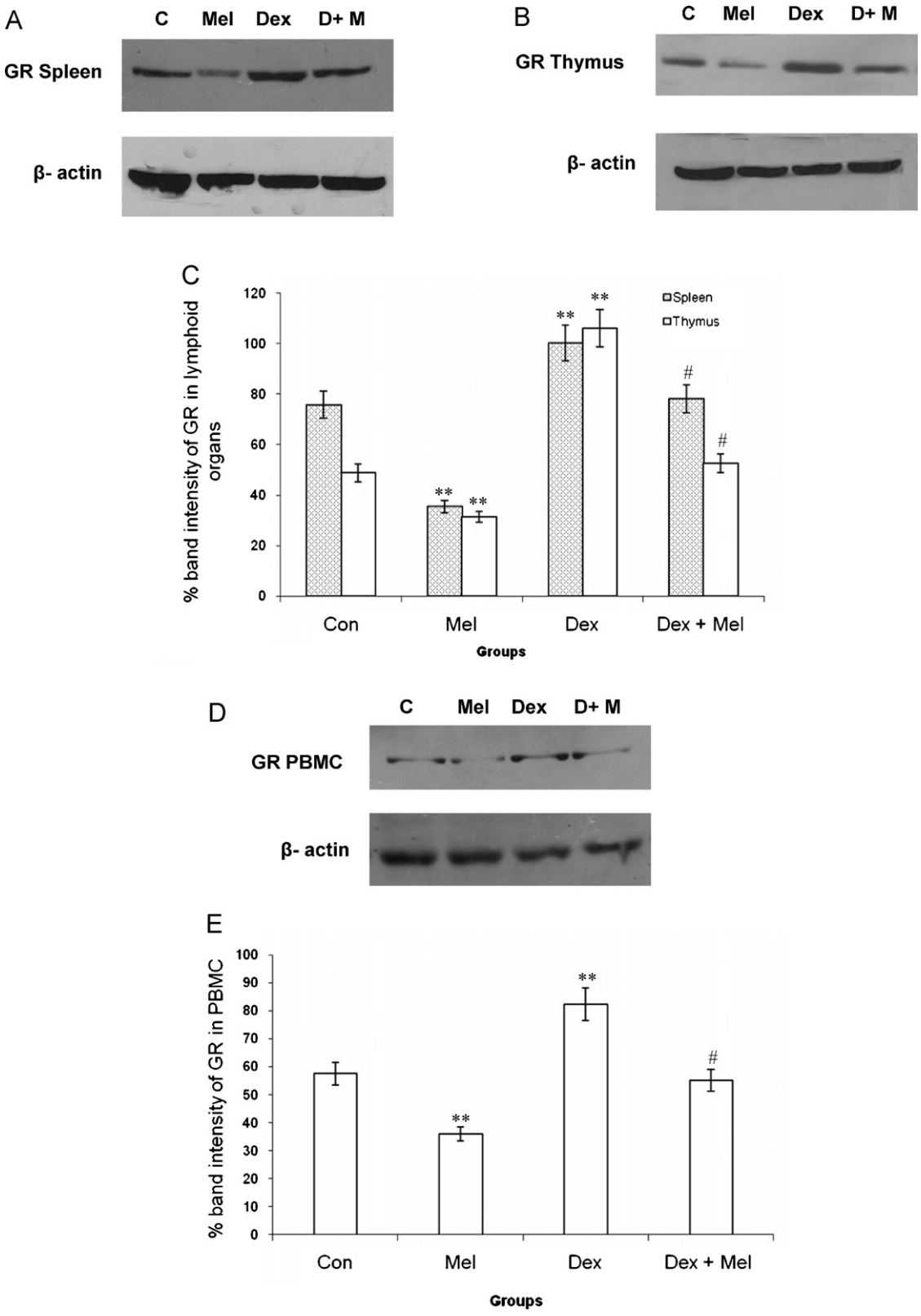

Fig. 5. Effect of melatonin and dexamethasone treatment on Glucocorticoid receptor (GR) expression. Western blot analysis of GR in (A) spleen (B) thymus and (D) PBMC. The data are expressed as % band intensity of GR expression in (C) lymphoid organs (spleen and thymus) and (E) PBMC in Con, Mel, Dex, and Mel + Dex treated groups of *F. pennanti*. β-Actin expression was used as loading control. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* Mel + Dex.

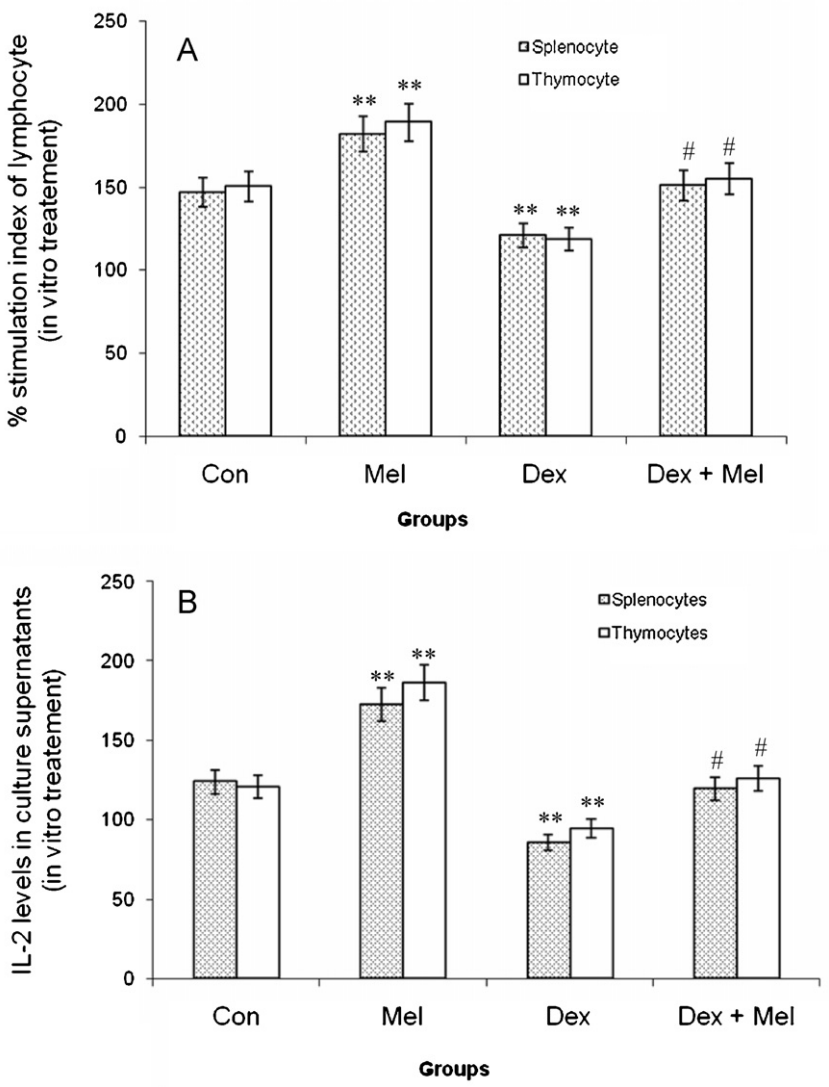

Fig. 6. Effect of *in vitro* melatonin and dexamethasone supplementation on splenic and thymic lymphocytes stimulated with Con A. (A) Blastogenic response (%SR) of splenocytes and thymocytes (B) IL-2 concentration in culture supernatant of splenocytes and thymocytes after *in vitro* supplementation with Mel, Dex, and Mel + Dex. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* Mel + Dex.

4.2. Results: in vitro study

4.2.1. Splenocyte and thymocyte proliferation

Splenocyte and thymocyte proliferation measured in terms of % stimulation ratio was increased significantly ($p < 0.01$) following melatonin supplementation, while dexamethasone decreased it significantly ($p < 0.01$) when compared with the control plates. Co-supplementation of melatonin along with dexamethasone significantly ($p < 0.01$) increased the splenocyte and thymocyte proliferation when compared with dexamethasone supplemented plates suggesting for the restorative effects of melatonin on the Con A challenged splenic and thymic lymphocyte proliferation (Fig. 6A).

4.2.2. ELISA of IL-2 in culture supernatant

IL-2 levels in the culture supernatant followed the pattern of splenic and thymic lymphocyte proliferation in response to *in vitro* treatment. Melatonin supplementation significantly ($p < 0.01$) increased the IL-2 level in culture supernatant when compared with control group while, dexamethasone supplementation significantly ($p < 0.01$) decreased the IL-2 production. Supplementation of melatonin along with dexamethasone significantly increased ($p < 0.01$) the IL-2 level in the culture supernatants when compared to dexamethasone supplemented groups (Fig. 6B).

4.2.3. Western blot analysis of splenocytes and thymocytes

Melatonin membrane receptor MT1 and MT2 expression was observed to significantly increase ($p < 0.01$) in both splenocytes and thymocytes supplemented with melatonin when compared with the control group. Dexamethasone however, significantly ($p < 0.01$) reduced and down regulated the MT1 and MT2 receptor expression in cultured splenocytes and thymocytes compared to control. Melatonin supplementation with dexamethasone significantly ($p < 0.01$) upregulated the MT1 and MT2 receptor expression when compared with dexamethasone alone treated group (Fig. 7A–D).

Gulcocorticoid receptor expression on the other hand decreased significantly ($p < 0.01$) following the melatonin supplementation in splenocyte as well as thymocytes when compared with control. Dexamethasone supplementation however, significantly ($p < 0.01$) increased the GR expression. Co-supplementation of melatonin along with dexamethasone resulted in significant ($p < 0.01$) decrease in the expression of GR in both splenocyte and thymocyte when compared with the dexamethasone treated groups (Fig. 8A–C).

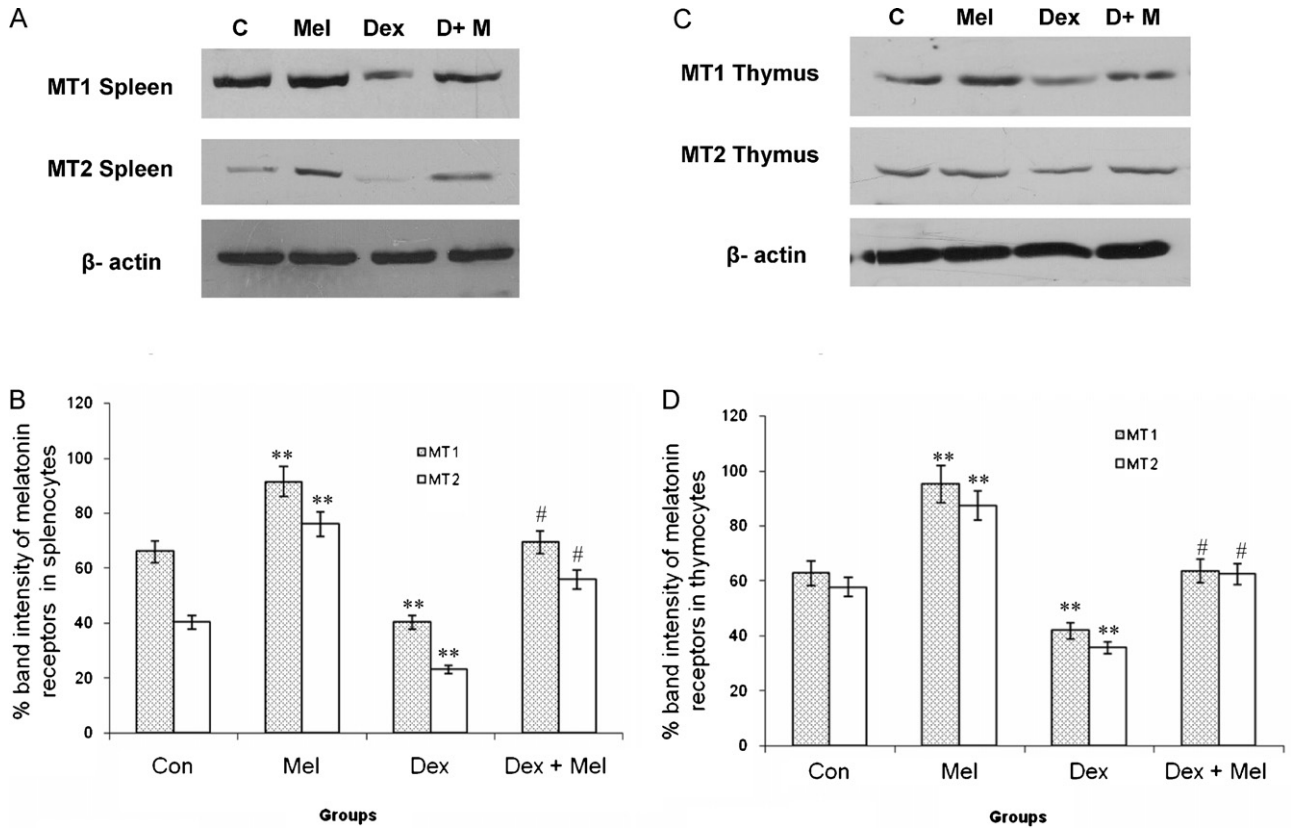

Fig. 7. Effect of *in vitro* melatonin and dexamethasone supplementation on melatonin membrane receptor MT1 and MT2 expression. Western blot analysis of MT1 and MT2 in (A) splenocytes and (C) thymocytes. The data are expressed as % band intensity of receptor expression in (B) splenocytes and (D) thymocytes after Mel, Dex, and Mel + Dex supplementation. β-Actin expression was used as loading control. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* Mel + Dex.

4.3. *In vitro: splenocytes treated with luzindole*

Splenocyte proliferation assessed as % stimulation ratio, was significantly ($p < 0.01$) decreased by dexamethasone and luzindole treatment when compared with the control and dexamethasone treated group. Co supplementation of luzindole along with dexamethasone and melatonin further significantly ($p < 0.01$) decreased the splenocyte proliferation when compared to control and dexamethasone treated group. Dexamethasone treatment along with melatonin significantly ($p < 0.01$) increased the splenocyte proliferation when compared to dexamethasone treated group alone but showed a non-significant ($p > 0.05$) decrease when compared with the control group (Fig. 9A).

4.3.1. *Western blot analysis of GR in splenocytes treated with luzindole*

Dexamethasone alone or in combination with luzindole significantly ($p < 0.01$) upregulated GR expression when compared with the control group. Melatonin treatment along with dexamethasone significantly decreased the GR expression when compared to the dexamethasone treated group, while luzindole supplementation along with dexamethasone and melatonin further increased the GR expression significantly ($p < 0.01$) when compared to control or dexamethasone treated group of splenocytes (Fig. 9B and C).

5. Discussion

The present study highlights the role of melatonin membrane receptors (MT1 and MT2), that mediates the immunoenhancing role of melatonin while counteracting the immunocompromised state induced by corticosterone in a wild tropical rodent *F. pennanti*. Melatonin administration upregulated the expression of its membrane receptors (MT1 and MT2) while, dexamethasone treatment decreased it significantly. Dexamethasone increased the GR expression and diminished T-helper cell mediated immune functions by decreasing the lymphocyte proliferation which was successfully restored upon melatonin administration.

The splenocyte, thymocyte and circulatory mononuclear cells (PBMC) proliferation in response to T cell specific mitogen Con A was enhanced by treatment of melatonin *in vivo* as well as *in vitro*. Melatonin administration perhaps mimicked the short day like conditions by raising the plasma level of melatonin and at the same time significantly ($p < 0.01$) upregulating the expression of melatonin membrane receptors MT1 and MT2. High melatonin level thereby counteracted the circulatory corticosterone level and simultaneously decreased the GR expression which resulted in enhanced T cell mediated (%SR and %DTH) and humoral immune response in seasonally breeding rodent *F. pennanti*. Our results are in line with the earlier report of Konakchieva et al. [32], suggesting that melatonin administration was associated with diminishing the corticosterone secretion thus protecting from the glucocorticoid induced deteriorations.

Previous reports suggest that mitogen induced lymphocyte proliferation was significantly depressed following the treatment of corticosteroid, either *in vivo* or *in vitro* [21,33,34]. Melatonin on the contrary is known to play an important role in immunomodulation by rescuing the immune cells from the immunocompromising effects of environmental stressors which ultimately leads to elevation of plasma corticosterone level [3,21,35,36]. These immunomodulatory effects of melatonin might dependent on activation of membrane melatonin receptors (MT1/MT2) by melatonin. There could be two possibilities for the increased melatonin

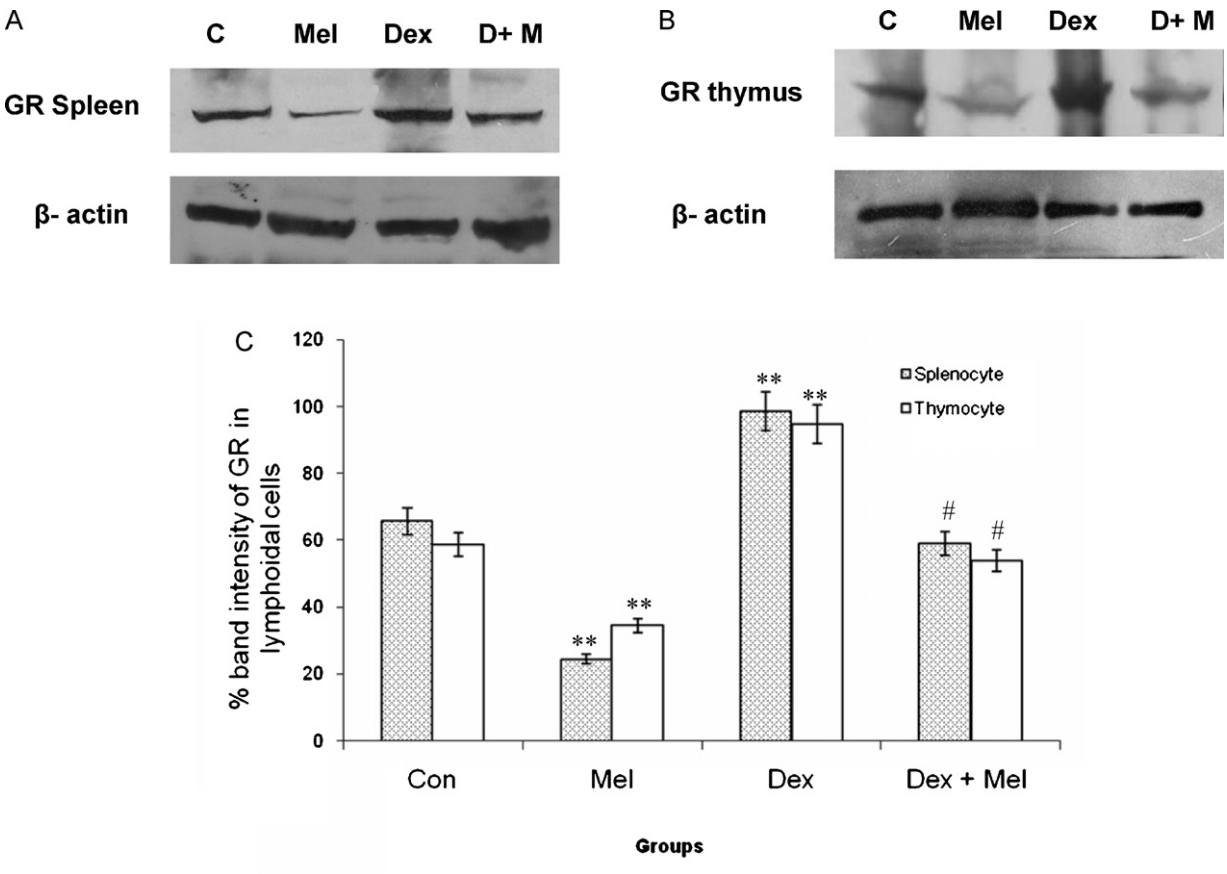

Fig. 8. Effect of *in vitro* melatonin and dexamethasone supplementation on GR expression. Western blot analysis of GR in (A) splenocytes and (B) thymocytes. The data are expressed as % band intensity of GR expression (C) in lymphoidal cells after Mel, Dex, and Mel + Dex supplementation. β-Actin expression was used as loading control. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* Mel + Dex.

membrane receptor expression. First, melatonin injection elevated circulatory melatonin level that might have enhanced the melatonin biosynthesis in lymphoid organs and circulatory mononuclear cells, which might have upregulated the receptor expression (MT1 and MT2) in lymphoid tissues. Our result gets support from previous reports [37–39] suggesting additive role of endogenous melatonin on extrapineal sources in amplifying the tissue level of melatonin. However, in the present study we have not measured the tissue level of melatonin to verify the above hypothesis. Since, we have already reported upregulation of MT1 and MT2 receptor following injection of physiological dose of melatonin [23]. Therefore, the second possibility that increased peripheral melatonin level might have upregulated the melatonin membrane receptors expression independently, thereby rescuing the immune repertoire by suppressing the apoptotic and antiproliferative effects of glucocorticoids. However, to be more precise in interpretation of dynamics of melatonin membrane receptor expression, further study needs to be undertaken by focusing on extrapineal biosynthesis of melatonin, following exogenous melatonin injection.

Delayed type hypersesitivity response, a Th1 type reaction is a direct measure of antigen specific cell mediated immune response. Melatonin treated squirrels showed robust DTH response while the dexamethasone treatment significantly ($p < 0.01$) decreased the DTH response compared with control and as noted earlier by Dhabhar and McEwen [40], suggesting that chronically elevated corticosterone or dexamethasone administration tends to suppress DTH response. DTH reaction has been demonstrated to be mediated primarily by $CD4^+$ T-helper cells [41,42], secreting IL-2 and IFN-γ [43]. So we measured IL-2 production which was found to be inhibited in the dexamethasone treated group that might have suppressed the generation of antigen specific T cells in the squirrels thus, decreasing the DTH response.

Production of anti-KLH-IgG is a T cell dependent phenomenon. Immunization with KLH in the dexamethasone pretreated groups resulted in suppression of circulatory anti-KLH-IgG which was restored in the groups pretreated with melatonin and dexamethasone. Melatonin pretreatment was found to be immunostimulatory and considerably increased the antibody titer. As KLH is a T cell specific antigen, so stress like levels of corticosterones after dexamethasone treatment might have resulted in reduction in proliferation of anti-KLH T cells [44], resulting in reduced anti-KLH-IgG antibody level.

Melatonin may influence immune function by altering the circulatory concentrations of other hormones such as gonadal and adrenal steroids [21,32,45,46], or by activation of melatonin receptors present on the cell surface of lymphoid organs [23,47]. We observed that physiological concentration of melatonin successfully counteracted the immunosuppressive effects of glucocorticoids by up regulating the expression of melatonin membrane receptors thus modulating the cell mediated immunity on one hand and down turning the sensitivity of immunocompetent cells towards glucocorticoid on other, by attenuating GR expression in lymphocytes. The observation implies for a possible involvement of melatonin in an adaptive bidirectional "trade off" communication between neuroendocrine hypothalamo–hypophyseal–adrenal (HPA) axis and immune system in this tropical rodent.

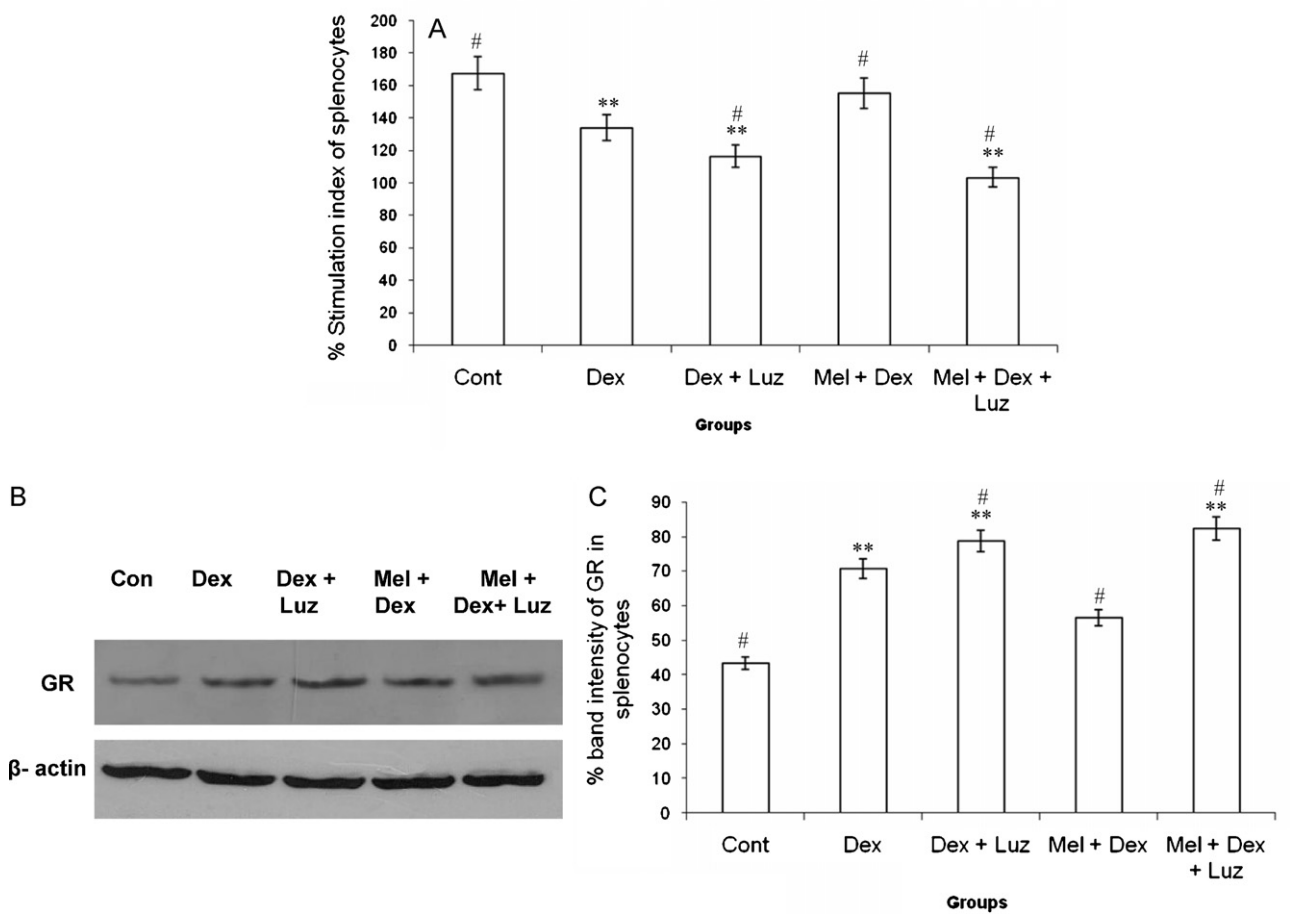

Fig. 9. Role of melatonin membrane receptors in amelioration of glucocorticoid induced immunosuppression. (A) Blastogenic response (%SR) of splenocytes after *in vitro* supplementation. (B) Western blot analysis of GR in splenocytes. (C) The data are expressed as % band intensity of GR expression in splenocyte after Dex, Dex + Luz, Mel + Dex and Mel + Dex + Luz supplementation *in vitro*. β-Actin expression was used as loading control. Values are expressed as mean, vertical bar on histograms represents ± SEM, $n = 5$. Significance of difference; $^{**}p < 0.01$, Con *vs.* all experimental groups and $^{\#}p < 0.01$, Dex *vs.* all experimental groups.

Immunomodulation by glucocorticoid and melatonin seems to be regulated by their effects on expression of cytokines. Melatonin independently stimulates IL-2, IL-6 and IFN-γ production by cultured lymphocytes [48,49] but, the physiological effects of melatonin at the level of immune system are largely mediated by modulation of IL-2 production by lymphocytes [49]. Our results demonstrated a parallel relationship between melatonin treatment and IL-2 level both *in vivo* and *in vitro*. Glucocorticoid induced suppression of IL-2 level was also significantly restored upon treatment of melatonin. Dexamethasone in fact, is known to inhibit IL-2 transcription and affecting IL-2 mRNA stability [50] and is involved in down regulation of the IL-2 R expression thereby compromising the activation of T cells.

Recent reports from Lardone et al. [51] and Carrillo-Vico et al. [37,49], emphasized on the role of melatonin synthesized from lymphocytes in modulation of IL-2/IL-2 receptor system in the lymphocytes. This physiological interplay involves both the membrane and nuclear melatonin receptors present in immunocompetent cells. Upon stimulation by melatonin T cells mainly produce IL-2 which usually serves to induce autocrine proliferation response [52]. Our previous studies also support that melatonin enhances IL-2 production by activating membrane melatonin receptor MT1, which was shown to be blocked upon luzindole treatment, suppressing the cell proliferation [23].

Further, to document whether the effect of melatonin in counteracting the glucocorticoid induced immunosuppression was a receptor dependent/independent phenomenon, we treated the splenocytes with a nonselective melatonin receptor antagonist [53], luzindole. We observed that luzindole treatment along with dexamethasone suppressed the lymphocyte proliferation significantly ($p < 0.01$) compared to dexamethasone treatment alone suggesting that luzindole further augmented the immunosuppressive effects of glucocorticoids. Supplementation of melatonin along with luzindole and dexamethasone suppressed the lymphocyte proliferation to the highest extent suggesting that melatonin could not counteract the inhibition of lymphocyte proliferation induced by glucocorticoids in presence of luzindole. Rather, in presence of melatonin the antagonistic effects of luzindole on membrane melatonin receptors (MT1 and MT2) was amplified and antiproliferative effect of dexamethasone was further heightened, suggesting that increased melatonin level itself promotes the antagonistic effect of luzindole [23].

To support our view we also checked GR expression in the splenocytes following luzindole treatment and found that, luzindole treatment along with dexamethasone increased the GR expression in cultured splenocytes. Melatonin supplementation along with luzindole and dexamethasone resulted in further upregulation of GR expression. In other words, luzindole enhanced the glucocorticoid induced inhibition of cell mediated immune response, possibly by attenuation of IL-2 secretion. Activation of MT1 and MT2, *via* melatonin resulted in downregulation of GR expression thereby prohibiting their immunosuppressive effects on immune mediators, which in the present study was reversed by luzindole treatment. Our results were in accordance with the earlier

report of Persengiev [54], which suggested that functional domains of melatonin receptor molecule MT1 are involved in regulation of GR induced transcription. However, our observations contrasted the most recent findings of Presman et al. [55] suggesting luzindole as an antiglucocorticoid in new born hamster kidney cells (BHK21).

The antagonizing effects of melatonin upon GR expression are also evident from the reports where chronic melatonin treatment resulted in considerable alterations in binding activity of GR leading to its differential sub-cellular localization [56,57]. The molecular mechanisms behind these interactions are still poorly understood however, it is proposed that melatonin attenuates the transcriptional activity of GR [54]. Recently, melatonin has also been proposed to impair the dissociation of HSP90 from GR which retards its nuclear translocation and hence its biological activity [56]. However, Presman et al. [56] reported inhibitory effect of melatonin on the GR translocation to be a tissue specific phenomenon as the inhibitory effect of melatonin on GR activity was not observed in mouse fibroblasts L929, HC11 mouse mammary epithelial cells or Cos-7 kidney cells. In addition, there are reports which suggest that melatonin does not inhibits nuclear translocation of GR, it rather impedes the interaction of GR with transcriptional intermediary factor-2 (TIF-2) [55] that leads to suppression of GR induced transcriptional activity. Most importantly, all of these reports culminate to a common assumption that melatonin can be helpful in counteracting the deleterious effects of glucocorticoids on central nervous system and immuno-competent cells.

In conclusion, our study suggests the existence of a reciprocal 'cross talk' between two hormones, of antagonistic nature, melatonin and corticosterone. The reciprocal interactions noted among their receptors may critically account for a dynamic and intricate physiological response required for maintenance of seasonal immune homeostasis in wild conditions. Our view gets support from previous reports of Sainz et al. [58], where melatonin while mediating its effects down regulated the GR expression in dexamethasone treated thymocytes while glucocorticoid always tended to upregulate the same. Thus, the pineal gland *via* its hormone and receptors seems to play a significant role in regulation of the HPA-lymphoid axis, maintaining an immunological link for significant adaptability in the wild species and thus increasing their chances of survival against all the ecological odds.

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