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# Receptor mediated counteraction of melatonin in splenocytic proliferation of lipopolysaccharide challenged mice

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## ABSTRACT

The activation of the spleen lymphocyte can be measured by spleen lymphocytic proliferation assay. The pineal gland secretion melatonin hormone is well known for its immune modulation in mammalian physiology. This hormone performs the various activities including the immune responsiveness through binding of its two receptors MT1 and MT2. The aim of the present study is to focus on the MT1 and MT2 receptor mediated impacts of melatonin in bacterial toxic component LPS affected spleen lymphocytic proliferation of Swiss Albino mice. Immunohistochemical study revealed the localization of melatonin receptors on spleen. The western blotting analysis was done to carry out the melatonin receptors expression in spleen and splenocytes proliferation was assayed for analyzing the splenocytes proliferation during different experimental situations. The increased expression of melatonin receptors of spleen and the decreased proliferation of splenocytes were observed due to melatonin treatment in LPS challenged mice. The observing results finally conclude that melatonin counteracted the splenocytes proliferation through activation of its MT1 and MT2 receptors in spleen of LPS challenged mice.

**Keywords:** Melatonin; Splenocytes proliferation; LPS; MT1 and MT2 receptors.

## INTRODUCTION

The spleen is the largest secondary lymphoid organ containing about one-fourth of the body's immune responses to antigens (Kuper et al., 2002; Nolte et al., 2002; Balogh et al., 2004). The spleen is a primary site of extramedullary hematopoiesis and removes degenerated or aged red blood corpuscles, particulate materials and circulating bacteria from the blood. Splenic lymphocytic activation as well as cell mediated immune responses can be measured by splenic lymphocyte proliferation assay. B cells undergo stimulation and proliferation when they encounter with their specific antigen with the help of T cells. T cells undergo proliferation when they are activated by antigen presenting cells and cytokines.

Lymphocyte proliferation by mitogenic stimulation is an accepted method for cell mediate immunity and also for immune toxicology evaluation (Snyder and Valle, 1991).Researchers used this technique for evaluating the immune toxicological responses by using MTT assay (Bao-An *et al.*, 2010, Małaczewska, 2011, Yu *et al.*, 2012).

Melatonin is produced from the mammalian pineal gland by melanocytes and it is known for its distinct versatility on the variety of tissues. A large body of evidence supports the immunoenhancing role of melatonin (Nelson RJ and Drazen DL, 1999). *in vitro* administration of melatonin enhances the proliferative ability of splenocytes (Drazen et al., 2000; Kriegsfeld et al.) and enhances the mitogenic response of peripheral blood T lymphocytes (Kliger et al., 2000). Melatonin also acts as a protective agent in the treatment of pathological remodeling. Lipopolysaccharide (LPS) is the major molecular component present in the cell wall of gram negative bacteria (Alexander C and Rietschel ET, 2001; Raetz CR and Whitfield C, 2002). Induction of such bacterial endotoxin emerges the septic shock and creates the immune challenged conditions. *in vitro* study suggests the protective effects of melatonin against LPS induced myocardial hypertrophy through down regulation of the TNF-alpha expression and also retains the intracellular calcium ion homeostasis (Lu Q et al., 2015) . Melatonin decreases the level of oxidative stress and the production of proinflammatory cytokines, chemokines, and IgG in LPS-stimulated mouse mammary tissue *in vitro*, which mimicked the local immunity (Yu GM and Tan W, 2019).

In mammalian physiology, melatonin performs the various activities through binding with its two cell membrane bound receptors, MT1 and MT2. High-affinity melatonin receptors have been localized on various lymphoid tissues including spleen. The aim of the present study is to delineate the receptors MT1 and MT2 mediated action of melatonin in splenocytic proliferation of bacterial endotoxin lipopolysaccharide induced immune challenged condition of mice.

## MATERIALS AND METHODS

All the experiments on the animals were conducted in accordance with institutional practice and within the framework of the revised Animal (Specific Procedure) Act of 2007 of Govt. of India on animal welfare. The study was approved by institutional animal ethics committee (IAEC) with ethical clearance no. TU/IAEC/2013/V/5-3.

### Animal procurement and Maintenance

Healthy Swiss albino mice colonies were housed at animal house in ambient laboratory conditions having temperature of 25±2°C with alternative maintenance of light/dark cycle (12L:12D). Mice were kept in groups of seven (n=7) in polycarbonate cages (43cm x 27cm x 14cm) to avoid the crowding effect and fed with mice feed and water ad libitum.

### Experimental Design

For observing the effects of melatonin in lipopolysaccharide challenged condition in pituitary-thyroid axis, mice were divided into four groups having 5 mice in each group as follows:

Control (Con) group: Mice of this group were received subcutaneous injection of ethanolic saline (0.01% ethanol), 0.1 ml/day for consecutive 30 days.

Melatonin (Mel) group: Mice of this group were received subcutaneous injection of melatonin (Sigma-Aldrich Chemicals, St. Louis, USA), 25 µg/100 g BW/day for consecutive 30 days at evening (16:30-17:00) hours.

Lipopolysaccharide (LPS) group: Mice of this group were received single intra-peritoneal injection of Lipopolysaccharide (LPS) (Sigma-Aldrich Chemicals, St. Louis, USA), 250 µg/100g BW. Experimental mice were sacrificed after 4 days of LPS administration (Zhang et al., 1994).

Lipopolysaccharide + Melatonin (LPS + Mel) group: Mice of this group were received both LPS and melatonin. LPS was injected 4 days prior to the completion of melatonin treatment.

### Sample collection and processing

After 24 hours of last administration, experimental mice were sacrificed under anaesthesia (pentobarbital, 15mg/Kg, intraperitoneal injection). Experimental tissue (spleen) was dissected out immediately and a few parts of it stored at -20°C for western blot analyzing and a few parts were kept in Bouin's fixative for immunohistochemical staining. The half part of each dissected experimental tissue (spleen) was

immediately processed for single cell suspension preparation for the study of spleen T-cells proliferation.

**Immunohistochemical Staining**

Immunohistochemical staining was done to observing the melatonin receptors localization (Savaskan et al., 2002) in spleen. Paraffin sections (5µm) fixed on 3% gelatine coated slides were deparaffinised and rehydrated with alcohol grades. The sections were placed in PBS for 30 minutes and endogenous peroxide activity was blocked by 0.3% $H_2O_2$ in methanol for 30 minutes at room temperature ($25^0$C). Sections were washed thrice with phosphate buffered saline (PBS: 0.1M $Na_2HPO_4$, $NaH_2PO_4$, 0.9% NaCl, pH=7.4) and were placed in blocking solution (horse blocking serum, diluted 1:200 in PBS, PK -6200, Vector Laboratories, Burlingame, CA) for 2 hrs. Sections were incubated with primary antibodies [Mel1AR (MT1); sc13186 and Mel1BR (MT2); sc13177, goat polyclonal, Santacruz Biotech, USA, diluted 1:200] overnight at $4^0$C. Next day, sections were washed thrice with PBS and incubated with biotinylated secondary antibody (Vectastain ABC Universal Kit, PK-6200, Vector Laboratories, Burlingame, CA, dilution 1:1000). Sections were again washed thrice with PBS and incubated with preformed AB (Avidin-Biotin) reagent for 30 minutes. The antigens were visualized using the 0.03% peroxidase substrate 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Chemicals, St. Louis, USA) in 0.01M Tris-Cl (pH=7.6) and 0.1% $H_2O_2$ and counterstained with Ehrlich's haematoxylin. The sections were dehydrated and mounted with DPX. Microphotographs of the stained sections were taken under 40X objective of Olympus microscope BX 41. To test the specificity of the used antibodies, the primary antibodies were not added in control sections which were treated as negative control and incubated with same dilution of normal serum for overnight at $4^0$C. Next morning the immunohistochemical protocol was followed under the same conditions.

**Western Blot analysis**

Tissue samples were homogenized and lysed in RIPA buffer [1% (v/v) NP-40, 0.1% w/v] sodium dodecyl sulphate (SDS) in PBS containing aprotinin, sodium orthovanadate and phenyl methyl sulphonyl fluoride (PMSF) and total sample protein was quantified by Lowry method (1951). Aliquots containing 100 µg proteins were resolved by 10% (w/v) SDS polyacrylamide gel electrophoresis followed by electro transfer to nitrocellulose membrane (Santa Cruz Biotech, USA). Immune detection was carried out by using primary antibodies [Mel1AR (MT1); sc13186, Mel1BR (MT2); sc13177, goat polyclonal, Santacruz Biotech, USA, diluted 1:100] and β-actin antibody (sc-130656, rabbit polyclonal, Santacruz Biotech, USA, diluted 1:500) diluted in PBS contained 5% skimmed milk and 0.01% Tween-20 followed by incubation with horse-radish peroxidase conjugated secondary antibodies (goat anti-rabbit IgG for β-actin antisera; diluted 1:1000 and rabbit anti-goat IgG for MT1, MT2 antisera; diluted 1:1000). The immune interactions were detected by using Super Signal West Pico Chemiluminescent Substrate (#34080, Thermo Scientific, Rockford, USA). Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation, MD, USA). Values were expressed as ratio of the density of the specific signal to -actin signal and expressed as the % control value (Treeck et al., 2006). Each sample corresponds to tissue from a single animal and at least five gels corresponding to each subunit and experimental conditions were analyzed.

**Splenocytes proliferation**

Spleens of experimental mice were dissected out on chilled PBS and processed for preparation of single cell suspension of splenocytes. Erythrocytes of splenic cell suspension were lysed with 1:10 solution of cold 0.5% Tris and 0.84% $NH_4Cl$ (pH 7.2). Cell suspension was washed thrice with chilled PBS. Cell viability was determined by trypan blue exclusion method. Viable cells (which exceeded 95%) number was adjusted to $1x10^7$cells/ml in culture medium [RPMI-1640 medium supplemented with Streptomycin (100g/ml), Penicillin (5000U/ml), L-glutamine (2mM/ml), 0.1% 2-mecaptoethanol ($5 \times 10^{-2}$mM/ml) and 10% FCS). 100µl splenocytes suspension was added to the wells of sterile flat bottom 96 well culture plates. Mitogen concanavalin-A (Con A; T-cell mitogen; Sigma-Aldrich Chemicals, St. Louis, USA) solution was prepared at the concentration of 5µg/ml in the culture medium. 50µl mitogen solution was added to the wells containing splenocytes suspension and yielded a volume of 150µl/well (in duplicate). Finally a volume of 200µl/well was made by adding complete culture media 50µl/well in mitogen containing and 100µl/well in without mitogen containing wells of culture plate. Culture plate was incubated in a humidified 5% $CO_2$containing chamber at $37^0$C for 44 hrs. 20l MTT solution (3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyl-

tetrazolium bromide, SRL, Mumbai, India, 5mg/ml in PBS) was added in each well and incubated at 37$^0$C in 5% $CO_2$for additional 4 hours. After 4 hours 100 l of acidified propanol (0.04 mol/LHCl in isopropanol) was added in each well and the optical density was determined with a microplate reader (ECIL, India) at 570 nm wave length (Ahmad and Haldar, 2010). Mean OD values for each set of duplicates were used in subsequent statistical analysis. Response was calculated as percent (%) stimulation index representing the ratio of absorbance of the mitogen stimulated cultures to control cultures.

**Statistical Analysis**

Statistical analysis of the data was performed with one way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) multiple range test. The differences were considered significant when $p< 0.05$. Microsoft Excel program and Statistical Package for the Social Sciences (SPSS) were used for calculation and graph preparation.

## RESULTS

**Immunohistochemical localization of MT1 and MT2 receptors in spleen**

Both MT1 and MT2 receptors' immunoreactivity were observed throughout the spleen section. Immune reactions were noted dominantly in PALS region of white pulp (Figure 1).

**MT1 receptor protein expression in spleen**

MT1 receptor expression was unaffected in melatonin treated group. But it was significantly ($p<0.01$) decreased in LPS treated group in comparison to control group. In (LPS+Mel) treated group, MT1 receptor expression was significantly ($p<0.01$) increased in comparison with LPS treated group of mice (Figure 2).

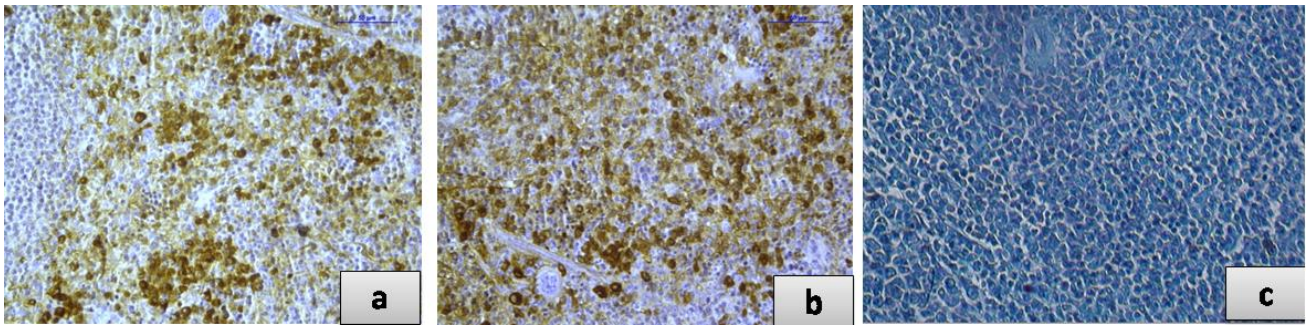


**Figure 1.** Immunohistochemical staining of melatonin receptors, MT1 receptors (a) and MT2 receptors (b) in spleen tissue of mice. DAB reaction was not detected in negative control section (c). Microphotographs were taken by Olympus Microscope under 40X objective.

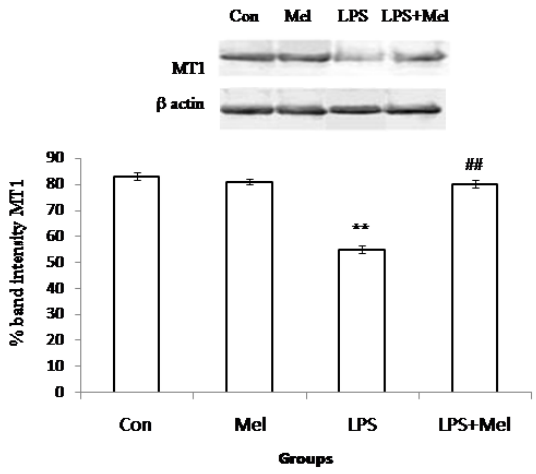


**Figure 2.** Western blot analysis of MT1 receptor protein expression in spleen. β-actin was used as loading control. Lower panel shows percent expression of protein following Scion Image analysis. Histogram represents Mean ± SEM. The mean differences were considered significant when $p< 0.05$. **$p<0.01$: Con vs LPS; ## $p<0.01$: LPS vs LPS+Mel.

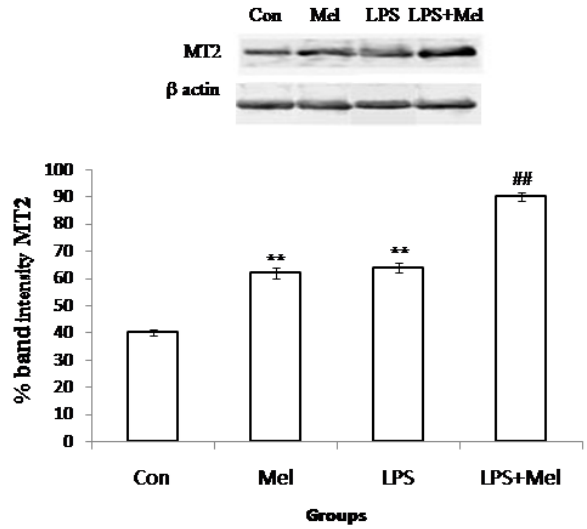


**Figure 3.** Western blot analysis of MT2 receptor protein expression in spleen. β-actin was used as loading control. Lower panel shows percent expression of protein following Scion Image analysis. Histogram represents Mean ± SEM. The mean differences were considered significant when p< 0.05. **p<0.01: Con vs Mel, Con vs LPS; ## p<0.01: LPS vs LPS+Mel.

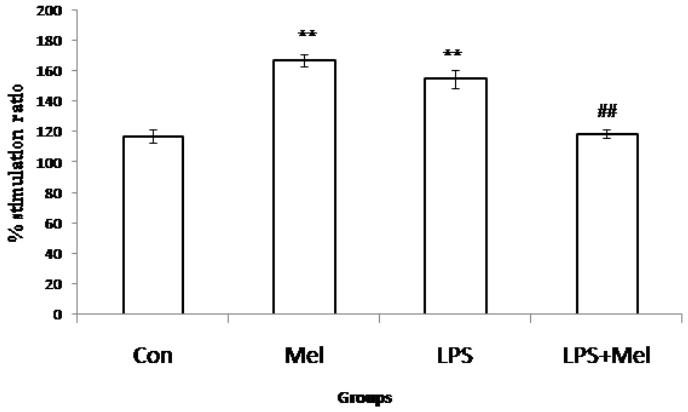


**Figure 4.** Percent stimulation ratio (% SR) of splenocyte. Histogram represents Mean ± SEM. The differences were considered significant when p< 0.05. **p<0.01: Con vs Mel, Con vs LPS; ## p<0.01: LPS vs LPS+Mel.

**MT2 receptor protein expression in spleen**

MT2 receptor expression was significantly (p<0.01) increased for melatonin treated as well as for LPS treated group of mice as compare to control group of mice. In (LPS+Mel) treated group, MT2 receptor expression was significantly (p<0.01) increased in comparison to LPS treated group of mice (Figure 3).

**Effect of melatonin on splenocyte stimulation index (%SR)**

Splenocyte stimulation index (% stimulation ratio) was significantly (p<0.01) increased in both melatonin treated group and LPS treated group of mice as compare to control group of mice. But in (LPS+Mel) treated group, % stimulation ratio of splenocyte proliferation was significantly (p<0.01) decreased in comparison with LPS treated group of mice (Figure 4).

**DISCUSSION**

Melatonin receptor expression in the lymphoid tissues supports the notion of the immunomodulatory role of melatonin. In the present study, the receptors of melatonin are observed in the studied lymphoid tissue, spleen. Immunohistochemical staining was showed the localization of MT1 and MT2 receptors on the splenocytes. The melatonin receptors immune reactivity was observed mainly in the periarteriolar

lymphoid sheaths (PALS) of white pulp of the spleen. The PALS is typically associated with the arteriole supply of the spleen that contains T lymphocytes. Earlier studies done by the other researchers described the binding sites of iodinated melatonin in the rat spleen (Rafii-El-Idrissi et al., 1995). The binding of melatonin receptor antibody on immune organ such as spleen may suggest the involvement of MT1 and MT2 receptors in melatonin mediated immunomodulation.

After the treatment of melatonin the melatonin receptors expression were found to be expressed differentially. In this study, melatonin administration causes the increased expression of MT2 receptor whereas, MT1 receptor was remaining unchanged. In our previous report it was documented that melatonin differentially modulate the expression of MT1 and MT2 melatonin receptors in spleen in age and sex dependent manner (Singh et al., 2015). After the treatment of LPS decreased expression of MT1 and increased expression of MT2 were observed. Due to LPS induction, MT1 receptor density was reduced whereas MT2 receptor density was increased in spleen. It was appeared that LPS does change the number of melatonin receptors in 2-day cultured monocytes as reported by investigators (Barjavel et al., 1998). In (LPS+Mel) treated group of mice, melatonin receptors MT1 and MT2 expression were significantly increased as compare to LPS treated group. Melatonin counteracted the LPS caused effects by activation or up regulation of its two high affinities membrane bound receptors in spleen.

Splenocytic proliferation is used as an index of immune function in many clinical applications. In this study the splenocytes proliferation was found to be increased in melatonin treated mice as compare to control. A stimulatory effect of melatonin on immune responses was well established by many researchers. Melatonin stimulates the immune activity through elevation of T-cell numbers in spleen as depicted by investigators (Demas and Nelson, 1998). The clinical relevance of the multiple functions of melatonin under different immune challenged conditions including infection was also reviewed (Carrilo-Vico et al., 2013). Due to LPS treatment increased splenocytes proliferation was observed in this study. Earlier reports were also suggested that *in vivo* treatment of LPS increased the T-cell proliferation (Tough et al., 1997). In this study it is also observed that melatonin supplementation along with LPS decreased the stimulation rate of splenic T-cell as compare to the LPS alone treated group of mice. Exogenous melatonin encounters the LPS induced pathological hazards and counteracted the splenocytic proliferation to control level. The other studies are also revealed that generally melatonin inhibited the LPS induced inflammation in cultured mouse mammary tissue that mimicked the local immunity (Yu and Tan, 2019).

## CONCLUSION

From the above discussion we may conclude that melatonin through binding on white pulp of the spleen and through activation of its two receptors, MT1 and MT2 in spleen normalize the spleen cells proliferation which was affected due to bacterial endotoxin LPS. So, it may reveal that melatonin counteracted the LPS stimulated immune function which was mainly observed in this study through splenocytes proliferation through up regulation of its receptors present in the spleen. Further studies are also required to elucidate the LPS stimulated immune function through observing the levels of pro-inflammatory cytokines and also needed to replenish the cellular and molecular mechanism of receptors mediated impact of melatonin in this facet.

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**Conflict of Interest**

The author declares that there is no conflict of interest

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