Protective effects of melatonin in endosulfan induced immunomodulation and their association with oxidative stress markers in rats

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Endosulfan toxicity affects the nervous system as well as immunological functions. It also causes oxidative stress and subsequent mitochondrial dysfunction. In the present study, we tried to evaluate the protective effects of melatonin on endosulfan (END) induced immunological and biochemical changes in rats. Wistar rats (200-250 g, n=8/group) were immunized with fresh SRBC (0.5×10⁹ cells/kg) and were exposed to END (4-16 mg/kg, orally), simultaneously exposed animals were treated with vehicle or melatonin (10 and 50 mg/kg) for 14 days. On day 15, their blood and spleen was collected for immunological assays and oxidative stress markers. Endosulfan (8 and 16 mg/kg) significantly suppressed (i) anti-SRBC antibody titer; (ii) footpad thickness; (iii) spleen PFC counts; and (iv) Th₁ (IFN-γ) & Th₂ (IL-4) and significantly increases serum TNF-α level as compared to controls (P <0.05 in all parameters). Endosulfan induced immunological changes were found associated with changes in oxidative stress markers as evidenced by the results of this study. Endosulfan, while significantly decreased GSH, SOD and CAT activity (P <0.05), it increased serum TBARS activities (P <0.001). These endosulfan induced changes in immunological and biochemical parameters were found significantly reversed by the treatment with melatonin (10 and 50 mg/kg) in a dose dependent manner by differential degrees. Results of the present immunological and biochemical data suggest the protective role of melatonin in endosulfan induced immunomodulation which is associated with oxidant/antioxidant imbalance.

Keywords: Cytokine, Immunomodulation, Melatonin, Oxidative stress, Pesticide toxicity

Environmental stressors like pesticide are known to cause severe pollution and influence health and diseases1. Pesticide induced oxidative stress is the final manifestation of the multi-step pathways and may result in the imbalance between the pro-oxidant and antioxidant defense mechanisms2,3. Further, pesticide exposure induces a derangement of certain antioxidant mechanisms in different tissues, including alteration of the antioxidant enzymes and the glutathione-redox system4. The organochlorine pesticides are known to inhibit acetyl cholinesterase activity in the target tissues and affect Na⁺, K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ activities of cells5.

Endosulfan, a common organochlorine insecticide of cyclodiene group, was recommended for global ban by the Stockholm Convention in April 2011 due to its toxicological effects on humans. Since then, more than 80 countries have announced complete ban on endosulfan or have decided to phase it out. It has been listed as priority pollutant by US Environmental Protection Agency (EPA). However, India and China are two major countries where endosulfan is still used extensively6,7. Endosulfan gets absorbed into the body through several routes such as skin, respiratory and oral routes, and gets stored in adipose tissue8. It affects physiological functions of body and has toxicological effect on various organ systems including nervous system and immune function9,10. Singh et al.11 observed teratogenic effect of endosulfan in rats.

Due to its property of mitochondrial dysfunction and oxidative stress endosulfan is widely used in toxicological studies12. Acute exposure to endosulfan leads to immunotoxicity, which is a major concern of its exposure13. Saiyed et al.14 has documented the role of endosulfan exposure causing delay in sexual maturity, especially in male children. Several subacute and chronic toxicological studies of endosulfan have
suggested that liver, kidneys, testes and immune system are the main target organs affected by endosulfan toxicity\textsuperscript{25,26}. Toxicity of endosulfan in immunity is well known and several studies have indicated the involvement of free radical mediated mechanism in its toxicity\textsuperscript{27,28}. The role of free radicals and antioxidants defense mechanism in psychological stressors like emotional and environmental stress (endosulfan) induced immunomodulation is still an interesting subject of study.

Melatonin a pineal gland hormone has strong antioxidant property. Various studies have been done to prove its antioxidant property and protective effect in various stress induced disorders\textsuperscript{18}. Melatonin plays an important role in oxidative stress due to its free radical scavenging property and by enhancing the transcription of the antioxidant enzymes levels in various organs of the body\textsuperscript{19}. It acts as a direct free radical scavenger and indirect antioxidant and have protective role in various diseases\textsuperscript{20,21}.

The role of melatonin as immunomodulatory agent during old age is well known\textsuperscript{22,23}. 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)\textsuperscript{24}. Additionally, it directly neutralizes reactive oxygen species (ROS), such as hydroxyl radical (OH\textsuperscript{·}), superoxide radical (O\textsuperscript{2-}), peroxyl radical (LOO\textsuperscript{·}), singlet oxygen (\textsuperscript{1}O\textsubscript{2}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})\textsuperscript{25,26}.

Some researchers have reported in their study that long term administration of melatonin down regulates pituitary gonadal axis along with reduced sperm motility\textsuperscript{27,28}. However, role of melatonin in reproductive toxicity is contradicted by several researchers. In one such study, Bornman et al.\textsuperscript{29} have shown that, seminal plasma melatonin plays no important role in sperm motility. Chabra et al.\textsuperscript{30} have shown that melatonin has protective effect in people undergoing chemotherapy against reproductive toxicity. Simultaneously, Luboshitzky et al.\textsuperscript{31} reported that long term melatonin administration alters semen quality in healthy men. However, conclusion of this study is contradictory as 6 out of 8 patients did not report any change in semen quality or in serum and seminal plasma hormone levels during the 6 months long study. Serum gonadotropin levels were also unchanged during period of study in all 8 men. Also, the two subjects who showed decreased sperm production had lower sperm concentrations at baseline than 6 non-responders\textsuperscript{31}. Further, melatonin has been shown to improve the sperm quality in forced swimming test induced oxidative stress in nandrolone treated wistar rats\textsuperscript{32}.

In this study, we explored the possible protective role of melatonin in endosulfan induced immunomodulation in relation to oxidative stress in rats.

**Material and Methods**

**Drugs and Chemicals**

Melatonin (Sigma chemical Co., USA), endosulfan (technical grade, Cat. No P-435N, α and β stereoisomer’s, 70:30 ratio was obtained from Accustandard, RFCLTM Limited India), ether, pentobarbitone sodium (May & Baker) and diethyl ether (SD Fine Chemicals). potassium phosphate buffer, disodium hydrogen potassium phosphate, DTNB, NaN\textsubscript{3}, EDTA, FAD, Zn\textsubscript{SO\textsubscript{4}}, heparin, glutathione reductase, sodium carbonate buffer, tween-20, H\textsubscript{2}SO\textsubscript{4}, Hank’s balanced salt solution (HBSS), Gentamicin, Trypan blue, PBS, pyrogallol, chloroform, n-butanol (All from Central Drug House Pvt. Ltd.), RPMI-1640 (Hi-Media), Na\textsubscript{3}N, EDTA, 1,1,3,3 Tetramethoxy propane, potassium dichromate, thiobarbituric acid, Drabkin’s reagents, HCl, fetal calf serum, HRPO complex (Sigma USA), Sheep RBC and bovine serum albumin. ELISA kits (Diaclone, France) were used for cytokine assay

**Experimental animals**

Inbreed male Wistar rats of (200-250 g) and n =8 animals 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.

**Endosulfan exposure**

Male Wistar rats (200-250 g) were randomly divided in to six groups with n =8/group. Rats were then exposed to endosulfan (4-16 mg/kg) by orally feeding endosulfan (technical grade) dissolved in ground nut oil in the volume of 5 mL/kg once daily. Simultaneous to endosulfan exposure rats were treated with melatonin (10 and 50 mg/kg \textit{i.p.}) daily for 14 consecutive days. Control group received only groundnut oil (5 mL/kg orally).

**Experimental procedures**

Haemagglutination assay

Haemagglutination assay was performed as per the procedure described by Clarke and Casals\textsuperscript{33}. To
perform the experiments, fresh blood of the healthy sheep was collected by venipuncture and was centrifuged at 1500 rpm for 10 min. The supernatant thus obtained was discarded and RBCs were washed with 0.9% normal saline and diluted to 0.5 × 10⁹ 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 × 10⁹ 134 cells on day ‘0’. The serum of each rat was collected in the Eppendorf tube. The serum samples were kept at −80°C until analyzed for primary anti-SRBC antibody titre by haemagglutination (IHA) technique. For secondary antibody response, animals were given a booster dose of 0.1 × 10⁹ cells and again exposed to endosulfan. After 48 h 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 h. 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 was assayed for statistical significance.

**Splenic Plaque Forming Cell (PFC) assay**

This assay was carried out by using the method of Li *et al.* The animals were immunized with 0.5 × 10⁹ SRBC on day ‘0’. At the end of experiments rats were sacrificed under high dose of anaesthesia (Pentobarbitone 50 mg/kg i.p.) and their spleen was dissected out and rinsed in 2 mL of RPMI-1614 (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 min at 4°C. The whitish interface of the lymphocytes between bottom red pellet and uppermost layer was taken and adjusted to a volume of 1 million lymphocytes per milliliter (1 × 10⁶ 158 cells/mL). SRBC was washed with PBS (150 mM/L, pH 7.4) and the cells count was adjusted to 800 × 10⁶ cells/mL. Guinea pig serum was taken as complement and diluted 5 times in PBS. For assay 1 mL of SRBC suspension, 1 mL of lymphocytes and 0.5 mL of guinea pig serum were taken in the micro tube, from this mixture 200 μL was loaded into a Cunningham’s chambers and incubated for 1 h at 37°C. The plaques were counted under light microscope and result is expressed as plaques/ spleen cells.

**Cell-mediated Immunity assay**

The delayed type hypersensitivity response was measured by the method previously described. Animals were immunized with SRBCs as for antibody titre on day 0. 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 receive normal saline. Simultaneous to endosulfan exposure rats were treated with melatonin (10 and 50 mg/kg). On day 13 rats were challenged with the same antigen in right hind foot and left foot receive only normal saline. After 24 h the footpad edema was measured and difference in the footpad volume (Rt-Lt) was measured by a dial caliper (Mututoyo, Japan) and result is expressed as percentage difference in DTH response.

**Cytokine assay**

The cytokines assays were carried out by the commercially available cytokine assays kits (Diaclone, France). Briefly, the Th1 (IFN-γ) and Th2 (IL-4) and TNF-α cytokines assays were performed by using solid phase sandwich enzyme linked immunosorbant assay (ELISA). Monoclonal antibody specific for IL-4, IFN-γ and TNF-α was coated on to the wells of the microtitre strips provided. Antigen and antibodies (biotinylated polyclonal antibody specific for IL-4, IFN-γ and TNF-α) were incubated simultaneously at 37°C for 1 h. The revelation steps include streptavidin horse sera dish peroxidase and TMB as chromogen. Rest of the protocol was followed as described in the assay kit. The plates were read on Microscan-5405A (ECIL) and result is expressed in pg/mL.

**Biochemical studies**

**Thio-barbituric acid reactive substance (TBARS) marker in serum**

TBARS level in serum was estimated using the method described by Lowry *et al.* In this method, 0.5 mL of the serum samples and 2.5 mL TCA (20%), was taken in the test tubes and was centrifuged 3500 rpm for 10 min. The supernatant thus obtained was decanted and the precipitate was washed once with 2.0 mL of 0.05 M H₂SO₄. Washed precipitate was then dissolved in 2.0 mL of the H₂SO₄ and 3.0 mL of
thiobarbituric acid (0.68% TBA in Na₂SO₄) was added and mixture was vortexed. The tubes were incubated in boiling water bath for 90 min and then 4.0 mL of n-butanol was added to the incubated mixture with vigorous shaking. After addition of n-butanol mixture was again centrifuged at 3000 rpm for 10 min. The upper organic layer obtained after centrifugation was separated and absorbance was taken at 530 nm on spectrophotometer (UV 5704 SS). The calculation was carried out using extinction coefficient and result is expressed in nM/mL.

**Superoxide dismutase (SOD) activity**

Superoxide Dismutase activity in the serum samples was determined using the method described by Marklund et al. In this method, RBCs were washed with the normal saline and haemolysate was prepared by adding 3 mL of cold distilled water. This haemolysate was then used for SOD estimation. 0.5 mL of RBCs haemolysate was mixed with 3.0 mL of ice cold water, 1.0 mL of ethanol & 0.6 mL of CHCl₃. Haemolysate was mixed properly after each addition and finally shaken for 10 min and centrifuged at 3000 rpm for 30 min. The supernatant was used for SOD estimation by taking different aliquots. About 100 µL of pyrogallol was added in cuvette containing tris-HCl buffer (2.7 mL) EDTA (100 µL) and haemolysate (100 µL). The increase in the absorbance at 420 nm was recorded using spectrophotometer from 30 s to 3 min, the lag of 30 s/min was allowed for steady state or auto-oxidation of pyrogallol to be attained. The concentration of pyrogallol was so adjusted that rate of change of absorbance was approximately 0.020-0.023 per min. The change in the absorbance at 420 nm on spectrophotometer (UV 5704 SS) was observed. One unit of SOD was defined as the amount of the enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 mL of assay mixture, result is expressed in U/gm Hb.

**Catalase (CAT) activity**

Serum Catalase activity was determined using the method described by Marklund et al. A stock haemolysate was prepared by addition of four parts of ice cold triple distilled water to one parts 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) immediately assay was performed. The assay was carried out by taking 2 mL of haemolysate in a 3 mL cuvette and the reaction was started by adding 1 mL of 30 mM/L of hydrogen peroxide; decrease in the absorbance was recorded at 240 nm on spectrophotometer (UV 5704 SS) for 30 s. The results are expressed in U/g Hb.

**Glutathione (GSH) assay**

Total glutathione assay was carried out using Ellman’s reagent by the method of Tietze et al. The assay is based on the enzymatic recycling procedure in which glutathione is sequentially oxidized by the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase. The extent of 2-nitro-5-benzoic acid formation is monitored as an increase in absorbance at 412 nm on the spectrophotometer (UV 5704 SS) and the glutathione content in the sample was determined by the comparison of the rate observed with known amount of reduced glutathione. The results are expressed in mM/L.

**Statistical analysis**

The data was expressed as Mean ± SE. For statistical significance data was analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test for multiple group comparison. A p value of at least 0.05 was considered as the level of significance in all statistical tests.

**Results**

**Effects of melatonin on endosulfan induced changes in humoral and T-cell mediated immune responses**

Endosulfan at a dose of 4, 8 and 16 mg/kg or 10, 20 and 40% of LD₅₀, respectively affects both humoral and cell-mediated immune responses in the dose dependent manner, and no mortality was observed at these doses. Exposure to endosulfan (4 mg/kg) for 14 consecutive days does not have significant influence on the humoral and cell-mediated immune responses. Both humoral and cell mediated immune responses were significantly reduced by higher doses of the endosulfan (8 and 16 mg/kg) (P <0.0001 for all parameters). Endosulfan (8 and 16 mg/kg) significantly reduces primary (P <0.001) and secondary (P <0.01) anti-SRBC antibody titre, further, for the other specific test for the humoral immune response. The reduction in the primary anti-SRBC antibody was more as compared to the secondary anti-SRBC antibody titre as evidenced by the indirect haemagglutination assay (IHA). Further, assessment of the humoral immune defense mechanism by splenic lymphocytes plaque formation (PFC) assay also suggests that endosulfan (8 and
16 mg/kg significantly suppress humoral immune defense mechanisms ($P < 0.001$). PFC assay clearly indicates that endosulfan significantly reduces splenic lymphocytes plaques forming cells (PFC) (Table 1). It was also found that endosulfan significantly suppressed cell-mediated immune responses as evidenced by the SRBC immunized and locally challenged antigen in the footpad. The % change in the footpad thickness (paw volume) of the SRBC sensitized animals was significantly decreased as compared to the controls ($P < 0.001$) (Table 1).

Further, for T-cell mediated immune defense mechanisms, the levels of the Th1 & Th2 cytokines were assessed and it was found that both Th1 & Th2 cell mediated immune defense mechanisms were suppressed (Fig. 1). The suppression of the Th1 defense mechanism was more as compared to the Th2 cells dependent defense mechanism. It was evidenced from the result that endosulfan (8 and 16 mg/kg) exposure significantly suppresses Th1 (IFN-γ) and Th2 (IL-4) cytokines levels. It was also observed that endosulfan exposure induces changes in some observable parameters like weight loss, hair loss, sluggish behaviors and decreased food intake.

Endosulfan induced changes in both, humoral and T-cell-mediated immune parameters were attenuated by the treatment with melatonin ($P < 0.0001$ for all parameters). Endosulfan induced suppression in the humoral immune responses was reversed significantly with the treatment with melatonin. It was found that endosulfan induced reduction in both, primary and secondary anti-SRBC antibody titers were attenuated by treatment with melatonin (10 mg/kg) for 14 consecutive days ($P < 0.01$). These results are more pronounced at a higher dose of melatonin (50 mg/kg) ($P < 0.001$) (Table 1). The endosulfan induced changes in the PFC counts were restored to normal as compared to only endosulfan exposed groups after treatment with melatonin (10-50 mg/kg) in a dose dependent manner ($P < 0.001$) (Table 1).

For the cell-mediated immune responses it was found that decreased % change in the footpad thickness was reversed significantly with the treatment with the antioxidant melatonin at a lower and a higher dose of 50 mg/kg ($P < 0.001$) (Table 1). Further, sensitive marker for the T-cell mediated immune responses endosulfan induced, decrease in both Th1 & Th2 cytokines, were attenuated with the treatment with melatonin (10 mg/kg) and both these Th1 (IFN-γ) and Th2 (IL-4) cytokine levels were reversed back towards normalcy as compared to the only endosulfan exposed animal groups at higher doses of melatonin (50 mg/kg) ($P < 0.001$) (Fig. 1). Endosulfan induced decreased ratio of the Th1/Th2 balance was reversed with the treatments with the melatonin.

Exposure to endosulfan significantly increases serum TNF-α level ($P < 0.05$) which was restored

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**Table 1—Effect of melatonin on endosulfan induced immunological changes in rats**

<table>
<thead>
<tr>
<th>Treatment (mg/kg i.p.)</th>
<th>Anti-SRBC primary</th>
<th>Antibody titre secondary</th>
<th>Plaques/10^6 spleen cells</th>
<th>% Change in paw volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5.8±0.1</td>
<td>7.2±0.2</td>
<td>432±37.9</td>
<td>20.5±0.6</td>
</tr>
<tr>
<td>END (4)</td>
<td>5.0±0.2</td>
<td>6.3±0.5</td>
<td>395±31.8</td>
<td>16.2±1.3</td>
</tr>
<tr>
<td>END (8)</td>
<td>3.2±0.3</td>
<td>5.9±0.9</td>
<td>305±31.6</td>
<td>10.1±0.5</td>
</tr>
<tr>
<td>END (16)</td>
<td>2.9±0.3</td>
<td>5.2±0.3</td>
<td>287±32.2</td>
<td>9.2±0.3</td>
</tr>
<tr>
<td>MEL (10) + END (8)</td>
<td>6.9±0.3</td>
<td>8.2±0.2</td>
<td>445±34.0</td>
<td>19.7±0.5</td>
</tr>
<tr>
<td>MEL (50) + END (8)</td>
<td>7.3±0.3</td>
<td>8.9±0.3</td>
<td>467±34.7</td>
<td>21.3±1.9</td>
</tr>
</tbody>
</table>

[Treatment with melatonin (10 & 50 mg/kg) significantly reversed endosulfan induced changes in primary and secondary anti-SRBC antibody titre, splenic plaque forming cell count and % Change in paw volume. END, Endosulfan; MEL, Melatonin. All data are Mean ± SE. *P <0.001; †P <0.01 compared to vehicle; ‡P <0.001 compared to END8; and §P <0.01 compared to END8]
Effects of melatonin on endosulfan induced changes in oxidative stress parameters

Endosulfan at a dose of 10, 20 and 40% of LD50 or 4, 8 and 16 mg/kg, respectively influenced pro/antioxidant balance and causes toxicity mediated through free radical/antioxidant balance, and no mortality was observed. Endosulfan (4 mg/kg) exposure for 14 consecutive days does not significantly influence all oxidative stress markers. Endosulfan exposure at the dose levels of (8 and 16 mg/kg) significantly increases oxidative stress markers as compared to the controls (P <0.0001 for all parameters). Endosulfan (8 and 16 mg/kg) significantly disturbed the pro/antioxidant balance and both enzymatic and non-enzymatic antioxidant defense mechanism was found altered.

The activities of the enzymatic antioxidants were found significantly decreased as evidenced by decrease in the in activities of the red cell superoxide dismutase (SOD) and catalase (CAT) when compared to the controls (P <0.001). Lipid peroxidation was significantly increased as evidenced by the increased serum TBARS activity (MDA levels) as compared to the controls (P <0.001). Further, endosulfan exposure significantly decreases the levels of aqueous phase antioxidant glutathione (GSH) (P <0.001). These results suggest that endosulfan-exposure decreases antioxidant balance and increases the pro-oxidant status. Endosulfan induced changes in the oxidative stress markers were significantly attenuated by treatment with melatonin (10 and 50 mg/kg) in a dose dependent manner as compared to the only endosulfan-exposed control groups (P <0.0001 for all parameters). The endosulfan induced changes in the red cell activities of the antioxidant enzymes were significantly reversed by the treatment with melatonin (10 mg/kg) for 14 consecutive days as suggested by the reversal of the endosulfan induced changes in the activities of the red cell superoxide dismutase (SOD) and catalase (CAT) when compared to the only endosulfan exposed groups. Further, higher dose of melatonin i.e. (50 mg/kg) further significantly reversed these endosulfan-exposed changes in the antioxidants enzymes activities (P <0.001) (Table 2).

The endosulfan induced increase in the lipid peroxidation was significantly decreased as evidenced by the decreased serum TBARS activity (MDA levels) in a dose dependent manner with treatments with melatonin (10-50 mg/kg) as compared to the only endosulfan-exposed groups (P <0.001) (Table 2). Further, endosulfan-induced decrease in the levels of glutathione (GSH) were reversed back toward normalcy as compared to only endosulfan exposed animals treated with melatonin (P <0.001) (Table 2).

Discussion

Pesticide induced immunological changes have been a focus of toxicological research. In the present
study we have carefully evaluated dose dependant protective effects of melatonin on endosulfan-induced immunological changes in rats.

Endosulfan at lower dose of 4 mg/kg does not have any significant effect on the humoral and cell-mediated immune responses. However, at higher doses (8 and 16 mg/kg) Endosulfan significantly reduces both humoral and cell mediated immune responses ($P < 0.0001$). The primary and secondary anti-SRBC antibody titers as well as splenic lymphocytes PFC counts were reduced after exposure to higher dose of endosulfan (8 and 16 mg/kg). Endosulfan in a dose dependent manner decreases both cell-mediated immune markers like DTH reaction and the Th$_1$/Th$_2$ cytokines like IFN-$\gamma$ and IL-4 levels.

These immunological changes were associated with significant decrease in the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and significant increase in index of lipid peroxidation reaction (MDA levels) after exposure to endosulfan in a dose dependant manner. These changes in the oxidative stress parameters suggest that ROS is involved in endosulfan-induced immunotoxicity. Further, endosulfan induced decrease in the level of serum glutathione (GSH) also validate these results.

Exposure to endosulfan significantly suppressed percentage change in paw volume (footpad thickness) in a dose dependant manner. This percentage change in paw volume may be attributed to suppression of cell trafficking and inflammatory cytokines to the site of antigen challenge after endosulfan exposure. These endosulfan induced changes in footpad thickness were significantly reversed by treatment with melatonin in a dose dependant manner.

Endosulfan significantly suppresses humoral immune response, which is supported by decreased anti-SRBC antibody titre, which may be attributed to decreased formation of antibody cells during stress. After exposure to endosulfan splenic plaque forming cell count also decreases in a dose dependant manner significantly decreases ($P < 0.05$). These endosulfan induced immunological changes were significantly attenuated by treatment with the melatonin (10-50 mg/kg) in a consistent dose dependent manner ($P <0.05$).

Markers of cell-mediated immune responses, DTH reaction and Th$_1$ and Th$_2$ cytokine levels were significantly suppressed by exposure to endosulfan. Regulatory T (T$_reg$) cells secrete anti-inflammatory cytokines such as IL-10 due to increased production of pro-inflammatory cytokines predominantly of Th$_1$ subtypes$^{39}$, which results in the resolution of the inflammatory responses$^{40}$. Increased secretion of IL-10 significantly decreases the ability of antigen presenting cells to present antigen to T lymphocytes. As a result of inability of antigen presenting cells to present antigen to T lymphocytes, induction of Th$_1$ mediated response is inhibited. Decreased humoral and cellular immune response after endosulfan exposure may be attributed to increased oxidative stress or decreased antioxidant levels during stress. These endosulfan induced immunological changes were not only prevented by treatment with melatonin but also restored towards normalcy due to its free radical scavenging and anti-oxidant property. The results of the immunological studies are with agreements with our previous study which indicates that role of oxidative stress in endosulfan induced immunotoxicity in rat$^7$.

Further, to evaluate the role of free radical induced damage caused by endosulfan induced environmental stress in the brain, the brain homogenate was assessed for lipid peroxidation parameters. Significant increase in the levels of lipid peroxidation product malondialdehyde (MDA) was observed after endosulfan exposure, while significant decrease in the endogenous antioxidant enzymes SOD and CAT in the red blood cells and GSH was observed in serum of the endosulfan exposed animals. These endosulfan induced changes were significantly attenuated/reversed by treatment with melatonin (10 and 50 mg/kg). Treatment with melatonin (10 and 50 mg/kg) leads to marked increase in the activity of superoxide radical (O$_{2}^{-}$) scavenging enzyme SOD and CAT as well as GSH levels.

Taken together, results of the immunological and biochemical studies suggested that emotional and environmental stressors-induced immunosuppression may be associated with decreased antioxidants defense and increased oxidative stress. Hence, exogenous antioxidants treatments may delay or prevent such emotional and environmental stressors induced immunomodulation.

Conclusion

Taken together, results of the immunological and biochemical studies suggested that xenobiotic (endosulfan) induced immunomodulation may be associated with decreased antioxidants defense and increased oxidative stress. Hence, exogenous melatonin treatment may delay or prevent such environmental stressors-induced immunomodulation.
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Conflict of interest

All authors declare no conflict of interest.

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