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Protective effect of melatonin against propoxur-induced oxidative stress and suppression of humoral immune response in rats

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Effect of melatonin in attenuation of propoxur induced oxidative stress and suppression of humoral immune response was studied in rats. Oral administration of propoxur (10 mg/kg) increased lipid peroxidation in serum after 28 days treatment. Superoxide dismutase, catalase and glutathione were also altered following propoxur exposure. In addition propoxur exposure markedly suppressed humoral immune response as assessed by antibody titre and plaque forming cell assay. Simultaneous treatment with melatonin (5 mg/kg, ip) markedly attenuated the effect of propoxur on (a) lipid peroxidation, (b) oxidative stress parameters and (c) immunotoxicity. Results have been discussed in the light of possible immunopotentiating and antioxidant effects of melatonin to understand the influence of oxidative stress on propoxur induced immunomodulation.

Keywords: Carbamate, Humoral immunity, Immunotoxicity, Lipid peroxidation, Pesticide

Propoxur (2-isopropoxy phenyl *N*-methyl carbamate) is a well known carbamate insecticide. Carbamates quickly paralyze the nervous system of insects by blocking the production and action of cholinesterase thereby known for its rapid knockdown effect. Widespread application of propoxur in public health programmes and households (and to a lesser extent in agriculture) in many countries inevitably results in exposure of the population to higher (producers/distributors/hygiene workers) or lower (household) levels of the pesticide[1]. Several cases of suicidal and occupational poisoning have also been reported[2-5]. Hence, propoxur has generated considerable concern regarding its subtle health effects.

Possible effect of persistent exposure to propoxur on oxidative stress and functional integrity of the immune system has heightened interest in these parameters as additional indices to analyse potential long term health effects[3,6,7]. Recent studies on animals and human from our laboratory suggest that propoxur can cause oxidative stress and immunotoxicity [4,5,8,9]. Both, humoral and cell mediated immune responses were suppressed in rats exposed to subchronic doses (10, 30 and 90mg/kg body wt) of propoxur in a dose dependent manner[9]. At present, little is known about the mechanism of immunotoxicity of propoxur. We have earlier reported that propoxur (10, 30 and 90mg/kg body wt) exposure results in increased lipid peroxidation coupled with altered levels of oxygen free radical (OFR) scavenging enzymes and glutathione redox system in male albino rats[5], thereby causing oxidative stress, which may profoundly affect the immune system[10]. Hence, it is worthwhile to study the role of oxidative stress in propoxur induced immunosuppression. Since, melatonin (5mg/kg ip) has been reported to have a protective role on paraquat, phosphine and other pesticide induced oxidative stress[11-13], we have chosen this compound and the lowest dose of propoxur (10 mg/kg orally) known to cause immunosuppression as well as oxidative stress, in this study. If oxidative stress contributes to propoxur induced immunosuppression it is logical to expect that exogenous supplementation of melatonin, an antioxidant, may attenuate partially or completely the immune dysfunction associated with exposure to this carbamate.

Materials and Methods

Technical grade (99.4% purity) propoxur was obtained through the courtesy of M/s Bayer AG,

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Monheim, Germany. Melatonin was procured from M/s Dabur India Ltd., Delhi, India. Bovine serum albumin, *o*-phenylenediamine (OPD), were obtained from Sigma-Aldrich, New Delhi. Pyrogallol and 5,5-bis-dithionitrobenzoic acid (DTNB) were obtained from E.Merck (Mumbai). All other reagents used were of analytical grade and obtained either from Sisco Research Laboratories, Mumbai or Qualigens Fine Chemicals, Mumbai.

Wistar strain male albino rats, weighing 200-250 g, were used for the study. The animals were kept under standard laboratory conditions i.e. 12 hr. light/dark cycle, $22^{o} \pm 2^{o}$ C and RH 70 ± 10%. Necessary approval was obtained from the institutional animal ethical committee for the study. Food consumption, general condition and any other clinical symptoms were observed daily and body weights were recorded weekly. In conducting experiments on animals regards have been maintained to comply with the conditions as desired by Institutional Ethical Committee-Animal Research, University College of Medical Sciences, Delhi. Rats were randomly divided into four groups (eight animals in each). Group I received daily groundnut oil (orally) or normal saline intraperitoneally (ip). Propoxur was dissolved in groundnut oil of pharmaceutical quality and administered orally to Group II animals at a dose of 10mg/kg body wt/day using syringe and 20 gauge Ryle's tube. Melatonin was grounded in mortar-pestle, suspended in normal saline (NS) and administered to group III rats at a dose of 5 mg/kg body wt/day (ip). Group IV animals received daily dose of both propoxur (10mg/kg body wt, orally) and melatonin (5 mg/kg body wt; ip). The rats were treated with vehicle or test chemicals for 28 days. The animals were immunized with 2×10^{9} SRBC (ip in 0.5 ml PBS) on the 24^{th} day of treatment. Four days later, the spleen was removed and the plaque forming cells (PFC/10^{6} cells) were determined[14].

After overnight fasting, animals were sacrificed by decapitation and heparinized blood samples were collected and processed for erythrocyte isolation. Whole blood samples were also collected and serum was separated for various biochemical investigations. Hemoglobin concentration in hemolysate was estimated spectrophotometrically at 540 nm with Drabkin's reagent. Serum protein was estimated by the method of Lowry *et al*[15]. Tsuchihasi extract was prepared as described by Banerjee *et al*[3]. Malondialdehyde (MDA) level in serum (as an index of *in vivo* lipid peroxidation) was determined as per the method described by Satoh[16] using thiobarbituric acid reagent (TBA). MDA-TBA adduct formation was measured spectrophotometrically at 532 nm. Concentration of MDA was expressed as nmol/ml. The activity of superoxide dismutase (SOD) in Tsuchihasi extract was measured by the method of Nandi and Chatterjee[17]. The unit of activity has been defined as the amount of enzyme that inhibits the rate of autooxidation of pyrogallol by 50% under standard conditions. Catalase (CAT) activity in Tsuchihasi extract was determined as described by Sinha[18]. Activity of SOD and CAT was expressed as U/gHb. Total glutathione (GSH) content in blood was measured by the method of Beutler[19] using DTNB. The concentration of blood GSH was expressed as mg/g Hb.

The serum antibody titre to SRBC was measured by haemagglutination technique[20]. The antibody titre was expressed as $\log_2$ of the reciprocal of the first dilution where no visible agglutination was observed.

Data was analyzed by ANOVA test using SPSS version 5 statistical program and compared each treatment using Tukey's multiple comparison procedure. Spearman's test was used for correlation analysis. *P* value at least 0.05 was considered as significant.

Results and Discussion

Melatonin, a major secretory product of the pineal gland has been reported to scavenge hydroxyl radical, peroxynitrite, singlet oxygen and possibly peroxyl radicals which are generated during oxidation of unsaturated lipids and lead to propagation of lipid peroxidation[21-23]. Melatonin may reduce oxidative stress by stimulating some important antioxidative enzymes, i e, SOD, glutathione reductase (GR), etc. The effectiveness of melatonin is facilitated by its combined lipophilic and hydrophilic character[24-25]. Keeping this in view, the present study was initiated to determine whether oxidative stress is involved in reducing humoral immunity in propoxur exposed albino rats with melatonin as an antioxidant of choice.

Effects of propoxur and melatonin treatment on primary antibody response and direct splenic plaque forming cells against SRBC in rats are shown in Table 1. The rats exposed to propoxur alone showed a significant decrease in primary antibody titre as well as direct splenic PFC (IgM-producing cells) as compared to control. Administration of melatonin

alone had no significant effect on the immune response. However, co-administration of melatonin along with propoxur significantly nullified the effect of the pesticide and brought the level of antibody as well as splenic PFC to near control/normal values.

Effect of propoxur and melatonin on lipid peroxidation, antioxidant enzymes and glutathione content are depicted in Table 2. These results are consistent with our previous reports of increased oxidative stress parameters following propoxur exposure[2,5]. Co-treatment with melatonin, reduced the level of oxidative stress and normalized antibody titre level and IgM-PFC response to SRBC significantly, indicating a reversal of propoxur induced effects on humoral immune function. It is to be mentioned here that we opted for melatonin co-treatment rather than pretreatment, as previous studies have reported lack of protective effect of melatonin pretreatment on oxidative injury, possibly due to its short half-life and quick excretion from the body[26]. Our results indicated that melatonin alone increased the activities of SOD and CAT, but did not alter MDA or GSH levels as compared to control. These results were in agreement with Gultekin *et al.*[13] who have shown that synthesis of SOD and CAT is increased by melatonin. Melchiori[11] has reported that melatonin alone does not change the level of lipid peroxidation and GSH. The present results clearly showed that significant increase in MDA, SOD, CAT levels as well as concomitant decrease in GSH concentration elicited by propoxur were recovered by melatonin co-treatment. It is well known that melatonin can directly suppress oxidative stress through its ability to scavenge hydroxyl and peroxyl radicals[27] and by enhancing the production of endogenous antioxidants[28]. Hence, it can be proposed that protection conferred by melatonin against propoxur induced oxidative stress is probably due to both these abilities of this compound.

The hypothesis that the effect on humoral immune response may be a manifestation of increased oxidative stress is supported by the finding that propoxur intoxication is accompanied by increase in serum MDA level (indicative of increased lipid peroxidation) and a decrease in total GSH content in erythrocytes. The significant negative correlation between MDA and antibody response ($r = -0.76$, $P<0.001$), between SOD and antibody titre ($r = -0.69$, $P<0.001$), and CAT and antibody titre ($r = -0.84$, $P<0.001$) along with a positive correlation between GSH content and antibody titre ($r = +0.91$, $P<0.001$) further support the above contention. Melatonin, *per se* did not have any effect on antibody production or GSH content in the present study (Tables 1, 2), nevertheless, melatonin attenuated both immunotoxic and oxidative stress effects of propoxur, which further suggested a possible nexus between propoxur, free radical generation and immunotoxicity. The immune system is regulated by an intricate mechanism and free radicals play an important role in immune regulation[3]. Increased OFR generation due to pesticide exposure can exert deleterious effect on immune function as OFR has many molecular and cellular targets in the immune system[5]. Thus, attenuation of propoxur induced oxidative stress and immunosuppression by melatonin, an antioxidant with potent hydroxyl and peroxyl radical scavenging activity, supported our hypothesis that oxidative stress contributed to propoxur induced immune alteration/suppression. Further, confirmatory studies using other antioxidants such as vitamin E, and C etc. need to be undertaken to understand the influence of oxidative stress on propoxur induced immunotoxicity.

Table 1— Effect of propoxur and melatonin on humoral immune response in albino rats[1]

[Values are mean ± SD of 8 rats/group]

Treatment (mg/kg body wt)	Antibody titre($-\log_2$)	PFC/10^6 splenic cells
Control	8.01 ± 0.5	2600 ± 340
Propoxur (10)	5.00 ± 1.0[a]	1150 ± 400[a]
Melatonin (5)	8.50 ± 1.0[b]	2800 ± 315[b]
Melatonin (5)+Propoxur (10)	7.50 ± 0.5[b]	2200 ± 250[b]

Animals were immunized with SRBC. Significantly different from [a] control and [b]propoxur treated group; ($P<0.05$).

Table 2— Effect of propoxur and melatonin on serum level of malondialdehyde, activity of antioxidant enzymes and glutathione content in erythrocytes of albino rats[1]

[Values are mean ± SD of 8 rats/group]

Treatment (mg/kg body wt)	MDA (nmol/ml)	SOD (U/gHb)	CAT (U/gHb)	GSH (mg/gHb)
Control	2.90 ± 0.19	742.64 ± 22.8	2.26 ± 0.18	2.25 ± 0.14
Propoxur (10)	3.82 ± 0.22[a]	1216.22 ± 34.6[a]	3.92 ± 0.29[a]	1.10 ± 0.05[a]
Melatonin (5)	2.88 ± 0.14	863.55 ± 40.8[a]	2.50 ± 0.21[a]	2.00 ± 0.17
Propoxur (10) + Melatonin (5)	3.00 ± 0.13[b]	885.86 ± 62.8[a,b]	2.62 ± 0.22[a,b]	2.18 ± 0.20[b]

Significantly different from [a]control and [b]propoxur treated group; ($P<0.001$).

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