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AMELIORATIVE EFFECT OF MELATONIN ON 2,4-DICHLOROPHENOXY ACETIC ACID (2,4-D) EXERTED HEPATOTOXICITY IN MICE

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ABSTRACT
The present study was undertaken in order to evaluate ameliorative efficacy of melatonin (10mg/kg) against the toxic effects of 2, 4-dichlorophenoxyacetic acid (2,4-D) at a dose of 75 mg/kg body weight for 45 days in liver of mice (*Mus musculus*). The parameters studied were biochemical indices followed by antioxidant markers in liver of all groups. Alterations of the liver dysfunction were confirmed by decrements in most of them like succinate de hydrogenase (SDH), adenosine tri phoshatase (ATPase), acid phosphatase (ACPase) and alkaline phosphatase (ALPase), superoxide dismutase (SOD), catalase(CAT), glutathione (GSH), total ascorbic acid (TAA) and total protein levels followed by an increased lipid peroxidation (LPO) levels. This indicated that 2,4-D had definite toxic effect on this vital organ with respect to its structure, metabolism and function in intoxicated mice. These 2,4-D induced effects were effectively mitigated by supplementation of melatonin, as noticed in this study. Melatonin therefore mitigates these toxic effects exerted by this toxicant due to its free radicals scavenging cascade which other wise has no toxicity at this dose level.

INTRODUCTION

India is an agricultural country. About 75% of population depends on agriculture. One of the most commonly used herbicide is 2, 4-dichlorophenoxyacetic acid (2,4-D). The most common form of 2,4-D is the acid form, which is toxic. Once absorbed into the circulation, the various salts and esters of 2,4-D are hydrolyzed from the parent compound, which move freely into most tissues. As soon as intake stops, 2,4-D moves out of tissues to the kidneys and is excreted unchanged within a short time. This behavior has been studied in humans Sauerhoff *et al*., 1977) as well as experimental animals. In a two-year study with rats, 2,4-D caused kidney lesions (WHO, 1997). Chronic effects of 2,4-D have also been reported in people. Several physicians have published reports of liver disease (hepatitis) associated with exposure to it. The 2,4-D is known to provoke generation of free radicals, increases the lipid peroxidation process, depletion of ATP, NADPH and GSH concentrations and also modulates the activity of antioxidant system. It also affected reproduction leading infertility in rats (Joshi *et al*., 2012). Melatonin is a hormone and has direct scavenger of reactive oxygen species (ROS) such as O_2,-ROO, H_2O_2. (Poeggeler *et al*., 1994; Kumar *et al*., 2013). It on the other hand, once oxidized, cannot be reduced to its former state because it forms several stable endproducts upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan *et al*., 2000) and also found in animals and plants and known to be a strong antioxidant (Paredes *et al*., 2009). However, its protective role on hepato toxicity induced by 2,4-D in rodent model is scanty. Hence, this study has been proposed in mice.

MATERIALS AND METHODS

Healthy adult male albino mice of Swiss strain, weighing between 30-50g were obtained from Zydus-Cadila Pharmaceutical, Ahmedabad, Gujarat, India, under the Animal Maintenance Registration No. 167/1999/CPCSEA from the Ministry of Social Justice and Empowerment, Govt. of India. The animals were housed in an air conditioned animal house at a temperature of 26 ± 2^{o} C and exposed to 10-12 hours of day light and relative humidity of 30-70%. Animals of different experimental groups were caged separately and maximum of five animals per cage were maintained on a standard animal diet, obtained from Pranav Agro Industries (Baroda), containing wheat-70%, gram-20%, fish meat-5% and yeast powder-5% and distilled water was given. The control and all treated groups of animals were given free access to standard diet. The animals were acclimatized seven days prior to the commencement of the treatment and were divided into four major groups and the treatments administered were as shown in Table 1.

The compound 2,4-D was obtained from Loba Chemie, Mumbai, India with 99% purity. Melatonin (H≈99.0% pure) was also obtained from Hi media Laboratories, Mumbai, India. All the other chemicals used in different assays were procured from Sigma Chemicals (USA) and Hi Media (Mumbai). The dosage of 2,4-D (Fig. 1) was decided based on LD50 370 mg/kg in mice (Gervais *et al*., 2008). The dosage of melatonin (Fig. 2) was decided based on earlier work of Rao and Rajendra

(2012).The treatments were given orally (2,4-D) or intraperitoneally (Melatonin) to experimental animals. The animals were provided only standard diet with water ad libitum.

The control and treated animals were sacrificed by cervical dislocation after each treatment. The liver was excised carefully, blotted free of blood before weighing and used for carrying out antioxidant studies on superoxide dismutase (SOD) (Kakkar *et al*., 1984), lipid peroxides (LPO) (Ohkawa *et al*., 1979), catalase (Luck, 1963), glutathione (GSH) (Gru¨nert and Philips, 1951), total ascorbic acid (Roe and Ku¨ether, 1943) in all groups were done using standard methods. Other biochemical parameters studied were succinate dehydrogenase (SDH); (Beatty *et al*.,1966), adenosine triphoshatase (ATPase); (Quinn and White,1968), acid phosphatase (ACPase); (Bessey *et al*., 1946), and alkaline phosphatase (ALPase); (Bessey *et al*., 1946) and total protein (Lowry *et al*.,1951) along with gravimetric analysis. All enzymes were expressed as per mg proteins.

Statistical analysis

The data were statistically analyzed by student's't' test and analysis of variance (ANOVA) (Ipsen and Feigel**,** 1970). Data were also analyzed using Graph Pad Prism 5.0 statistical software. P values <0.05 were considered significant.

RESULTS

Gravimetric indices

Body weight

A significant ($p<0.01$) reduction was marked in whole body weight of toxicant treated mice as compared to control group. Melatonin and 2,4-D co-administration to male mice indicated restoration in the body weights, where as melatonin alone had no effect as compared to control groups. Same pattern was also noticed with respect to percent relative weights. (Table 2).

Organ weight

The liver weightdecreasedsignificantly($p<0.05$) by the treatment as compared to controls for 45 days. However, supplementation of melatonin to treated mice restored the weight's of liver .Melatonin alone treatment had no effect. Percent relative weight also exhibited the same trend. (Table 2).

Antioxidant enzymes, proteins and lipid peroxidation

The antioxidative enzymes SOD and catalase were significantly ($p<0.001$) reduced by 2,4-D treatment. In combination, these enzyme activities were significantly ($p<0.05$) recovered as compared to 2,4-D treated groups and were comparable to control groups. Same result was noticed in the level of total proteins comparatively. LPO levels were recorded significantly ($p<0.001$) high in treated groups as compared to controls. Supplementation of melatonin to treated groups, a significant ($p<0.05$) amelioration was noticed and were comparable to control and melatonin alone treated groups (Table3).

Non-enzymatic antioxidant indices

In non-enzymatic antioxidant indices, GSH level was decreased ($p<0.001$) in liver of 2,4-D treated group as compared to control and melatonin alone treated mice. Supplementation of melatonin, a marked ($p<0.05$) recoveryin GSH level was noticed as comparedto treated groups. The totalascorbate level was also significantly ($p<0.01$) reduced by 2,4-D treatment.In combination (2,4-D +Melatonin), its level was significantly ($p<0.05$) mitigated as compared to 2,4-D treated and comparable control groups. Melatonin supplementation alone had no effect (Table 4).

Other biochemical indices

The changes in biochemical indices in the liver were confirmed by decrements ($p<0.01$) in SDH and ATPase in treated group of mice as compared to control and melatonin alone groups. There was a significant increase ($p<0.05$)in SDH level by the supplementation of melatonin along with toxicant fed group and was comparable to other groups (Graph 1). In ATPase activity, amelioration ($p<0.05$) was noticed in melatonin supplemented 2,4-D fed mice as compared to 2,4-D treated groups and was similar to normal mice (Graph 2).

The ACPaseand ALPase levelswere significantly ($p<0.01$) reduced by the treatment.In combination (2,4-D +Melatonin) and melatonin alone supplementation groups, there were no significant reduction occurred as comparable to control group.However, significant mitigation ($p<0.01$) was noticed by the combined treatment with respect to group III (Graphs 3 and 4).

DISCUSSION

The present study was undertaken in order to evaluate ameliorative efficacy of melatonin against toxic effects of 2,4-D in liver of adult male mice *(Mus musculus)* at a dose of 75 mg/kg body weight for 45 days. The various parameters studied at the end of the treatment were body and organ weights. The levels of total proteins, phosphatases, succinate dehydrogenase and ATPase were biochemically evaluated. In addition the activities of some antioxidants indices were also evaluated in the vital organ liver.

Gravimetry index is one of the parameters useful for assessment of toxic nature of the toxicant. Numerous herbicides and pesticides are known to induce alterations in body and organ weights, affecting growth and appetite due to less intake of food. Few like 2,4-D are also known to act as endocrine disrupters (Rao and Bhatt, 2012).The loss of organ and body weights in our study indicate protein synthesis inhibition which is the builder of tissue and body growth, and may also be due to less food intake and loss of appetite leading as cited by Rao *et al*. (2010). Growth is dependent on biomolecules synthesized in animals, under hormonal control and inhibition of theirsynthesis in these animals subsequently might led to a loss of their body and organ weights by 2,4-D feeding. (Joshi *et al*, 2012).

In the present study too revealed a significant decrement in total protein levels in this hepatic tissue after 45 days. In support of our data Caroline (1999) reported that it inhibits an enzyme involved in the metabolism of lipids and protein synthesis. The overall decline in protein levels in our study due to the treatment of 2,4-D would affect the activities of various enzymes in the cell and organ metabolism in liver of fed mice. Reduction in the total protein levels has been

Table 1: Distribution of animal groups

Groups	Treatment and dose	Duration (days)	Day of autopsy	No. of animals used
I	Control (untreated)	45 days	46th day	10
II	Melatonin alone (10 mg/kg body weight)	45 days	46th day	10
III	2,4-D treated + Melatonin (75 mg/kg body weight)	45 days	46th day	10
IV	2,4-D treated + Melatonin	45 days	46th day	10

Table 2. Body (gm) and organ weights (gm) of control and treated groups of mice

Group	Body weight		Organ weight	
	Body weight(gm)	Relative weight(%)	Organ weight(gm)	Relative weight(%)
I	41.60 ± 1.43	100	2.26 ± 0.29	100
II	43.00 ± 1.55^{NS}	103.36	2.41 ± 0.30^{NS}	106.63
III	$33.00 \pm 1.2^{**}$	61.06	$1.38 \pm 0.17^{*}$	61.06
IV	37.60 ± 0.75^{NS}	84.51	1.91 ± 0.19^{NS}	84.51

Values are Mean ± S.E., Gr. = Group, NS = Non significant (Gr. I vs II & IV), **p<0.01, *p<0.05; (Gr. I, II & IV vs III).

Table3. Enzymatic antioxidant indices, proteins and lipid peroxidation (LPO) in liver of control and experimental groups

Parameters	Control (Gr.-I)	Melatonin(Gr.-II)	2,4-D (Gr.-III)	2,4-D + melatonin(Gr.-IV)
Total Proteins[a]	15.86 ± 0.18	15.96 ± 0.48^{NS}	$7.89 \pm 0.84^{***}$	$13.89 \pm 0.40^{NS,*}$
LPO (TBARS[b)]	30.08 ± 0.75	28.91 ± 0.34^{NS}	$48.09 \pm 0.85^{***}$	$35.01 \pm 0.98^{NS,*}$
SOD[c]	1.78 ± 0.02	1.89 ± 0.51^{NS}	$0.98 \pm 0.03^{***}$	$1.65 \pm 0.56^{NS,*}$
Catalase[d]	38.45 ± 0.89	40.23 ± 0.45^{NS}	$28.45 \pm 0.49^{***}$	$36.42 \pm 0.86^{NS,*}$

ValuesareMean ± S.E., Gr - Groups. NS = Non significant (Gr. I vs II & IV), ***p<0.001 (Gr. I, II &IV vs III), *p<0.05 (Gr. IV vs III); TBARS- Thiobarbituric acid reactive substances. a = mg/100mg tissue weight, b = µ moles of MDA formed/100 mg tissue weight, c = units/mg protein, d = ì moles of H_2O_2 consumed/min/mg protein

Table4: Non-enzymatic antioxidant indices in liver of control and experimental groups

Parameters	Control(Gr.-I)	Melatonin(Gr.-II)	2,4-D (Gr.-III)	2,4-D + melatonin (Gr.-IV)
Glutathione[a]	60.08 ± 0.55	60.19 ± 0.28^{NS}	$48.03 \pm 0.45^{***}$	$53.86 \pm 1.36^{NS,*}$
Total ascorbic acid[b]	2.89 ± 0.45	2.108 ± 0.08^{NS}	$1.66 \pm 0.05^{**}$	$2.01 \pm 0.08^{NS,*}$

Groups I or II or IV compared with Group III or individually. Values are Mean ± S.E., Gr. = Group, NS = Non Significant (Gr. I vs II & IV); *** p<0.001; ** p<0.01 (Gr. I, II, IV vs III), *p<0.05(Gr. IV vs III). a = µ moles/100mg tissue weight, b = mg/gm tissue weight, c = mg/100mg tissue weight.

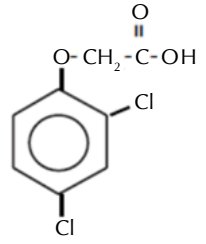


Figure 1: Chemical structure of 2,4-D

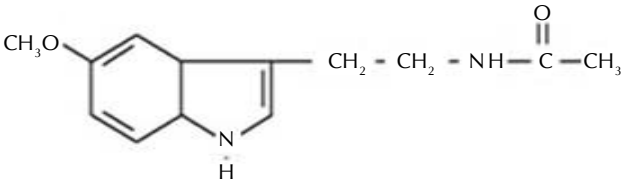
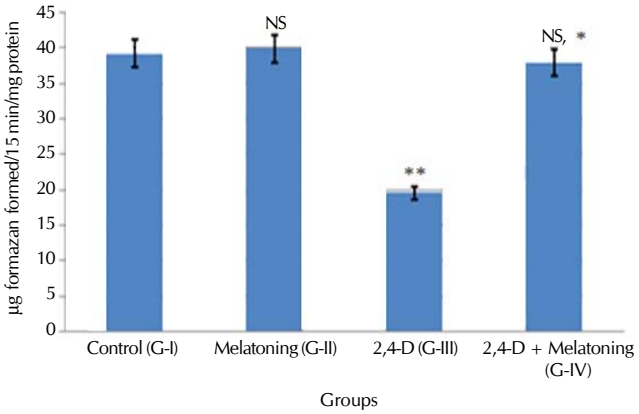


Figure 2: Chemical structure of Melatonin

reported in treated rodents and fish model too by it (Anusaya and Hemlatha, 2013; Vigario *et al.*, 2014). Phosphatases (acid and alkaline) in our study also indicated a decreased trend affecting liver metabolism. Alkaline phosphatase is a membrane stabilizing enzyme and its alteration leads to cell membrane damage. These altered biochemical indices can be related to a loss of liver function also. Altered energy metabolic effects were indicated by a decline in succinate dehydrogenase, a mitochondrial enzyme leading to a reduction in ATP molecules produced from mitochondrial Krebs cycle. Further, energy loss is also supported by ATPase enzyme reduction in herbicide treated mice. Overall the energy output was decreased and was correlated to mitochondrial dysfunction by its treatment which supported our investigation (Bukowiska**,** 2003).

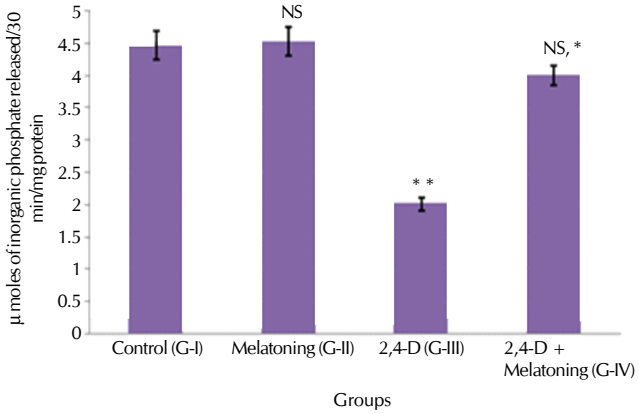
The formation of lipid free radicals and lipid peroxides is considered an important feature of cell injury (Rao and Damore, 2007). The major fatty acids that undergo lipid peroxidation in the cell membranes are all polyunsaturated fatty acids (PUFAs). Abundance of free radicals could undergo uncontrolled chain reactions and lipid peroxidation (Mark *et al.*, 1997). Studies carried out in our laboratory have also revealed an increase in LPO levels by 2,4-D treatment in mice liver.

The enzyme superoxide dismutase is catalyses dismutation of superoxide radical leading to production of hydrogen peroxide which in turn detoxified by an enzyme catalase (Rzeuski *et al.*, 1998). It is an efficient inhibitor of LPO when hydrogen peroxide accumulates in a cell containing free ferrous ions. The enzyme , SOD is considered as a first line of defense against free radical (oxygen) toxicity and central regulator of ROS levels by hydrogen peroxide, which in turn is detoxified by an enzyme catalase to molecular oxygen



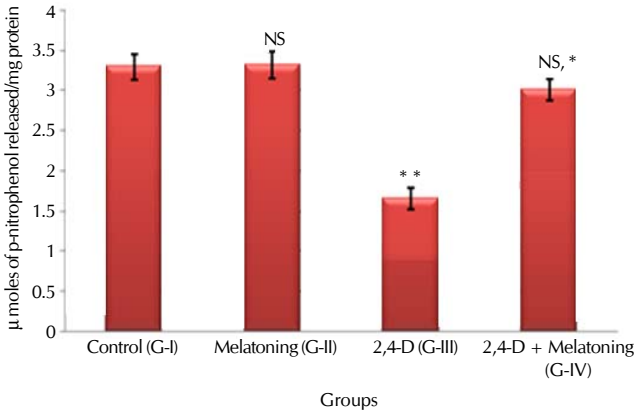
Values are Mean ± S.E., NS = Non significant, Gr.- Group. **p < 0.01 (Grs. I , II and IV vs Gr. III). *p < 0.05, (Gr. IV vs Gr III)

Graph1: Succinate dehydrogenase in liver of control and treatedgroups of mice



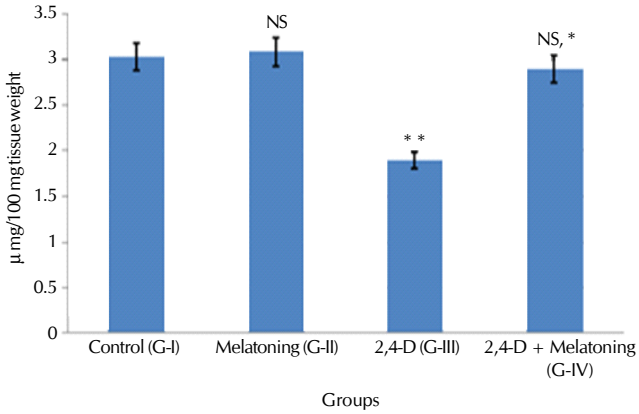
Values are Mean ± S.E., NS = Non significant, Gr.- Group. **p < 0.01 (Grs. I, II and IV vs Gr. III). *p < 0.05, (Gr. IV vs Gr III)

Graph 2: Adenosine triphosphatase in liver of control and treatedgroups of mice



Values are Mean ± S.E., NS = Non significant, **p < 0.01 (Grs. I ,II &IV vs Gr. III; Gr. IV vs Gr. III)

Graph 3: Acid phosphatase in liver of control and treated group of mice



Values are Mean ± S.E., NS = Non significant, **p < 0.01 (Grs. I ,II &IV vs Gr. III; Gr. IV vs Gr. III)

Graph 4: Alkaline phosphatase in liver of control and all treated groups of mice

(Acharya *et al*., 2008). In the present investigation, 2,4-D caused a decline in the activities of free radical scavenging enzymes *viz*., superoxide dismutase (SOD) and catalase in liver tissue studied. Anusaya and Hemalatha(2014) reported that catalase enzyme altered in blood parameter of *Channa Striatus* which tends the fishes to get prone to ancillary stress. Similarly Tayeb *et al*. (2012) observations on Oxidative stress induced by this toxicantalso support our data.

Glutathione (GSH) comprises upto 90% of the non-protein thiol content of mammalian cells. It acts as a nucleophilic 'scavenger' of many compounds and their metabolites via enzymatic and chemical mechanism, converting electrophilic centres to ether bonds. Total thiol(-SH) groups too play an important role in balancing the structure of protein and also the cellular equilibrium. In the present study there is a decrement in the level of GSH in treated mice liver. This might lead to cell injury and death. Glutathione conjugation also helps in detoxification by binding to electrophiles (Chouhan and Flora; 2008). Reduced glutathione is converted to oxidized glutathione (GSSH) by glutathione peroxidase (GPx) by consuming H_2O_2 and again this oxidized glutathione gets converted back to reduce glutathione (GSH) by glutathione reductase (GR) through conversion of NADPH to NADP. Kubrak *et al*. (2013) documented that in gold fish *Cacarious auratus L*. muscle glutathione (GSH) significantly reduced after 2,4-D treatment. These significant changes mentioned above indicated an evidence of oxidative stress exerted by 2,4-D in treated mice liver.

Ascorbic acid (AA) is known to be a powerful reducing agent which acts as an antioxidant by detoxifying several toxic substances and helps in activating several enzymes (Kutsky, 1973; Lewin, 1976). Its reduction in liver indicates its involvement in overcoming stress. The 2,4-D intoxication to mice also brought a significant decline in total ascorbic acid in the liver indicating stress leading to a rapid utilization of it. This suggests that the stored ascorbic acid is rapidly oxidized in the tissue under 2,4-D induced stress and converted it to its dehydro form in this present study. According to Lewin (1976) maintenance of proper levels of ascorbate is essential for protection during stress condition. It also increases C-AMP

levels by hindering the PDE activity in cell physiological changes. This explains for a reduction in hepatic AA levels also. These effects were supported by degenerative changes in treated mice liver in support of earlier observations documented in fish liver (Ortiz *et al*., 2003).

Co-administration of antioxidants like melatonin to treated mice, however body and organ weights did not reveal any significant change. Amelioration of biochemical parameters were observed in our study with melatonin along with 2,4-D to mice. The antioxidant indices were also mitigated. It was further documented that besides melatonin's ability to scavenge ROS, it has been demonstrated activation of antioxidative enzymes by it (Pal and Chatterjee, 2006). Its supplementation also led to an increase in SOD activity in accordance with the finding of Antony *et al*. (2008) who reported that its administration induced an increase in the mRNA levels of dismutase. These beneficial effects of melatonin might be due to its property as an electron donor. Reports also suggested that melatonin detoxifies numerous ROS including H_2O_2, singlet oxygen and also reactive nitrogen species. In support of our data Bongivanni *et al*. (2007) too reported that melatonin decreases the oxidative stress produced by 2,4-D in mice cerebellar granule cells. This capacity to absorb free radicals extends at least to the quaternary metabolites of melatonin, a process referred to as the free radical scavenging cascade (Tan *et al*., 2007) exerting the best protective role against toxicity imposed by the toxicant in this study. It is also known to be better antioxidant than others who documented earlier with other toxicants in rodents (Bindu and Rao, 2008: Rao and Damore, 2007). In conclusion, melatonin supplementation to 2,4-D in toxicated mice brought about a definite restoration in metabolism and function of this vital organ liver in a mice model reported in this study and may be useful to human exposed to such toxicants in agriculture.

Conflict of interest statement

There are no conflicts of interest.

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