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Melatonin Attenuates 2,4-Dichlorophenoxyacetic Acid Toxicity in Kidney of Mice

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Abstract

Protective effects of melatonin against 2,4-Dichlorophenoxyacetic acid (2,4-D) induced nephrotoxicity in adult albino male mice of Swiss strain were evaluated in the present study. Twenty adult male albino mice were assigned into four groups with five mice in each group. Group I served as control; Group II - melatonin alone (10 mg/kg body weight); Group III - 2,4-D alone (50 mg/kg body weight) and Group IV - 2,4-D + melatonin was given orally for 45 days. Ameliorative effects of melatonin against oxidative stress of 2,4-D were evaluated by estimation of total protein, Malondialdehyde (MDA), Total Glutathione (GSH) and Total Ascorbic Acid (TAA) level as well as Superoxide Dismutase (SOD) and Catalase (CAT) activity parameters. Effect of melatonin and 2,4-D alone and in combination on energy metabolism were also studied by assessment of Succinate Dehydrogenase (SDH), Adenosine Triphosphatase (ATPase), Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP) activity. Along with this, the marker for kidney function i.e. creatinine was also evaluated in all studied groups. The significant value was considered at $p<0.05$. Significant reduction in levels of total protein, GSH, and TAA whereas an increase in MDA and creatinine levels were observed after 2,4-D treatment. Moreover, there was a decrease in the activity of SOD, CAT, SDH, ATPase, ACP, and ALP due to the herbicide treatment. These values were comparable to control when melatonin was given alone and in combination with 2,4-D. The study demonstrated nephroprotective potential of melatonin against oxidative stress and altered energy metabolism due to the toxicity of 2,4-D.

Keywords: 2,4-Dichlorophenoxyacetic Acid, Kidney, Melatonin, Metabolic Status, Mitigation, Mouse, Oxidative Stress

1. Introduction

Over the last few decades, agricultural pesticides have become a common household item in rural areas of the developing countries[1]. Farmers are the population most exposed to the pesticide. Occupational exposure to pesticides often occurs in the case of agricultural workers in open fields and greenhouses, workers in the pesticide industry, and home pest exterminators. India being the second largest producer of fruits and vegetables in the world, farmers being ignorant and use harmful pesticides. Nonetheless, it is not just farmers who are exposed to pesticide but along with them, farmer households are also exposed to this chemical as they live near or on the farms and spend much of their time in close proximity to areas where pesticides are applied on a regular basis[2,3]. Pesticide poisoning from occupational, accidental and intentional exposure is a major public health problem of the world, especially in low-income countries[4]. Chlorophenoxy herbicides have been suggested as a potential environmental endocrine disruptor and oxidative damage inducer[5]. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a systemic chlorophenoxy herbicide widely used in the world for broadleaf weed control. This herbicide exerts physiological effects similar to those of auxin (a natural plant hormone), resulting in overstimulation of plant growth and culminating in death[6,7]. Since 2012, 2,4-D has been the most commonly used herbicide in the residential market at 7 to 9 million pounds annually and is the fifth most commonly used herbicide in the agricultural market ranging from 30 to 40 million pounds annually[8]. A previous study evaluating tissue residues of some chlorophe-

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noxy acids in sheep and cattle following oral doses has reported that chlorophenoxy acids and the related phenolic metabolites are found in muscle, fat, liver, and kidney tissues of sheep and cattle; with the highest residues in the kidney[9]. Metabolism of 2,4-D in laying hens and lactating goats has been studied and the most abundant tissue residue of 2,4-D was found in kidneys[10]. Therefore, there are clinical and economic reasons to identify novel and effective therapies against 2,4-D induced nephrotoxicity. Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine mainly produced by the pineal gland and also by many other peripheral tissues and organs[11,12]. Melatonin participates in the regulation of many physiological processes. It is a potent free radical scavenger that possesses antioxidant and anti-inflammatory properties[13,14]. When scavenging free radicals, melatonin is transformed into a series of metabolites that are also free radical scavengers[15]. Melatonin can reduce oxidative stress via stimulating the activities of the anti-oxidative enzymes; Superoxide Dismutase (SOD) and glutathione peroxidase (GSH-Px)[16]. Protective effects of melatonin have been demonstrated in various animal models[17–23], where oxidative stress causes tissue damage, such as sepsis, streptozotocin-induced diabetes, renal ischemia-reperfusion injury, acute pancreatitis, pyelonephritis, renovascular hypertension, and drug-induced toxicity. Moreover, melatonin was beneficial against 2,4-D in preserving histopathological damage of kidney in mice[24]. Because of these previously reported protective effects of melatonin, the present study aimed to investigate against 2,4-D induced nephrotoxicity in mice with respect to oxidative stress and energy metabolism constraints.

2. Materials and Methods

2.1 Chemicals

The herbicide, 2,4-D (CAS No. 94-75-7; Purity: ≥ 98.0%) was obtained from Loba Chemie, Mumbai, India. Melatonin (CAS No. 73-31-4; Purity: ≥ 99.0%) was obtained from HiMedia Laboratories, Mumbai, India. All the other chemicals (AR grade) utilized in different assays were procured from Sigma Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany) and HiMedia Laboratories (Mumbai, India).

2.2 Experimental Animals

Healthy, adult male albino mice of the Swiss strain (*Mus musculus*) obtained from Zydus Cadila Pharmaceutical, Ahmedabad, Gujarat, India were used for all experiments. Animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions (temperature 22 ± 3°C, 12 hours light/dark cycle, and relative humidity 50-55%). They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and water *ad libitum*. All the experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg-167/1999/CPCSEA), New Delhi, India. Animals were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India (2000)[25].

2.3 Treatment

As shown in Table 1, twenty mice were divided into four groups with five mice in each group for 45-day treatment. Group I served as control; Group II was given oral administration of melatonin alone (10 mg/kg body weight), while Group III was given oral administration of 2,4-D (50 mg/kg body weight) and Group IV was treated by 2,4-D along with melatonin at the same dose level as those in Group III and II, respectively.

After completion of the 45-day treatment, on the 46th day, animals were sacrificed by cervical dislocation. The kidney was dissected out carefully, blotted free of blood and utilized for determination of biochemical parameters.

Total protein levels were determined by the method of Folin phenol reagent[26]. Lipid peroxidation was assayed

Table 1. Experimental groups

Groups	Treatment and dose	Duration (days)	Day of autopsy	No. of animals used
I	Control (untreated)	45 days	46th day	5
II	Melatonin alone (10 mg/kg body weight)	45 days	46th day	5
III	2,4-D (50 mg/kg body weight)	45 days	46th day	5
IV	2,4-D + Melatonin	45 days	46th day	5

by determining the level of Malondialdehyde (MDA) by measuring thiobarbituric reactive species (TBARS)[27]. The activities of Superoxide Dismutase (SOD)[28], Catalase (CAT)[29], Succinate Dehydrogenase (SDH)[30], Adenosine Triphosphate (ATPase)[31], Alkaline Phosphatase (ALP)[32], and Acid Phosphatase (ACP)[32] were analyzed by the standard protocols. Levels of Glutathione (GSH)[33], Total Ascorbic Acid (TAA)[34] and creatinine[35] were also quantified by the standard methods.

2.4 Percentage Amelioration

Percentage amelioration in all studied parameters was calculated by using the following formula:

$$\text{Percentage Amelioration}(\%) = \frac{\text{Prooxidant Group} - \text{Respective Antioxidant Group}}{\text{Prooxidant Group} - \text{Control}} \times 100$$

2.5 Statistical Analysis

All the data were expressed as mean±standard error (SE). Statistical analysis was performed using GraphPad Prism software version 7.04. The data were statistically analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey's test. The level of significance was accepted at $p<0.05$.

3. Results

3.1 Effect of 2,4-D and/or Melatonin on Oxidative Stress Indices in the Kidney of Mice

A significant reduction in levels of total protein ($p<0.0001$), GSH ($p<0.001$) and TAA ($p<0.001$) along with the activities of SOD ($p<0.001$) and CAT ($p<0.0001$) in the kidney of 2,4-D treated mice was observed. Moreover, the MDA level showed a significant increase ($p<0.001$) in the kidney of 2,4-D treated mice. However, when melatonin supplemented along with 2,4-D, it demonstrated a significant ($p<0.001$) amelioration in the kidney with respect to these parameters and results were comparable to control, whereas melatonin alone had shown no effect (Table 2).

3.2 Effect of 2,4-D and/or Melatonin on Energy Metabolism and Creatinine level in the Kidney of Mice

2,4-D treated mice showed a significant decrement in the activities of SDH ($p<0.0001$), ATPase ($p<0.001$), ACP ($p<0.001$) and ALP ($p<0.001$) in the kidney. Creatinine level of the kidney was increased significantly ($p<0.001$) in 2,4-D treated mice. However, combined treatment of melatonin and 2,4-D showed a significant ($p<0.001$) amelioration in the kidney with respect to these parameters

Table 2. Effect of 2,4-D and/or melatonin on oxidative stress indices in the kidney of mice

Parameters	Groups			
	I – Control	II – Melatonin	III - 2,4-D	IV- 2,4-D + Melatonin
Total protein	16.97±0.28	16.78±0.19^{NS}	14.88±0.20**	16.60±0.29$^{NS;\#}$ (82.30%)
MDA	26.15±0.34	26.08±0.33^{NS}	28.73±0.35*	26.42±0.33$^{NS;\#}$ (89.53%)
SOD	8.60±0.19	8.45±0.27^{NS}	6.81±0.19*	8.36±0.25$^{NS;\#}$ (86.59%)
CAT	23.28±0.26	23.21±0.22^{NS}	21.01±0.31**	23.00±0.26$^{NS;\#}$ (87.67%)
GSH	107.80±1.28	107.50±1.26^{NS}	98.39±1.48*	107.20±1.07$^{NS;\#}$ (93.62%)
TAA	4.25±0.11	4.20±0.11^{NS}	3.36±0.15*	4.17±0.09$^{NS;\#}$ (91.01%)

Values are Mean ± S.E.; values in parenthesis indicate percentage amelioration by Melatonin.
*p<0.001; **p<0.0001; NS = Non-Significant when groups II to IV were compared with control (group I); #p<0.001 when group IV was compared with group III.
Total protein (mg/100mg tissue weight); MDA (Malondialdehyde) (nano moles of MDA formed/mg tissue weight/60 minutes); SOD (Superoxide dismutase) (units SOD/mg protein); CAT (Catalase) (μ moles of H_2O_2 consumed/minute/mg protein); GSH (Total Glutathione) (μmoles/100mg tissue weight); TAA (Total Ascorbic Acid) (mg/gm tissue weight).

and results were comparable to control, whereas melatonin alone had shown no effect (Table 3).

4. Discussion

2,4-Dichlorophenoxyacetic acid largely uses as a selective herbicide in all over the world[8] and have similar physiological effects as compared to auxin. Many studies showed its adverse effects on various organs of the body and many enzymatic and non-enzymatic molecules present in the cells[5,6,9,36–41]. Amongst them, the protein, which is involved in the architecture and physiology of the cell as well as it seems to occupy a key role in cell metabolism[42] showed a decline in the kidney of 2,4-D treated mice in the present study. Our findings are in accordance with the earlier studies, which showed a reduction in total protein levels in serum of rat after 2,4-D and Desormone lourd intoxication[41,43]. The reduction in protein content in animals treated with pesticides could be attributed to changes in protein and free amino acid metabolism and their synthesis in the liver[44].

The present study also showed that the level of end product of lipid peroxidation i.e. MDA increased in the kidney of mice treated with 2,4-D. Similar results were also obtained by other researchers in various organs including the kidney of fish and rat[36–39]. The increased MDA content may result from an increase in hydroxyl radicals (•OH). However, it is conceivable that 2,4-D might be interacting with the kidney cells resulting in lipid peroxidation processes by the way of increasing in superoxide radicals, unbalance in the generation of these radicals and its scavenging activity. As a result of an increased in lipid peroxidation was observed in our cohort. MDA is a major oxidation product of peroxidized polyunsaturated fatty acids, and increased MDA content is an important indicator of lipid peroxidation. Antioxidant enzymes, such as SOD and CAT play an important role in tissue protection against its impairment by Reactive Oxygen Species (ROS). Hence, it limits the effects of oxidant molecules in tissues and acts in the defense against oxidative cell injury by means of their being free radical scavengers. These enzymes work together to eliminate active oxygen species[45]. The enzyme, SOD can reduce free radical-mediated cellular damage as a result of producing H_2O_2 by dismutasing O_2, and CAT converts H_2O_2 to water and oxygen. The results of present study indicated that 2,4-D exposure decreased SOD and CAT activities in the kidney of mice. Previous findings also showed similar changes in SOD and CAT activities in the kidney of 2,4-D exposed rat[37–39]. This depletion may be due to the decreased synthesis of enzymes or oxidative inactivation of enzyme protein.

Along with SOD and CAT, the GSH also actively participate in the antioxidant defense mechanism. GSH is considered as the essential compound, which maintains cell integrity due to its reducing properties and its participation in cell metabolism. The present result showed that treatment with 2,4-D led to a decrement in renal GSH levels of mice. Our results are in accordance with previous reports which demonstrated that 2,4-D causes a decrease in the level of GSH in human erythrocytes and kidney of adult rats[38–40]. This may be due to their consumption in the scavenging free radicals probably generated by 2,4-D[40]. Along with GSH, the ascorbic acid is also thought

Table 3. Effect of 2,4-D and/or melatonin on parameters related to energy metabolism and level of creatinine in the kidney of mice

Parameters	Groups			
	I – Control	II – Melatonin	III - 2,4-D	IV- 2,4-D + Melatonin
SDH	54.56±0.42	54.40±0.60^{NS}	49.41±0.55**	53.73±0.69$^{NS;\#}$ (83.88%)
ATPase	3.03±0.08	3.03±0.09^{NS}	2.49±0.07*	3.02±0.06$^{NS;\#}$ (98.15%)
ACP	1.08±0.04	1.08±0.04^{NS}	0.81±0.04*	1.06±0.04$^{NS;\#}$ (92.59%)
ALP	0.41±0.02	0.40±0.02^{NS}	0.25±0.02*	0.39±0.02$^{NS;\#}$ (87.50%)
Creatinine	60.18±1.02	59.61±1.02^{NS}	68.03±1.11*	60.92±0.86$^{NS;\#}$ (90.57%)

Values are Mean ± S.E.; values in parenthesis indicate percentage amelioration by Melatonin.
*p<0.001; **p<0.0001; NS = Non-Significant when groups II to IV were compared with control (group I); #p<0.001 when group IV was compared with group III.
SDH (Succinate Dehydrogenase) (µg formazan formed/15 minutes/mg protein); ATPase (Adenosine Triphosphatase) (µ moles of inorganic phosphate released/30 minutes/mg protein); ACP and ALP (Acid Phosphatase and Alkaline Phosphatase) (µ moles of p-nitro phenol released/30 minutes/mg protein); Creatinine (µg /100mg tissue weight).

to be an important water-soluble antioxidant, which neutralizes ROS and reduces the oxidative stress[46]. A decline of vitamin C levels in kidney tissue of the present study was found to be of major importance due to the main role of this antioxidant to protect kidney under chronic exposure to 2,4-D. Troudi and co-workers[38] also found a reduction in vitamin C level of the kidney of 2,4-D fed rat mothers and their pups.

The administration of 2,4-D to mice might led a reduction in glomerular filtration rate portrayed by the reduced creatinine clearance and a decline of creatinine excretion, which correlated in the present study with the increased creatinine level in kidney. These findings imitate the diagnosis of renal failure and support the previous data which showed a rise in plasma and reduction in urinary levels of creatinine in mice[37,38].

Regarding the energy metabolism, the SDH is an enzyme involved in the Krebs cycle, and any abridgment in the activity of this enzyme leads to impairment in ATP generation process of the cell[47]. The present study showed a decrement in SDH activity in the kidney of 2,4-D fed mice. The decrease in SDH activity indicates the impairment of oxidative metabolism in the mitochondria as a consequence of hypoxic conditions under pesticide exposure. Zhu and co-workers also showed a reduction in SDH activity exposed to various concentrations of pesticide triazophos fed *Gobiocypris rarus* embryo and larvae[48]. Another study also found a decrement in the activity of Succinate Dehydrogenase (SDH) in the liver of adult zebrafish after exposure to pesticide thifluzamide[49]. Besides SDH, the ATPase, ACP, and ALP are a class of enzymes that catalyze the decomposition of ATP into ADP and a free phosphate ion[47]. In our study, a reduction in ATPase activity of kidney after 2,4-D administration to mice was noted which is corroborated with the previous study of triazophos fed *Gobiocypris rarus* embryo and larvae[48]. This reduction in ATPase activity might be due to reduced energy metabolic rate in the kidney region. Moreover, ACP is a hydrolytic lysosomal enzyme for the hydrolysis of foreign materials and ALP is a membrane-associated enzyme which participates in the membrane transport mechanism. The present study showed a decline in activities of ACP and ALP in the kidney of 2,4-D fed mice which might be due to alterations in its lysosomal activity and membrane permeability, respectively[50].

Melatonin easily enters the cells as well as crosses the blood-brain barrier. The protective action of melatonin against LPO as a factor modifying membrane organization, may due to melatonin's ability to scavenge the LPO initiating agents, which produced during the peroxidation of lipids[51]. Melatonin is an effective scavenger of reactive oxygen and nitrogen species including singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite anion ($ONOO^-$). In addition, melatonin can inhibit the catalytic activity of nitric oxide synthase and expression of inducible nitric oxide synthase at the transcriptional level that reduces the amount of NO produced[52]. Besides direct scavenging activity, melatonin can stimulate gene expression and activities of several antioxidative enzymes including glutathione reductase, glutathione peroxidase, CAT, and SOD, and this indirect antioxidant activity of melatonin is related to its interaction with either membrane or nuclear receptors. These antioxidative enzymes can metabolize free radicals to less reactive species and thereby decrease the formation of highly toxic by-products. It has also been shown that melatonin prevents the loss of vitamin C[53] and by stimulating its rate-limiting enzyme, g-glutamylcysteine synthase, increases the synthesis of GSH which ultimately leads to strengthen the defense against free radicals. Due to the above mentioned mechanism, it is believed that melatonin can ameliorate the free radical toxicity generated by 2,4-D in the present study.

On the other hand, melatonin upregulates the production of ATP, which is vital for cellular functions including the repair of molecular damage created by free radicals[54–56]. Melatonin is also reported for decreases mitochondrial oxidation by normalizing SDH, Lactate Dehydrogenase (LDH), Glucose-6-Phosphate Dehydrogenase (G6PDH), Aspartate Aminotransaminase (AST), Alanine Aminotransaminase (ALT), and Glutamate Dehydrogenase (GDH) activities and hence it plays a crucial role in restoring mitochondrial function[57]. Melatonin was also reported to be an effective protective agent in preventing the 2,4-D effect on redox balance of rat cerebellar granule cells *in vitro*[58]. Hence, it can be postulated that melatonin can also restore the impaired energy metabolism. In the present study the activity of energy metabolism related enzymes i.e. SDH, ATPase, ACP, and ALP were maintained after co-administration of melatonin and 2,4-D. Due to ameliorative effects on free radical toxicity and altered energy metabolism, the overall functions of the kidney also restore which was depicted by decreased in creatinine level in the kidney after co-treatment of melatonin and 2,4-D. In support of

our findings Ghaznavi and co-workers[59] also showed that melatonin was able to decrease significantly the higher creatinine value against gentamycin induced nephrotoxicity in rats. The overall results of the present study suggest that the protective effect of melatonin may be caused by its antioxidant properties.

5. Conclusion

Generation of free radicals due to the toxic effect of 2,4-D is a major concern to the places where it has been used the most. Our data indicated that 2,4-D altered the renal functions in the kidney by induction of free radical toxicity and by impairment of energy metabolism. The co-administration of melatonin exhibited ameliorative effects against renal dysfunctions by decreasing or scavenging the free radicals and improves the energy metabolism which also depicted by a decrease in kidney creatinine level. Hence, melatonin works as a good protective molecule against 2,4-D induced toxic effects in a mouse model.

6. Conflict of Interest

The authors declare that there is no conflict of interest.

7. References

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