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Free radical-induced nephrotoxicity following repeated oral exposure to chlorpyrifos alone and in conjunction with fluoride in rats

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Background/aim: Chronic renal disorder is becoming a major health problem worldwide. The purpose of the present study was to investigate alterations in the renal antioxidant system in rats induced by repeated exposure to chlorpyrifos (CPF) alone and in conjunction with fluoride.

Materials and methods: Wistar rats were randomly allocated to seven groups, each consisting of six rats, and were subjected to different treatment regiments for 28 days.

Results: Significant increases ($P < 0.05$) in plasma protein, blood urea nitrogen, and creatinine levels indicated alterations in renal functions on repeated exposure to CPF or fluoride; moreover, these changes were more pronounced in animals exposed to both toxicants concurrently. A significant increase ($P < 0.05$) in malondialdehyde levels and decreases in superoxide dismutase, catalase, and glutathione peroxidase activities in renal tissue were noted, indicating renal damage on exposure to CPF, fluoride, or the combination of those.

Conclusion: Our observations suggested that the concurrent exposure to CPF and fluoride increased the extent of renal damage. These findings indicate that this damage is due to increased free radical formation and a reduced function of the antioxidant system in renal tissue. Thus, the application of CPF as an insecticide should be reduced in areas where the fluoride levels in ground waters are high in order to minimize renal damage in exposed populations.

Key words: Antioxidant status, chlorpyrifos, fluoride, nephrotoxicity

1. Introduction

Kidneys are vital organs that are essential for maintaining the composition and volume of body fluids, the acid–base balance, and the redox status. Chronic renal disorder is becoming a major health problem. Protein synthesis inhibition and glutathione depletion have been recognized as common pathophysiological mechanisms of renal tissue damage. Oxidative stress due to increased generation of free radicals in the renal tissue contributes to the depletion of glutathione levels and functional status disruptions in structural and/or transporter proteins (1–3).

Chlorpyrifos (CPF) is a conventional organophosphorus insecticide commonly used to control a variety of pests in agriculture and veterinary practices. Indiscriminate and nonselective applications of CPF have inadvertently polluted the environment (4,5). Similarly, endemic fluorosis has been reported in all inhabited continents (6) due to increased levels of fluoride in food and ground waters. Both of these toxicants are known to cause tissue damage due to increased generation of free radicals or reduced antioxidant defenses of the organism. Various studies have implicated oxidative damage as the central mechanism of toxicity of pesticides and other environmental contaminants. Fluoride, along with other environmental contaminants, is known to increase the production of reactive oxygen species (ROS)/free radicals (7,8). Free radicals/ROS-induced oxidative cellular damage results from the interaction of such radicals with the sulfhydryl group (-SH) of different cellular macromolecules and membrane lipids, as indicated by increased malondialdehyde (MDA) levels (the end product of lipid peroxidation) in different body tissues (9,10). The aim of the present study was to investigate the renal antioxidant status and extent of renal damage induced by repeated exposure to CPF, fluoride, or the combination of CPF and fluoride in Wistar rats.

2. Materials and methods

2.1. Experimental animals

Healthy Wistar rats weighing 175 ± 25 g were obtained from the Animal Breeding House of the Indian Institute of Integrative Medicine at the Council of Scientific and

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Industrial Research Lab in Jammu, India. The animals were maintained in clean plastic cages (6 rats/cage) in the divisional laboratory animal house at 23 ± 2 °C for acclimatization for 1 week prior to the start of experiments. The rats were fed standard pellet diet, tap water was available ad libitum, and they were kept under a 12-h dark/light cycle. The experimental protocol was approved by the university's Animal Ethics Committee (vide No AU/FVSc/C-11/2456-68).

2.2. Experimental design

The rats were randomly allocated to seven groups, each consisting of six rats, and were subjected to different daily treatment regiments for 28 days. The rats in group I served as controls and received only normal tap water for drinking. The animals in groups II and III were provided drinking water containing fluoride at the rates of 1 mg/L (1 ppm) and 10 mg/L (10 ppm) of water, respectively. The rats in groups IV and V were administered CPF (oral gavage) at the rates of 1 mg/kg and 10 mg/kg of body weight, respectively. The animals in group VI were provided, through oral gavage, both water containing fluoride at the rate of 1 mg/L and CPF at the rate of 1 mg/kg of body weight. The animals in group VII received higher levels of both, fluoride (10 mg/L) in their drinking water and CPF (10 mg/kg of body weight daily). In order to minimize their possible instability, both toxins were prepared freshly in water. All rats were weighed weekly to make necessary corrections in the CPF dosage as per body weight. In the present study the No Observed Adverse Effect Level (NOAEL) dose of fluoride (1 ppm) and 10 times more than the NOAEL dose (10 ppm) were used for induction of fluoride toxicity; 1 mg/kg of body weight was the minimum reported dose of CPF needed to induce neurotoxicity in animals and doses of 1 and 10 mg/kg of body weight were selected for the study.

2.3. Sample collection and analysis

After 28 days of daily treatment, blood samples were collected from the retro–orbital fossa using capillary tubes in aliquots containing heparin, while animals were under light anesthesia with diethyl ether. Animals were sacrificed and dissected; their kidneys were collected after washing with normal saline; and the renal capsule was removed and kept in ice-cold 0.1 M phosphate buffer (pH 7.5). A 10% (w/v) homogenate was prepared in phosphate buffer using Teflon-coated homogenizer. The homogenate was centrifuged at 3000 *g* at 4 °C for 15 min to remove cell debris; the supernatant was placed in aliquots and stored at –20 °C for assaying the various antioxidant enzymatic activities. The plasma catalase (CAT) activity was measured according to the method described by Aebi (11). In brief, 20 µL of 1% erythrocyte lysate was incubated in 1.0 mL of 30 mM H_2O_2 at 37 °C and a decrease in absorbance was noted every 10 s for 1 min at 240 nm in a UV visible spectrophotometer (U-1800, Hitachi, Japan). The CAT activity was expressed as µM of H_2O_2 decomposed/min per grams of tissue using 36 as the molar extinction coefficient of H_2O_2. The activity of superoxide dismutase (SOD) in 1% erythrocyte lysate was determined by the method of Marklund and Marklund (12). The assay was based on the ability of SOD to exhibit the autoxidation of pyrogallol in the presence of ethylene diamine tetra acetic acid. The values were expressed as units/g of tissue. The glutathione peroxidase (GSH-Px) activity was determined according to the method of Hafeman et al. (13). The rate of oxidation of GSH by H_2O_2 was used as a measure of GSH-Px activity and expressed as units/g of tissue. The activity of lipid peroxidation in renal tissue was determined according to the method described by Shafiq-ur-Rehman (14). Briefly, 1 mL of 10% TCA was added to 1 mL of 10% tissue homogenate. After vortexing, the mixture was centrifuged at 389 *g* for 10 min. One milliliter of 0.67% (w/v) thiobarbituric acid was added to 1 mL of supernatant and kept in boiling water bath for 10 min. The absorbance was recorded at 535 nm after the addition of 1 mL of distilled water. The amount of lipid peroxidation was expressed as nmol MDA formed/g of tissue using the molar extinction coefficient. The total plasma protein, albumin, blood urea nitrogen (BUN), and creatinine (CR) were determined by standard kits from Transasia Bio-Medicals (India) using a chemistry analyzer (Kyoto Electronics, Japan).

2.4. Statistical analysis

The results were subjected to ANOVA in a completely randomized design (CRD) and their significance was tested using Duncan's multiple range test (15).

3. Results

3.1. Blood biochemical parameters

Different biochemical parameters like BUN, CR, and protein profile indicative of functional alterations in renal tissue are presented in the Table. The significant ($P < 0.05$) increases in plasma BUN and CR levels in rats treated with fluoride at 10 ppm and in those treated with low and high doses of CPF indicated renal insufficiency during toxicant exposure. The simultaneous exposure to both toxicants at both low and high doses produced more pronounced increases in BUN and CR. A nonsignificant increase in the concentrations of total plasma proteins, albumins, globulins, and the A/G ratio was observed in all groups exposed to either low or high doses of a single toxicant. Significant ($P < 0.05$) increases were observed in animals concurrently exposed to higher doses of fluoride and CPF.

3.2 Antioxidant status of renal tissue

The antioxidant status and extent of renal tissue damage was assessed by determining MDA levels in renal tissue and alterations in the activities of SOD, CAT, and GSH-Px. MDA levels in renal tissue were significantly ($P <$

Table. Effects of repeated oral administration of chlorpyrifos (CPF) and fluoride alone and in combination on different blood biochemical parameters in wistar rats.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
BUN (mmol/L)	37.85 ± 0.75^{a}	38.85 ± 0.76^{a}	44.11 ± 0.19^{b}	42.02 ± 0.18^{b}	43.91 ± 0.28^{b}	44.23 ± 0.58^{b}	45.25 ± 0.57^{b}
CR (mg/dL)	2.40 ± 0.04^{a}	2.74 ± 0.15^{bc}	2.72 ± 0.06^{abc}	2.77 ± 0.02^{bc}	2.66 ± 0.08^{ab}	2.93 ± 0.04^{c}	2.90 ± 0.05^{bc}
Total protein (g)	5.78 ± 0.08^{a}	5.85 ± 0.17^{a}	6.09 ± 0.14^{a}	5.90 ± 0.03^{a}	6.19 ± 0.06^{ab}	6.07 ± 0.23^{a}	6.52 ± 0.01^{b}
Albumin (g/dL)	3.38 ± 0.06^{a}	3.42 ± 0.06^{a}	3.59 ± 0.10^{ab}	3.52 ± 0.10^{ab}	3.63 ± 0.11^{ab}	3.46 ± 0.06^{a}	3.37 ± 0.10^{a}
Globulin (g/dL)	2.17 ± 0.09^{a}	2.14 ± 0.19^{a}	2.50 ± 0.15^{ab}	2.16 ± 0.06^{a}	2.56 ± 0.13^{ab}	2.37 ± 0.25^{ab}	2.79 ± 0.11^{b}
A/G ratio (g/dL)	1.68 ± 0.09^{ab}	1.80 ± 0.17^{b}	1.47 ± 0.11^{ab}	1.76 ± 0.07^{ab}	1.44 ± 0.11^{ab}	1.67 ± 0.20^{ab}	1.35 ± 0.09^{a}

Values given are means ± SE of the results obtained from 6 animals unless otherwise stated.
Means with at least one common superscript in a row do not differ significantly at $P < 0.05$.

0.05) increased in fluoride- and CPF-treated animals at both dose levels; the increase was maximum in the group simultaneously exposed to higher doses of fluoride and CPF (Figure 1). Compared with the control group, the animals treated with lower and higher doses of fluoride manifested nonsignificant decreases in SOD activity in renal tissue. However, a significant ($P < 0.05$) reduction was observed in the high dose CPF-treated animals (10 mg/kg) as compared with the controls. Furthermore, the concurrent administration of fluoride and CPF at both low and high doses showed a significant ($P < 0.05$) decline in SOD activity (Figure 2). A significant ($P < 0.05$) decline was also observed in renal CAT activity in the groups exposed to either fluoride or CPF at low and high doses. Similarly, the coexposure to fluoride and CPF produced a significant decrease in CAT when compared with the control group (Figure 3). Additionally, a significant ($P < 0.05$) decline in the activity of GSH-Px in renal tissue was observed with both doses of fluoride and with the high dose of CPF. The concurrent exposure to fluoride and CPF at high doses resulted in significant ($P < 0.05$) inhibition of GSH-Px activity (Figure 4).

4. Discussion

Renal tubular necrosis and acute renal failure are the most common disorders associated with renal function. Renal tubular necrosis is a kidney disorder characterized by irreversible damage to renal tubular cells primarily due to ischemia or toxicant exposure in humans and animals (16). In the present study the increased BUN and CR levels following exposure to CPF and fluoride indicated an alteration in renal function. Similar glomerular and renal tubular degenerative changes were also reported in other studies following exposure of animals to CPF (17,18). Elevation in plasma protein levels may occur due to increased stress protein production in the body, which

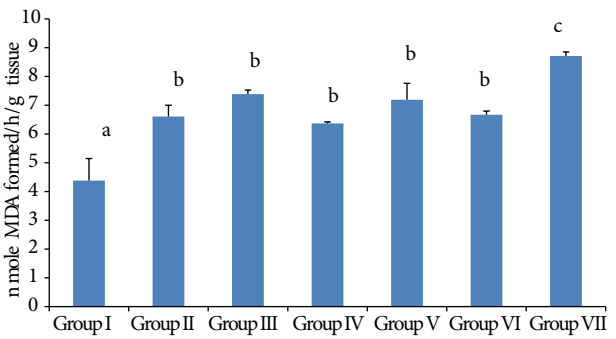

Figure 1. MDA (malondialdehyde) levels in renal tissue of different groups of rats exposed orally to different dosages of CPF and fluoride alone and in combination. (Means with different superscripts differ significantly at $P < 0.05$.

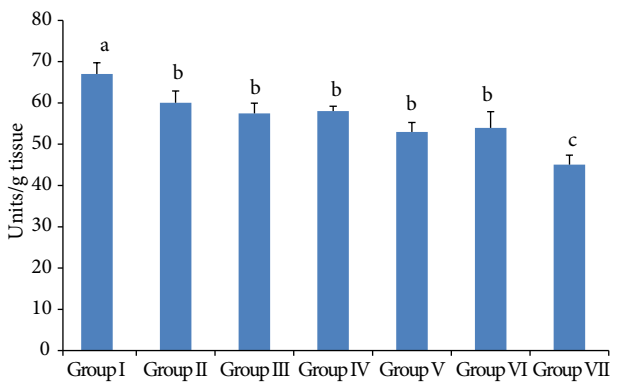

Figure 2. Alterations in superoxide dismutase (SOD) activity in renal tissue of different groups of rats exposed orally to different dosages of CPF and fluoride alone and in combination. Means with different superscripts differ significantly at $P < 0.05$.

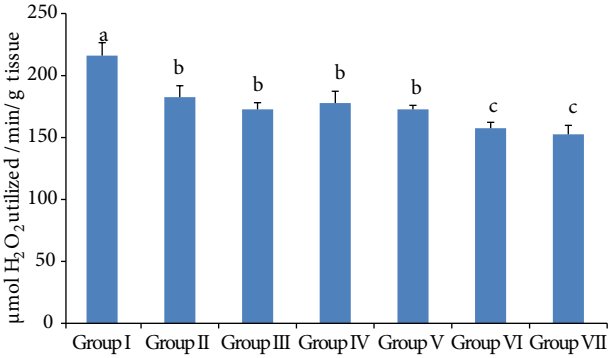

Figure 3. Alterations in catalase (CAT) activity in renal tissue of different groups of rats exposed orally to different dosages of CPF and fluoride alone and in combination. Means with different superscripts differ significantly at $P < 0.05$.

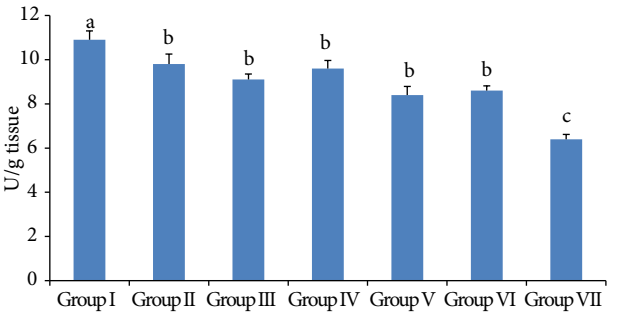

Figure 4. Alterations in glutathione peroxidase (GSH-Px) activity in renal tissue of different groups of rats exposed orally to different dosages of CPF and fluoride alone and in combination. Means with different superscript differ significantly at $P < 0.05$.

protects and buffers the cells from harmful compounds including toxins, pollutants, and poisons (18–20). Alterations in these biochemical parameters indicate increased catabolic activity, which leads to excessive formation of intermediate metabolites due to toxicant exposure (7).

Increased MDA levels in renal tissue are indicative of renal damage due to excessive production of free radicals during exposure to toxicants. Increased free radical levels may be due to fluoride and CPF metabolism or to reduced capability of the cellular antioxidant system to defend the organism against free radicals. The excess free radicals attack cellular macromolecules such as the -SH group of enzymatic/transporter proteins or polyunsaturated fatty acids of lipids in the cellular membrane (21). Increased MDA levels, the end product of lipid peroxidation, indicate increased peroxidation of membrane lipids leading to alterations in membrane functions (8).

SOD is the first line of defense against superoxide radicals produced in the mitochondria and endoplasmic reticulum as a consequence of autoxidation of electron transport chain components. Due to increased metabolic activities during repeated exposure of toxicants, an increase in the leakage of electrons leads to excessive production of superoxide radicals (22). In the present study, the reduced renal SOD activity indicates impaired ability of the renal antioxidant defense mechanism to handle the generated excess of superoxide radicals. Similar decreases in SOD activities following fluoride exposure were also reported in children, rats, and pigs (23,24). CAT is a heme-containing enzyme responsible for scavenging peroxide (H_2O_2) produced during biochemical reactions in the mitochondria and other cellular organelles. As observed in the present study, decreased CAT activity in renal tissue causes accumulation of H_2O_2 radicals at cellular sites, which in turn causes renal tissue damage and functional disturbance. Such decreases in the activity of CAT have also been reported in fluoride-treated pigs and CPF-treated rats (25,26). The inhibition of CAT and SOD activities was more pronounced in the groups exposed to both toxicants. The simultaneous exposure to both toxicants leads to more accumulation of free radical-induced cellular damage as indicated by higher MDA levels as compared with those in the groups exposed to a single toxicant (23,27).

GSH-Px constitutes a large family of selenium containing enzymes responsible for the detoxification of various peroxides using the reduced form of glutathione as an electron donor (28). At least four isozymes (GSH-Px1, 2, 3, and 4) have been identified in mammals; the cytosolic form, GSH-Px1, is widely distributed in tissues and has been most extensively investigated (29). GSH-Px1, like other antioxidative enzymes, prevents apoptosis induced by oxidative stress and other stimuli (30). The biological function of GSH-Px is to reduce the conversion of lipid hydroperoxides to their corresponding alcohols and to reduce free H_2O_2, thus protecting the tissue from oxidative damage (31). Significantly reduced GSH-Px activity, similar to the results of the present study in the fluoride and CPF exposed groups, was also observed in previously reported studies (32,33). The activity of GSH-Px is dependent upon the availability of reduced glutathione and selenium levels. Decreased GSH-Px activity in the groups exposed to both fluoride and CPF might be the reason for the decreased level of glutathione or selenium interaction with fluoride. Such changes in GSH-Px activity have also been reported in rats and pigs exposed to CPF (25). In the present study the toxicity induced by either CPF or fluoride alone or in combination, as indicated by a significant increase in MDA levels, was mainly due to alterations in the oxidant and antioxidant status of exposed rats. Various studies have suggested that exposure to CPF increases the expression of heat shock cognate proteins in different organs in experimental animals (33). Furthermore, studies have also reported that supplementation with natural antioxidants

like catechin, quercetin, or compounds endowed with high antioxidant potential limits the extent of CPF-induced damage by restoring the activities of antioxidant enzymes like CAT, SOD, and GSH-Px in experimental animals (34–39).

In conclusion, the results of the present study suggest that repeated exposure to CPF or fluoride alone or in combination produces renal damage either due to increased free radical generation, reduced antioxidant status of renal tissue, or both; such alterations were more pronounced in animals exposed concurrently to both toxicants. Thus, the application of CPF as insecticide should be limited in areas where fluoride levels in ground waters are high in order to minimize their effects on renal tissue.

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