Protective role of date fruit extract against chlorpyrifos-induced reproductive toxicity in albino male rats

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Abstract

Introduction: Chlorpyrifos (CPF) is a broad-spectrum organophosphorus insecticide; it causes adverse effects on the male reproductive system. The present study aimed to investigate the protective role of date fruit extract (DFE) on CPF-induced reproductive toxicity in male rats. **Materials and Methods:** Male rats were treated with CPF (orally with 6 mg/kg bw) or DFE alone (orally with 1000 mg/kg bw) or in combination for 45 days. Body weights, sperm parameters, lipid peroxidation, and levels of antioxidant enzymes were determined. **Results:** A significant decrease in the weights of testes and epididymis was observed in CPF-treated rats when compared with control rats. Treatment with CPF significantly decreased daily sperm count, epididymal sperm count, sperm motility, and sperm viability. A significant increase in the levels of lipid peroxidation with decreased activity levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) was observed in the testis of CPF-treated rats when compared to control rats, suggesting the CPF-induced oxidative stress in the testes of rats. No changes were observed after administration of DFE alone, whereas coadministration of CPF and DFE showed a significant improvement in body weights and weights of decreased reproductive organs and sperm parameters. Moreover, treatment of DFE to CPF significantly decreased lipid peroxidation with increased antioxidant enzyme levels in the testis compared with CPF-alone-treated rats. **Conclusion:** The results of the present study concluded that DFE administration protects the CPF-induced reproductive toxicity in male rats.

Key words: Antioxidant enzymes, chlorpyrifos, date fruit extract, oxidative damage, rat, spermatogenesis, testes

INTRODUCTION

Infertility among the population of reproductive age is one of the major problems globally, and several studies claimed that the decline in male infertility may be attributed to the environmental pollutants including pesticides.[1,2] Among a range of pesticides, organophosphorus (OP)-based pesticides are commonly used to control insects and pests, thereby protects agriculturally important crops. Like other pesticides, OP pesticides are not target specific and thus may pose serious threat to non-target species, including human.[3-5] Many studies reported that OP pesticides cause detrimental effects on the male reproductive system in humans[6] and also in experimental animals.[7,8] Chlorpyrifos (CPF) (O-O-diethyl-O-{3, 5, 6, trichloro-2-pyridyl}-phosphorothioate) is an organophosphate insecticide widely used for controlling a wide range of insects and pests in agriculture and household gardens.[9] Similar to other OP-insecticides, CPF also performs its mechanism through acetylcholinesterase (AChE) alterations in the central and peripheral nervous system.[10] Besides inhibition of AChE activity, it also induces oxidative stress[11-13] and exhibits endocrine disrupting effects by disrupting the pituitary–thyroid and pituitary–adrenal functions and also endocrine-dependent reproductive functions.[14,15] Numerous animal studies demonstrated the adverse effects of CPF on male reproduction.[16-21]

It is well known that the oxidative damage is a condition, wherein an imbalance occurs between the enzymatic and

non-enzymatic antioxidants and the excess reactive oxygen species (ROS) including free hydroxyl radicals. This in turn negatively affects the structural and functional integrity of organs. Pesticides are one of the major pollutants which have ability to interfere with the pro- and anti-oxidant balance in the tissues, thereby leading to excess generation of free radicals.[12,13] ROS oxidize hydrogen atoms from unsaturated fatty acids to initiate the membrane peroxidation of lipids.[22] Lipid peroxidation is associated with cellular injury and commonly used as an indicator of oxidative damage in the cells and tissues.[12,13,23] Many insecticides are hydrophobic that facilitates bind to biological membranes, accumulate in tissues,[24] and damage the membranes by inducing lipid peroxidation.[12,13] Several studies proved that toxicity with OP insecticides, including CPF, reflected the oxidative damage as evidenced by elevated levels of lipid peroxidation with decreased levels of antioxidant enzymes in the experimental animals.[11-13,19]

Date palm (*Phoenix dactylifera*) is a member of *Arecaceae* family. It is an ancient plant and its fruit supplemented as food for the past 100 years. The parts of date fruit are widely used in several therapeutic applications to control various diseases.[25,26] Date fruit contains various phytochemicals such as carbohydrates, alkaloids, steroids, flavonoids, tannins, ascorbic acid, thiamine, riboflavin, nicotinic acid, and Vitamin A.[27] It possesses antioxidant, anti-inflammatory, antiviral, antimicrobial, diabetic, and anti-tumor activities.[28,29] In addition, the date fruit is classically considered as a symbol of fertility. This is because of the presence of estrogen-like hormones and steroidal saponins and glycosides.[30] Several studies have shown that date fruit extract (DFE) inhibits lipid peroxidation and the hydroxyl radical scavenging activity showed recovery of liver, testes, and kidneys damaged from oxidative stress.[31-34] Therefore, the present study was carried out to investigate the protective role of DFE against to CPF-induced oxidative stress reproductive toxicity in rats.

MATERIALS AND METHODS

Maintenance of Animals

In this study, male Wistar Albino rats (180–200 g) were used as test animals and were purchased from authorized vendors (M/S Raghavendra Enterprises, Bangalore, Karnataka, India). The rats were maintained under temperature controlled environment (25°C ± 2°C) with a 12-h light and 12-h dark cycle. The rats were individually housed in polypropylene cages (18" × 10" × 8") lined with sterilized paddy husk and provided filtered tap water and rat food (purchased from HLL Animal Feed, Bangalore, Karnataka, India) *ad libitum*. All the experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India.[35]

Preparation of DFE

The dates were purchased from local markets in Tirupati, cleaned thoroughly, and dried at room temperature. After cleaning and drying, the flesh was manually separated from the pits and was soaked in sterile distilled water by keeping it in a shaker for 24 h at 4°C, and it was filtered through cheesecloth and the filtrate was treated orally with 1000 mg/kg body weight of DFEs daily for 45 days.

Experimental Design

Healthy male rats were randomly divided into four groups with each group consisting of eight animals. The rats in Group I served as control and received normal saline. Rats in Groups 2, 3, and 4 served as experimental animals. Animals in Group 2 were treated with DFE alone (1000 mg/kg body weight) daily for 45 days along with drinking water. Rats in Group 3 received CPF through gavage at concentration of 6 mg/kg bw daily for 45 days. Rats in Group 4 received the same experimental regimen as that of rats in Group 3; in addition, they received DFE (1000 mg/kg body weight) daily for 45 days along with drinking water. The experimental duration and the doses of test chemical and DFE were based on the previous studies.[18,36,37]

Necropsy

After completion of the experimental period (45 days), the rats were fasted overnight; on the 46th day, the rats were weighed and sacrificed by cervical dislocation. Testes, epididymis, seminal vesicles, vas deferens, and prostate were dissected out and weighed for the nearest milligram immediately using Shimadzu electronic balance after clearing off the adhering tissues. Body weight was determined just before killing each animal. Reproductive organ indices were determined using the formula:

$$TSI = [\text{weight of the tissue (g)/Body weight of the animal (g)}] \times 100$$

Sperm Parameters

Epididymal sperm analysis

Sperm parameters are the valuable indicators of male fertility. In this study, we analyzed sperm count, viable sperm, and motile sperm from the cauda part of the epididymis to assess the extent of pesticide toxicity and also the protective efficacy of the DFE on these sperm variables. The evaluation of sperm motility was performed according to the method described by Belsey *et al.*[38] Briefly, the cauda epididymis was minced in physiological saline (0.9% NaCl in distilled water) at 37°C, and the resultant epididymal fluid was used to analyze the selected sperm variables. To analyze the percent motile sperm, the diluted epididymal fluid was placed in Neubauer

hemocytometer, and total, motile, and non-motile sperms were counted. The number of motile and non-motile sperms was determined microscopically (Olympus CX41; Olympus Optical Co. Ltd, Japan) within 5 min following their isolation from the cauda epididymis at 37°C. For the analysis of the live and dead sperms, trypan blue reagent method[39] was used in this study. Briefly, one drop of the diluted epididymal sperm suspension was mixed with one drop of 1% trypan blue solution and incubated at 37°C for 15 min. The mixture was placed on a microscope slide, covered with a coverslip, and observed under a microscope. Sperms were considered as viable if they were unstained and considered as dead if they were stained with the trypan blue. The units for sperm count, viable sperm, and motile sperm were expressed as millions/ml, percentage of total non-motile sperm of the total sperm, and percentage of total unstained sperm of the total sperm, respectively.

Daily Sperm Production (DSP)

Testicular daily sperm count is a valuable indicator of spermatogenesis, which was analyzed in the testis of adult rats by the method of Blazak *et al*.[40] Briefly, the decapsulated was homogenized in 50 mL of ice-cold 0.9% NaCl solution containing 0.01% Triton-X 100 using a glass Teflon homogenizer. The homogenate was allowed to settle for 1 min followed by gentle mixing. After thorough mixing of each sample, the number of sperm heads was counted in four chambers of an improved Neubauer-type hemocytometer. The DSP was expressed as the sperm produced per gram testicular tissue per day.

Estimation of Lipid Peroxidation in Testes

Determination of lipid peroxidation levels in terms of thiobarbituric acid reactive substances (TBARS) provides valuable information about the cellular/tissue oxidative stress. In this study, the levels of TBARS were analyzed spectrophotometrically in the testis of controls and experimental rats based on the method described by Ohkawa *et al*.[41] Briefly, the testis (10% W/V) was homogenized in homogenization buffer containing 1.15% KCL solution. To the homogenate (2.5 ml), 0.5 ml of saline (0.9% sodium chloride) and 1.0 ml of (20% W/V) trichloroacetic acid were added and centrifuged for 20 min at $4000 \times$ rpm. To the resultant supernatant (1.0 ml), 0.25 ml of TBA reagent was added, and the mixture was incubated at 95°C for 1 h. Finally, an equal volume of n-butanol was added to the mixture and the contents were centrifuged for 15 min at 4000 rpm. The organic layer was carefully transferred into a clear tube, and its absorbance was measured spectrophotometrically at 532 nm. The absorbance was recorded and compared against the standard curve constructed with the known amount of malondialdehyde. The rate of lipid peroxidation was expressed as μ moles of malondialdehyde formed/gram wet weight of tissue.

Assay of antioxidant enzymes in testes

SOD (EC 1.15.1.1)

The activity of SOD was determined by the method of Mishra and Fridovich.[42] SOD was assayed in the microsomal fraction according to its ability to inhibit the auto-oxidation of epinephrine at alkaline medium. Briefly, the testes were homogenized (10% W/V) in 50 mM ice-cold sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,500 rpm for 60 min. After centrifugation, the supernatant (cytosol) fraction was used for the assay of enzyme activity. The reaction mixture contained 0.05 M carbonate buffer (pH 10.2), 30 mM epinephrine (freshly prepared), and the enzyme extract. Changes in absorbance were recorded at 480 nm and measured at 10 s intervals for 1 min in a spectrophotometer. The enzyme activity was expressed as units/mg protein/min.

CAT (EC 1.11.1.6)

The activity of CAT was determined by based on its ability to decompose hydrogen peroxide (H_2O_2), from the method of Chance and Machly.[43] The testes were homogenized (10% W/V) in 50 mM phosphate buffer (pH 7.0). Then, the homogenate was centrifuged for 60 min. The reaction mixture in a volume of 2.5 ml contained 0.05M phosphate buffer (pH 7.0), 19 mM H_2O_2, and appropriate amount of tissue homogenate. Then, the absorbance was read at 240 nm and measured at 10 s intervals for 1 min in a spectrophotometer. CAT activity was expressed as μM of H_2O_2 metabolized/mg protein/min.

GPx (EC 1.11.1.9)

The activity of GPx was estimated by the method of Mohandas *et al*.[44] Briefly, the reaction mixture contained 1.59 ml of 100 mM phosphate buffer (pH 7.6), 0.1 ml of 10 mM EDTA, 0.1 mL of 10 mM sodium azide, 0.1 ml glutathione reduced, 0.01 ml of 0.2 mM hydrogen peroxide, and 0.1 ml of enzyme source. Immediately, the contents were read at 340 nm against blank, at 10 s intervals for 3 min on a spectrophotometer. The activity of GPx was expressed as nanomoles of NADPH oxidized/mg protein/min.

Protein Estimation

Lowry *et al*.[45] method was used to quantitate the protein content in the enzyme source, wherein bovine serum albumin was used as a standard.

Statistical Analysis

Data are presented as mean ± standard deviation, and the values of $P < 0.05$ were considered statistically significant.

Statistical analysis between the control and experimental groups was analyzed using one-way analysis of variance followed by Tukey's multiple test as a *post-hoc* test (SPSS software for Windows, version 16.0).

RESULTS

Clinical Signs

In the present study, none of the animals from the control and experimental groups were excluded. No mortality was noticed in any of the control and treated rats. Interestingly, we noticed clinical signs in rats treated with CPF and CPF+DFE such as huddling, mild tremor, and diarrhea. This may be related to the cholinergic signs, a consistent sign in organophosphate poisoning. On the other hand, such clinical signs were not noticed in control and DFE-treated rats.

Body Weight and Reproductive Organ Weights

No significant change in the body weights was observed in controls and experimental animals [Table 1]. A significant ($P < 0.001$) decrease in the weights of testis and epididymis was observed in CPF-treated rats as compared to control rats. However, no significant changes were observed in the other reproductive organs, *viz*., seminal vesicles, vas deferens, and prostate gland after CPF treatment [Table 1]. Besides, coadministration of CPF and DFE significantly ($P < 0.001$) increased the weights of testis and epididymis as compared to CPF-alone-treated rats, whereas no significant changes in the body weights and in the relative weights of reproductive organs were observed in DFE-alone-administered rats [Table 1].

Sperm Parameters

The changes in the daily sperm production (DSP), epididymal sperm count, sperm motility, and sperm viability are shown in Table 2. Administration of CPF alone significantly ($P < 0.001$) decreased DSP, epididymal sperm count, sperm motility, and sperm viability when compared with controls. No significant

changes in the selected sperm parameters were observed in the DFE-alone-treated rats, whereas coadministration of CPF and DFE significantly ($P < 0.001$) increased the DSP, sperm count, motility, and viability [Table 2].

Effect of CPF and DFE on Lipid Peroxidation

The levels of malondialdehyde were significantly ($P < 0.001$) increased in the testis of CPF-treated rats when compared to control rats [Table 3]. A significant decrease ($P < 0.001$) in the activity levels of SOD, CAT, and GPx was observed in the testes of CPF-treated rats when compared with control rats [Table 3]. Coadministration of CPF and DFE significantly ($P < 0.001$) reduced the malondialdehyde level when compared with CPF-alone-treated rats, whereas activity levels of enzymatic antioxidants were significantly ($P < 0.001$) increased compared to CPF-treated rats; however, no significant increase was observed in DFE-alone-received rats [Table 3].

DISCUSSION

CPF is one of the most commonly used OP pesticides causing major toxic effects in the mammalian male reproductive system. Although there are several synthetic medicines are available to treat reproductive damage, due to their side effects, there is an increase in the importance of traditional medicine. The main objective of this study was to investigate the protective role of DFE on CPF-induced reproductive toxicity in adult male rats. In the present study, male rats were treated with CPF or DFE alone or in combination and assessed the changes in reproductive organ weights and testicular oxidative stress biomarkers.

In the present study, treatment of CPF did not show any significant change in the body weights. On the other hand, we found a significant decrease in the weights of testis and epididymis in CPF alone administered rats. It is well established that adequate supply of androgens sustains the structural and functional integrity of testis and the accessory sex organs.[46] Thus, the reduction in the weights of reproductive organs may be attributed to the inadequate supply of androgens. Moreover,

Table 1: Protective role of DFE on CPF-treated rat's body weight (g) and reproductive organ weights (W/W %)

Parameter	Control	DFE	CPF	CPF and DFE
Body weight	203.65[a]±2.33	204.05[a]±3.68 (0.19)	199.52[a]±3.66 (−2.02)	201.23[a]±3.85 (−1.18)
Testes	1.24[a]±0.04	1.29[a]±0.06 (4.03)	0.96[b]±0.02 (−22.58)	1.15[c]±0.04 (−7.25)
Epididymis	1.19[a]±0.04	1.20[a]±0.08 (0.84)	0.92[b]±0.05 (−22.68)	1.09[c]±0.02 (−8.40)
Seminal vesicle	0.37[a]±0.02	0.39[a]±0.04 (5.40)	0.34[a]±0.03 (−8.10)	0.35[a]±0.03 (−5.40)
Vas deference	0.13[a]±0.02	0.14[a]±0.02 (7.69)	0.11[a]±0.02 (−15.38)	0.12[a]±0.01 (−7.69)
Prostate gland	0.16[a]±0.01	0.18[a]±0.03 (12.5)	0.14[a]±0.02 (−12.5)	0.15[a]±0.01 (−6.25)

Values are mean±S.D. Of 8 individuals for each group. Values in parenthesis are percent change from that of control. Mean values with different superscripts in a row differ significantly at $P<0.05$. DFE: Date fruit extract, CPF: Chlorpyrifos. SD: Standard deviation

Table 2: Protective role of DFE on sperm parameters in control and CPF-administered rats

Parameter	Control	DFE	CPF	CPF and DFE
DSP (millions/g testis)	19.21[a]±1.34	19.43[a]±2.06 (1.14)	10.80[b]±1.84 (−43.77)	16.02[c]±1.44 (−16.60)
Sperm count (millions/mL)	65.25[a]±2.56	66.81[a]±1.88 (2.39)	44.76[b]±2.39 (−31.40)	58.24[c]±2.56 (−10.74)
Sperm motility (%)	72.75[a]±4.15	73.02[a]±2.65 (0.37)	50.22[b]±2.12 (−30.96)	64.89[c]±3.50 (−10.80)
Sperm viability (%)	68.16[a]±4.29	69.02[a]±4.49 (1.26)	46.84[b]±3.24 (−31.27)	65.02[a]±3.80 (−4.60)

Values are mean±S.D. Of 8 individuals for each group. Values in parenthesis are percent change from that of control. Mean values with different superscripts in a row differ significantly at *P*<0.05. DFE: Date fruit extract, CPF: Chlorpyrifos, DSP: Daily sperm production. SD: Standard deviation

Table 3: Protective role of DFE on lipid peroxidation and testicular antioxidant enzyme levels in control and CPF-administered rats

Parameters	Control	DFE	CPF	CPF and DFE
Lipid peroxidation (μ moles of malondialdehyde/g tissue)	12.24[a]±1.42	12.28[a]±1.45 (0.32)	21.15[b]±1.72 (72.79)	12.92[a]±1.62 (5.55)
SOD (n moles/mg protein/min)	9.12[a]±1.21	9.18[a]±1.15 (0.65)	5.37[b]±1.02 (−41.11)	7.04[c]±1.14 (−22.80)
CAT (n moles of H2O2 metabolized/mg protein/min	36.81a±3.16	36.94[a]±2.93 (0.35)	25.06[b]±2.88 (−31.92)	31.35[c]±3.63 (−14.83)
GPx (n moles NADPH oxidized/mg protein/min)	41.07[a]±2.34	41.34[a]±2.58 (0.65)	29.10[b]±3.11 (−29.41)	35.88[c]±3.64 (−12.63)

Values are mean±S.D. Of 8 individuals for each group. Values in parenthesis are percent change from that of control. Mean values with different superscripts in a row differ significantly at *P*<0.05. DFE: Date fruit extract, CPF: Chlorpyrifos. SD: Standard deviation, CAT: Catalase, SOD: Superoxide dismutase

size and weight of the testis are primarily dependent on the mass of the differentiated spermatogenic cells. In this study, a significant reduction in the sperm endpoints such as daily sperm production, sperm production, sperm motility and viability in the rats treated with CPF may suggest reduced spermatogenesis and deteriorated sperm maturation events. The present results are in agreement with earlier reports.[13,15-21] Previously it was reported that administration of CPF resulted in decreased levels of testosterone accompanied by a reduction in sperm count, viability and motility and spermatogenesis in rats. [13,15,21,47]

Oxidative stress is a condition wherein the intrinsic antioxidant defense system fails to negate the excessive generation of RO). Lipid peroxidation is one of the fundamental mechanisms of cellular damage caused by free radicals including malondialdehyde, hydrogen peroxide and hydroxyl radicals. The role of enzymatic antioxidant enzymes (SOD, CAT and GPx) as cellular defense system against the ROS is well appreciated. In the present study, significant increase in the lipid peroxidation associated with decreased activity levels of antioxidant enzymes, SOD, CAT and GPx in testes of CPF-treated rats may reflect testicular oxidative damage. These results are in line with the previous findings after treatment of CPF.[13,19,22]

Since the pesticide toxicity is mediated by induction of oxidative stress, the previous studies were done by selecting the antioxidant-rich natural sources such as Vitamin C and *Embilica* treatment to recover CPF-induced testicular toxicity.

[15,47,48] As DFE is also an effective antioxidant property source, earlier studies demonstrated the ameliorative effect of aqueous extract of date fruit against pesticides-induced oxidative stress in the testes.[8] In the present study, treatment with DFE alone did not show significant changes in the weights of reproductive organs, spermatogenesis, levels of lipid peroxidation, and activities of SOD, CAT, and GPx of rats when compared with the controls. However, coadministration of DFE and CPF restored the reduced reproductive organ weights, especially testes and epididymis. Administration of DFE improved sperm counts, sperm motility, and sperm viability as compared to CPF-treated rats. Further, coadministration DFE and CPF significantly decreased lipid peroxidation with increased levels of antioxidant enzymes, SOD, CAT, and GPx compared with CPF-alone-treated rats. The results showed that DFE treatment reduced CPF-induced increased lipid peroxidation level and enhanced the activity of antioxidant enzymes SOD, CAT, and GPx in CPF-treated rats. The results indicated that DFE improved the sperm parameters and restored the spermatogenesis in CPF-treated rats. Administration of DFE also recovered atrazine, an herbicide that caused oxidative damage in sperm parameters, reduced testicular antioxidant enzymes, GSH, SOD, CAT, and GPx levels in rats.[8] This study also noted the increased serum testosterone levels and decreased lipid peroxidation. The protective effect of DFE against dimethoate and chemical solvent CCl_4-induced oxidative stress in rat liver was reported,[31,32] and protective effect of DFE against diabetes-induced hepatic and nephrotoxicity was also studied.[33,34]

To conclude, the DFE with its antioxidant properties might negate the CPF-induced testicular oxidative damage, thereby restoring spermatogenesis and sperm maturation events in the rats.

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