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Research Article

Efficacy of Cow Urine against Chlorpyrifos Induced Toxicity in Male Wistar Rats

Shelly Sharma, Sukanya Mehra, Pooja Chadha*

Department of Zoology, Guru Nanak Dev University, Amritsar, India

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ABSTRACT

The present study's objective was to study the mitigating potential of cow urine against the detrimental effects of chlorpyrifos (CPF) on different organs of rat. For this purpose antioxidant potential of cow urine was measured using FRAP (Ferric (Fe3+) reducing antioxidant power) assay. Further after LD_{50} determination, $1/25^{th}$ i.e., 6.08 mg/kg b.wt, was selected and orally administered along with cow urine for 30 days to Wistar rats. After 30 days, blood, brain, liver and kidney tissues were collected and analysed for genotoxic (comet assay and chromosomal aberrations) and oxidative stress malondialdehyde(MDA), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) induced by CPF and ameliorative potential of cow urine. Noteworthy reduction in MDA and DNA damage and significant elevation GST, SOD, CAT and AChE activities reveals cow urine's alleviating effect.

Introduction

Agrochemicals being broadly applied all over the world as plant protection agents for boosting food production. As a result, huge amounts of these chemicals are discharged into the environment, causing significant negative effects on non-target organisms as almost 98% of insecticides, and 95% of herbicides sprayed in fields reach destinations other than the target species. Though pesticides carry wide applications but being highly persistent, pesticides have led to contamination of soil, water, and air. Organophosphate pesticides (OPs) being extensively pervasive in both developed, as well as developing countries, raises concern regarding the relative safety of the environment and human health against these chemicals.[1] The CPF is one of the widely used broad-spectrum conventional OP to control the number of pests in agriculture,[2] animal farms, and domestic purposes worldwide. According to the literature, CPF is a known toxic pesticide as it poses adverse effects on the activity of enzyme acetyl cholinestrase (AChE). Its decreased activity further prevents the smooth transmission of nerve impulses.[3-4] Because of extensive use and approachability, adulteration with CPF has become a major global health issue. The xenobiotic invasion in the tissue leads to the production of reactive oxygen species, which cause severe damage in biochemical constituents leading to oxidative stress. Oxidative stress is a harmful condition that prevails due to an increase in reactive oxygen species.[5] ROS cause severe damages by exerting a direct impact on lipids, DNA and proteins[6], leading to several diseases, including cancer. Therefore, to counteract the effect, antioxidants generated inside the body quench the free radicals and prevent substrates' oxidation, thus preventing oxidative stress and DNA damage.

Exposure to various environmental and food contaminants resulted in shifting the balance toward

***Corresponding Author:** Dr Pooja Chadha

Address: Cytogenetics laboratory, Department of Zoology, Guru Nanak Dev University, Amritsar-143005, Punjab, India

Email ✉: pooja.zoology@gndu.ac.in

Tel.: +91-9417312861

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the accumulation of free radicals, necessitating dietary supplements rich in antioxidants. Natural products being derived from nature such as animals, microorganisms, and plants[7] have persistently been of immense importance to researchers working on several diseases.[8] Scientists have found several new anticancer drugs, but side effects to these drugs pose a huge challenge to introducing these new drugs. Thus the modern research is primarily being focused on conventional medicines in combination with modern therapies. According to ancient Vedas, cow urine has always been considered holy and gets related to nectar as it carries medicinal values against various types of diseases. Due to its multiple therapeutic values, traditional healers practice its usage for the treatment of several diseases, including diarrhea, renal colic, gastric infection, anemia, jaundice etc.[9] It is also considered to produce bioenergy at a cellular level.[10-11] Studies states that cow urine consists of all elements which are required by the human body to function properly thus consider cow urine to be the natural remedy against several disorders.[12] It is also stated to be as neuro-protective agent and rich in antioxidants which hinder the generation of reactive oxygen species which are known to be responsible for DNA damage.[13] Cow urine has also been known to carry antimicrobial and lipase activity, which can be crucial for its application in the medicine area.[14] Jarald *et al.*[15] also revealed the antioxidant and antimicrobial activities of cow urine. Although there are uncountable benefits of cow urine, yet scientific studies are scarce. Moreover, its potential against pesticides induced toxicity is scarcely explored. Thus the present analysis was undertaken to fill the lacuna in this regard.

Materials and Methods

Experimental Model

Male Wistar rats having weight 110 ± 20 grams were used in the present experiment. They were fed on a commercial pellet diet and water *ad libitum* and were caged at room temperature with relative humidity and on 12 hours of light–darkness period. Animals were acclimatized for 15 days prior to the experiment

Ethical Note

The experiments were performed according to the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) number 226/CPCSEA2013/20.

Experimental Design

CPF (99%) was purchased from Sigma Aldrich, St Louis, US, and cow urine was purchased from Patanjali store, Amritsar. The antioxidant potential of cow urine was measured using FRAP assay. The experiment was further designed in two parts. In the first part, toxicity induced by chlorpyrifos was studied. For this, 6.08 mg/kg b.wt ($1/25^{th}$ of LD_{50}) of CPF was administered orally to the rats for 30 days consecutively. In the second part, the ameliorative potential of cow urine was studied in which rats were fed with cow urine prior to the exposure of CPF. For the experiment, three groups were made; each group were containing 10 male albino Wistar rats. One group fed with corn oil was taken as control, whereas second group was administered orally with 6.08 mg/kg b.wt ($1/25^{th}$ of LD_{50}) of CPF; the 3^{rd} group was pre-treated with 0.5 mL of cow urine for consecutive ten days prior to the exposure of CPF. After 30 days, blood, brain, liver and kidney tissues were collected after sacrificing rats and analyzed for studying genotoxic and oxidative stress-inducing potential of CPF and alleviating potential of cow urine against it.

Biochemical Analysis

Protein estimation

The protein content of the tissue homogenates mentioned earlier was determined by following the method described by Lowry *et al*.[16] using bovine serum albumin as a standard.

Determination of MDA (malondialdehyde) in rat tissues

The extent of MDA was measured according to the method of Draper and Hadley.[17] MDA concentrations of the samples were calculated using the extinction co-efficient of MDA, which is $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Determination of glutathione S-transferase (GST) activity in rat tissues

GST was measured according to the method of Chein and Dauterman.[18] One unit of GST activity is defined as 1 mol product formation per minute.

Determination of catalase (CAT) activity in rat tissues

The enzyme catalase converts H_2O_2 into water. The CAT activity in blood, kidney and liver supernatant was measured spectrophotometrically at 240 nm by calculating the rate of degradation of H_2O_2, the substrate of the enzyme.[19] Activity was monitored at 240 nm for 60 s. Data is expressed as U/mg protein.

Determination of superoxide dismutase (SOD) activity in rat tissues

Superoxide dismutase was measured according to Kono.[20] Data is expressed as U/mg protein.

Acetylcholinestrase (AChE) Activity

The activity of AChE was determined according to the method of Ellman's *et al*.[21] with some modifications.

Determination of genotoxicity

Genotoxicity was assessed with the help of two parameters.

Comet assay

Comet assay was determined by following the basic protocol for the Comet Assay with some modifications.[22]

Chromosomal aberrations

Chromosomal aberrations in bone-marrow metaphase cells were performed according to the technique described by Preston *et al*[23] with some modifications.

Statistical analysis

The data was analysed using ONE WAY -ANOVA (SPSS 11).

RESULTS

FRAP (Ferric (Fe^{3+}) Reducing Antioxidant Power) Assay

To quantify the reductive potential of cow urine the ferric ion-ferrous ion transformation in the presence of cow urine in the ferric reducing antioxidant power (FRAP) assay was carried out as the reducing ability indicates sample's potential antioxidant activity. The reductive potential of cow urine was estimated to be 60.05 ± 0.56% which was relatively more significant than the ascorbic acid.

Biochemical assays

Sub chronic exposure for 30 days to 6.08 mg/kg b.wt of CPF resulted in significant alterations in all the oxidative stress analysis parameters studied in all the tissues (Fig. 1). MDA levels in case of liver were found to be raised as compared to control. Whereas GST, CAT, SOD activities which were found to be reduced symbolically revealed by values which were 26.02 ± 0.37, 14.43 ± 1.39, 36.60 ± 1.30 and 59.76 ± 0.22 values as compared to the control values of 67.22 ± 0.65, 47.19 ± 1.79, 208.70 ± 14.7 and 193.01 ± 2.23 respectively. Similar trend was found in all the other tissues with the highest effect observed in brain. Cow urine pre-treated groups showed remarkable decrease in MDA levels and increase in GST, CAT, SOD and AChE activities for all doses and all durations revealing that cow urine modulated the genotoxic and oxidative stress effect induced by CPF. Pre-treatment with cow urine led to improvement in the levels of MDA levels and SOD, CAT, GST and AChE activities. 15%-80% and 30.35-293% elevation in the activity of SOD was found in brain and liver respectively. Likewise the CAT and GST activities have also been observed to boost up in cow urine pre-treated groups. The recovery in the activities of all the biomarkers was up to 90% in brain and 200%-300% in liver and kidney. Therefore it is apparent that the cow urine counteracts toxic effects induced by CPF by observing decrease in detoxifying enzyme activities and hike in MDA levels. The pre-treatment with cow urine was found to be efficacious for all the organs considered for the experiment which includes brain, liver, kidney and blood.

Fig. 2 also shows the percentage of chromosomal aberrations in bone marrow cells of rats after exposure of 6.08 mg/kg b.wt of CPF. Significant increase in the mean value of percentage chromosomal aberrations was observed in exposed group as compared to control group (ANOVA). The mean percentage chromosomal aberrations values elevated from 11.38 ± 0.38 (control) to 50.24 ± 0.24 after subchronic exposure of CPF. Fig. 1 shows microphotographs of chromosomal aberrations found in bone marrow.

Prominent changes occurred in all the parameters of comet assay such as tail length, % tail DNA , tail moment and olive tail moment after 30 days of exposure

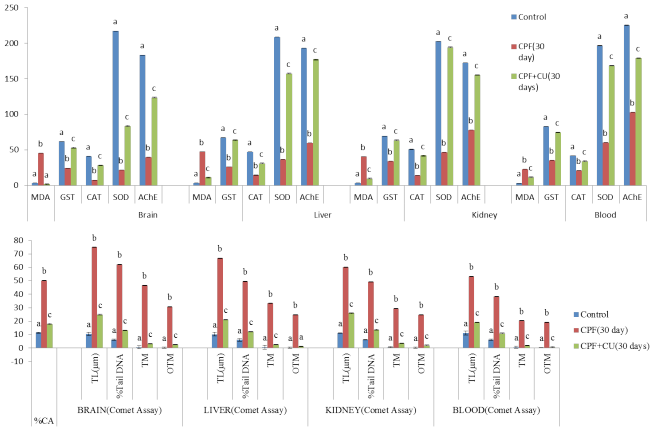

Fig. 1: Effect of 6.08 mg/kg b.wt of CPF and CU+CPF on different oxidative stress biomarkers and genotoxicity biomarkers after 30 days of exposure. (A) Brain (B) Liver (C) Kidney (D) Blood. Results are shown as means ± SE * significantly different (p≤0.05).

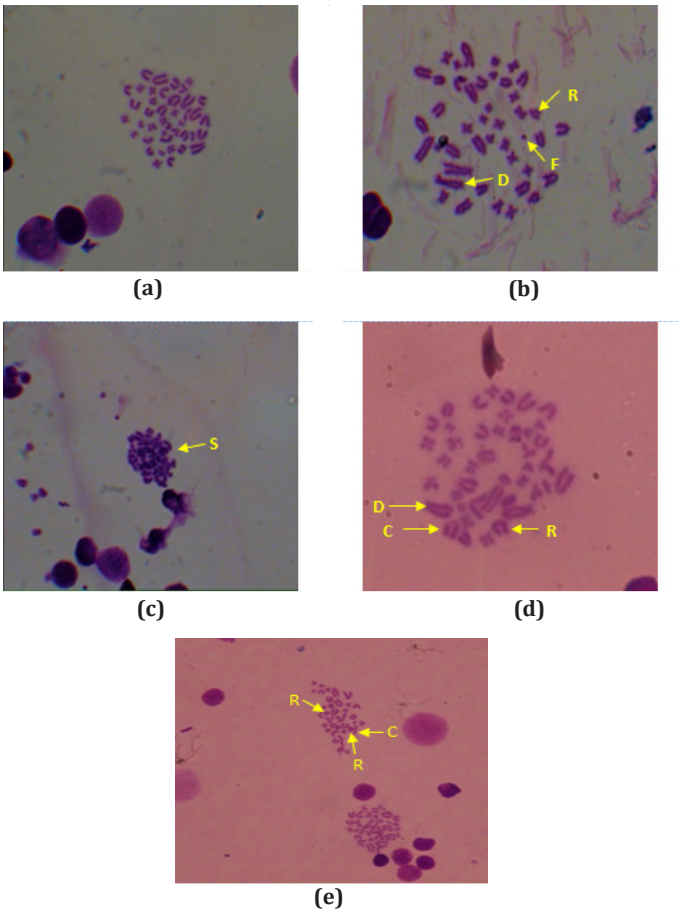

Fig. 2: Photomicrographs showing (a) control, (b-e) different types of chromosomal aberrations in rat bone marrow cells i.e. dicentric (D), ring (R), fragment (F), chromosomal break (C), stickiness (S).

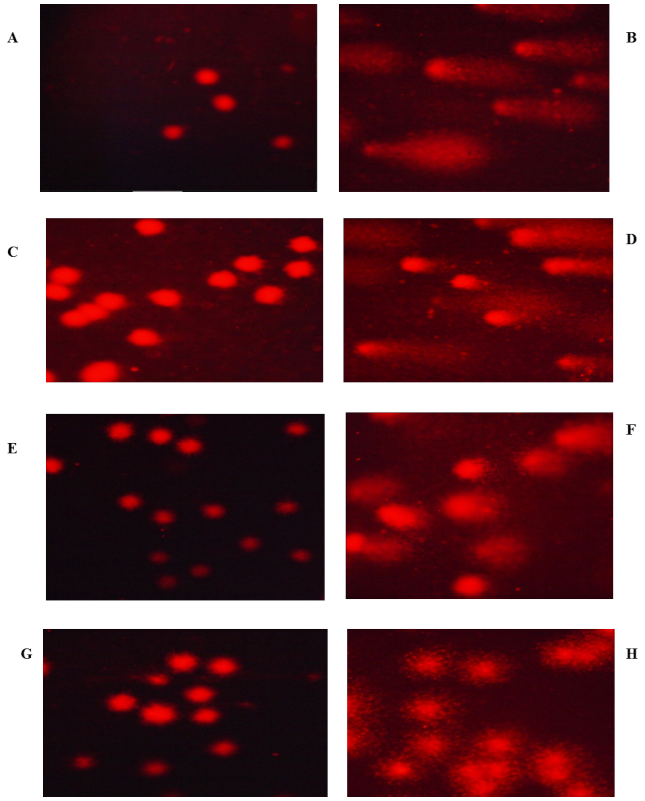

Fig. 3: Microphotgraphs of DNA extracted from (A) control brain (B) brain of group treated with CPF (C) control liver (D) liver of group treated CPF (E) control kidney (F) kidney of group treated with CPF (G) control blood (H) blood of group treated with CPF

to 6.08 mg/kg b.wt of CPF. Comparative analysis of DNA damage in all tissues of rats after 30 days of exposure of CPF showed that the longest tail length of 74.96 ± 1.23 µm for brain tissue. Lowest TL, TI, TM and OTM were observed for blood cells which were 53.14 ± 1.55, 38.31 ± 0.45%, 20.36 ± 0.83 and 19.26 ± 0.23 respectively. The overall results of DNA damaging effect of CPF can be outlined as Brain>Liver>Kidney>Blood. However CU+CPF groups depicted noteworthy reduction in all the comet assay parameters highlighting the mitigating ability of cow urine. Fig. 3 represents microphotographs of DNA extracted from different organs of rat.

Discussion

According to literature, pesticides have been suggested as potent producers of reactive oxygen species[24] and excessive exposure to these radicals makes our body highly susceptible to free radical-mediated tissue and genetic damage. These free radical species are involved in causing lipid peroxidation along with DNA damage prior to carcinogenesis.[25-27] Abundant free radicals lead to an imbalance in antioxidant levels and enzymatic activities, leading to defacement of biochemical roles. Similar alterations in enzymatic potentials were noted in the present analysis after subchronic exposure of CPF. The study performed by Ahmed *et al.*[28] stated that continuous exposure of CPF raised MDA levels along with antioxidant enzymes in different organs of rats when treated with CPF. Raina *et al.*[29] also revealed 98.27% increase in MDA levels in rat organs when treated with 10 mg/kg b.wt CPF for 28 days.

Reduction in the specific activities of CAT, GST, and SOD, further worsen the condition. These enzymes provide a first line of defense to the organism being exposed to a xenobiotic. These enzymes have proven to carry a crucial role against cellular damage. SOD and CAT are known to quench ROS and act as the important players against the induced oxidative stress.[30] A study performed by Singh *et al.*[31] revealed a reduction in SOD and CAT activities of rats after subchronic exposure of CPF, which further supports the outcome of the present study in which CPF intoxication inhibits the activities of intracellular antioxidant enzymes CAT and SOD in all the studied tissues of rat. SOD and CAT are known to work in correlation as SOD helps in catalyzing the transformation of superoxide radicals to hydrogen peroxide, which is further converted to water by CAT. Therefore the reduction in SOD and CAT activities due to an abundance of free radicals leads to amassing of H_2O_2, which foster lipid peroxidation, DNA modification and cell apoptosis.[32] GST's belong to the family of detoxification enzymes thus have a crucial role in the degradation and metabolism of toxins.[33] The detectable reduction in GST activity in CPF exposed groups show inadequate detoxification of electrophiles.[34] The reduced GST activity reveals cellular oxidative stress due to CPF intoxication. In accord with the present outcome, noteworthy reduction in GST activity was observed after the intoxication of rats with cypermethrin and lambda-cyhalothrin.[35] Few other studies also show that pesticides including dithiocarb, cypermethrin, and dimethonate also led to consequential inhibition of GST activity.

Studies revealed that even small doses of pesticides could activate the mechanism of apoptosis in lymphocytes by damaging DNA.[36] Similar results were observed in the present analysis. However, in the present investigation cow urine shows a significant reduction in various parameters studied while assessing CPF induced toxicity revealing its beneficial function in healing the damage. Khan and Srivastava[37] also stated the antitoxic potential of cow urine due to the occurence of Zn^{2+}, which attributes to the bio enhancing the ability of cow urine. Mice exposed to cadmium chloride showed zero percent fertility, whereas the mice treated with cow urine prior to cadmium chloride exposure showed 88% rise in fertility, thus supporting the antitoxic ability of cow urine. Thus keeping this in mind present study was performed to evaluate the ameliorative potential of cow urine against toxicity induced by CPF. Biomarkers used to study the mitigating potential were MDA, GST, CAT, SOD, and AChE, chromosomal aberrations and comet assay. Variability in oxidative stress biomarkers was also sound after CPF intoxication for all the organs of rat. Oxidative stress occurs when an imbalance is

created between the antioxidant system and ROS.[38-39] The reduction in SOD, CAT, GST and AchE levels for all the organs. Prior treatment with cow urine tends to improve the activities of SOD and CAT. 15-80% and 30.35-293% rise in the activity of SOD was observed in brain and liver, respectively. Similarly, the activities of CAT and GST have been observed to enhance in cow urine pre-treated rats. 90% and 200%-300% recovery was observed in brain, liver, and kidney, respectively. The rise in antioxidant enzymes' activities might be due to the interaction of bioactive compounds present in cow urine. This enhanced enzymatic activity could be a potential therapy against in liver/kidney disorders caused by oxidative stress. The outcome of the present study reveals the efficiency of cow urine against oxidative stress induced by CPF. The supplementation of cow urine and the treatment of CPF led to a noteworthy decrease in MDA levels, which was 90, 775, 80, and 70% for brain, liver, kidney and blood, respectively the beneficial role of cow urine. MDA levels due to cisplatin in mice[40] were also observed. Likewise, study performed by Nagda and Bhatt[41] shows the antitoxic effect of cow urine against lindane-induced nephrotoxicity and oxidative stress.

After subchronic exposure of 30 days the value of percent chromosomal aberrations was also found to be decreased to 64.28% in rats pre-treated with cow urine. Likewise, results were noted after mitomycin treatment, where supplementation of cow urine was found to reduce the chromosomal aberrations remarkably in the treated group.[42] Similarly, a reduction in TL, TI, TM, and OTM values was also seen in the present study after prior treatment of cow urine. In a study on apoptosis of avian lymphocytes due to pesticides, cow urine has been found to be beneficial by protecting it against pesticide-induced damage.[43] Although the percentage varies in biomarkers, the decline in these biomarkers of genotoxicity as well as oxidative stress analysis in all the target tissues was observed, indicating the ameliorative potential of cow urine.

The mechanism behind its protective effect might be its antioxidant property which was measured in the present study using FRAP assay. It was estimated to be 60.05 ± 0.56%. Antioxidant potential contributed mainly due to the occurrence of volatile fatty acid and its free radical scavenging property[43] as volatile fatty acids was reported to activate the third complement component in serum. This activation's product carries histamine releasing effects, which further leads to a decrease in monocyte and neutrophil count in blood within 4hrs of treatment. Further, copper in cow urine has also been considered as having a crucial role in its lipid-lowering process. Gosavi *et al.*[44] highlight the presence of high amounts of vitamin C in cow urine, which is known to be responsible for its immunomodulatory potential and its antioxidant status restoration ability. The natural occurrence of vitamins in cow urine further supports cow urine antioxidant efficacy, which increases its ameliorative potential against toxins.

Conclusion

The present study concludes the antioxidant potential as well as the protective effect of cow urine against chlorpyrifos-induced toxicity, emphasizing the therapeutic importance of cow urine against various disorders. Also, farmers being regularly exposed to such pesticides are recommended to consume sufficient cow urine to protect themselves against pesticide-induced damage.

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