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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(7): 62-67

www.thepharmajournal.com
Received: 22-05-2023
Accepted: 26-06-2023

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Biochemical and free radical-mediated nephrotoxicity induced by imidacloprid (IMI) and Chlorpyrifos (CPF) in Male *Wistar* Rats

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Abstract
The experimental study was designed to evaluate the nephrotoxicity produced by Imidacloprid (IMI) and Chlorpyrifos (CPF) in rats. A total of 24 *Wistar* rats were grouped into 4 groups. The group 1 rats served as control, group 2, 3 and 4 rats received oral doses of Imidacloprid (@ 10 mg/Kg body weight), Chlorpyrifos (@ 7.5 mg/Kg body weight) and IMI+CPF (@ 10 mg/Kg body weight + 7.5 mg/Kg body weight) respectively for 28 days. At the end of the experiment, the serum and kidney were collected for analysis. The serum levels of BUN and Creatinine were increased from group 1 to group 4. The Total Protein and albumin levels were decreased from group 1 to group 4. The rate of increase and decrease was further pronounced in group 4 rats. There was an increase in TBARS and a decrease in GSH, SOD values from group 1 to group 4 in tissue homogenates of kidney. Gross examination revealed severe congestion of kidneys in group 4 rats. Microscopically, kidney sections of group 2 rats revealed tubular dilatation and epithelial degeneration. Group 3 rats showed an increase in intertubular space and round cell infiltration. Group 4 rats showed marked inter and intra-tubular vacuolation, nuclear degenerations and distortion in the tubular structure.
In conclusion, the study suggested that IMI and CPF in combination and alone could be responsible for cellular alteration in kidneys supported by serum and tissue biochemistry, and histopathology and the toxicity was more pronounced in group 4 than in groups 2 and 3.

Keywords: Nephrotoxicity, Imidacloprid, Chlorpyrifos

Introduction
Pesticides are being extensively used worldwide [1]. To meet the growing food demand, use of pesticides is a common practice in agriculture now a days but extensive use of pesticides has resulted in global contamination of the environment and only 0.1% of the applied pesticides reach the pests and the remaining 99.9% find their way to various components of environment [2]. Because of their systemic character and high efficiency to control insects, neonicotinoids are one of the fastest growing and the biggest-selling insecticides in the market [3, 4].
Imidacloprid (IMI) is the first neonicotinoid registered for use as a pesticide by the United States Environmental Protection Agency (US EPA). It is mainly applied in agriculture and in many veterinary formulations for flea control in pet animals. IMI causes oxidative stress and genotoxicity in specific species and hepatotoxicity and nephrotoxicity at a dose much lower than the LD_{50} in mice [5]. The organophosphates are extensively used in agriculture and domestic purposes and their share is more than 50% of all insecticides used in the world [6]. The CPF is a broad-spectrum chlorinated insecticide that is easily absorbed from the intestine and lungs in humans and laboratory animals [7].
In view of above-mentioned observation, this research work was designed to study nephrotoxicity of the chlorpyrifos (CPF) and imidacloprid (IMI) in male *Wistar* rats with the following objectives.

1. To study the effects of Imidacloprid and Chlorpyrifos on Kidneys in male *Wistar* rats.
2. To study the combined toxic effects of chlorpyrifos (CPF) and imidacloprid (IMI) on kidneys.

Materials and Methods
A total of 24 male *Wistar* rats weighing between 180- 220 g, were purchsed from Jeeva life sciences, Hyderabad (CCSEA registration number 1757/PO/RcBiBt/S/14).

The rats were housed in polypropylene cages at laboratory animal house facility in College of Veterinary Science, Rajendranagar, Hyderabad, Telngana and were maintained at 20-22 °C throughout the experiment. Sterile corn cob was used as bedding material for the rats. The rats were given standard pellet diet and deionized water *ad libitum* throughout the experimental period. The animals were acclimatized for 7 days. The rats were closely monitored thrice daily for clinical signs and mortality, if any, during the entire period of study.

The experimental protocol was approved by Institutional Animal Ethics Committee, College of Veterinary Science, Rajendranagar, Hyderabad (6/25/C.V.Sc,HYD.IAEC-Rats/02-07-2022).

Imidacloprid (IMI) was obtained from Insecticides (INDIA) Ltd. Hyderabad under trade name Victor Super IMI 30.5 per cent suspension concentration. Chlopyrifos (CPF) was purchased from Insecticides (INDIA) Ltd. Hyderabad under trade name Lethal CPF 20 per cent emulsified concentration.

Table 1: The experimental design of the study

Groups	No. of animals	Treatment protocol
Group 1	06	Sham
Group 2	06	Imidacloprid @ 10 mg/ Kg body weight per orally daily for 28 days.
Group 3	06	Chlorpyrifos @ 7.5 mg/ Kg body weight per orally daily for 28 days
Group 4	06	Imidacloprid @ 10 mg/ Kg body weight+ Chlorpyrifos @ 7.5 mg/ Kg body weight per orally daily for 28 days

Collection of blood for serum biochemical profile

Approximately, 2 ml of blood was collected from (retro-orbital plexus using capillary tubes into a plain serum vaccutainer (BD Vacutainer, Serum, 13mm x 75mm, 4mL) and allowed to clot for 3-4 hours. The clotted blood was later subjected to centrifugation at 4500 RPM for 10 minutes. After centrifugation, serum was separated and collected into clean Eppendorf tubes and stored at -20 °C. The samples were later used for serum biochemistry by using a semiautomatic ELISA reader by using Erba Mannheim biochemical kits. Serum creatinine and total protein (TP) were estimated by modified Jaffes reaction as per the alkaline picrate technique [8] and standard Biuret procedure [9] respectively. Serum albumin evaluated by BCG (Bromocresol Green Albumin) dye method by using Erba Albumin Assay Kit {(Erba diagnostics Mannheim GmbH, Mumbai), BUN as per Glutamate dehydrogenase (GLDH)} – Urease method [10].

Collection of tissue for Histopathology and antioxidant profile

At the end of the experiment, rats were sacrificed by cervical disarticulation. Detailed necropsy examination was carried out as per standard procedure [11]. Gross lesions in kidneys were recorded. Small slices of respective tissue samples were collected for histopathology in 10% neutral buffer formalin (NBF). The samples were processed, sectioned (5 µm) and stained with haematoxylin and eosin (H and E) for histopathological examination as per the standard procedure [12]. The kidney samples were also collected in liquid nitrogen and stored at -20^0 C. The tissue homogenates were prepared to study the organ oxidative stress parameters like reduced glutathione (GSH) [13], superoxide dismutase (SOD) [14] and Thiobarbituric acid reactive substances (TBARS) [15].

The data obtained were subjected to statistical analysis i.e. one way Analysis of variance (ANOVA) using GraphPad Prism 5, version 5.01 (GraphPad software, California, United States of Amrica). Differences among the means were tested by using Turkey's test, and the significance level was set at $p<0.05$ [16].

Results and Discussion

Effect on Serum Biochemistry

Blood Urea Nitrogen

The mean blood urea nitrogen (mg/dL**)** values in the rats of group 4 (28.07±0.70) were significantly ($p<0.05$) increased as compared to rats of groups 1, 2 and 3 (13.31±0.94, 17.87±0.74 and 20.56±1.05) respectively (Table 2).

Serum Creatinine

The mean serum creatinine (mg/dL**)** values in the rats of group 4 (3.22± 0.14) were significantly ($p<0.05$) increased as compared to groups 1, 2 and 3 (0.58 ± 0.04, 1.17±0.13 and 1.82 ± 0.20) respectively (Table 2).

Total Proteins

The mean total protein (g/dL**)** values in the group 4 rats (6.01±0.26) were significantly ($p<0.05$) decreased as compared to mean TP of groups 1, 2 and 3 rats (8.49±0.12, 7.35±0.30 and 7.04±0.24) respectively (Table 2).

Albumin

The mean albumin (g/dL**)** values in the group 4 rats (2.94±0.06) were significantly ($p<0.05$) decreased as compared to groups 1, 2 and 3 rats (4.25±0.14, 3.72±0.18 and 3.67±0.11) (Table 2).

Increase in values of BUN and creatinine indicate renal damage. Renal tubular damage alters glomerular filtration, increases the production of ROS and renal damage [17]. Decrease in TP and albumin in the current study is attributed to loss of biosynthesis function, inhibition of protein synthesis and increased protein loss *via* kidneys.

Table 2: Serum biochemistry in different groups of rats

Group	Treatment	BUN (mg/dl)	Creatinine (mg/dl)	Total Proteins (g/dl)	Albumin (g/dl)
Group 1	Control	13.31^{a}±0.94	0.58^{a}±0.04	8.49^{a}±0.12	4.25^{a}±0.14
Group 2	IMI@10 mg/Kg b.wt.	17.87^{b}±0.74	1.17^{b}±0.13	7.35^{b}±0.30	3.72^{b}±0.18
Group 3	CPF@7.5 mg/Kg b.wt.	20.56^{b}±1.05	1.82^{c}±0.20	7.04^{b}±0.24	3.67^{b}±0.11
Group 4	IMI@10 mg/Kg b.wt. + CPF@7.5 mg/Kg b.wt.	28.07^{c}±0.70	3.22^{d}±0.14	6.01^{c}±0.26	2.94^{c}±0.06

Values are Mean ± SE (n=6); One way ANOVA

Means with different superscripts in a column differ significantly at $p<0.05$

Tissue Antioxidant Profile

Thiobarbituric Acid Reacting Substances (TBARS-µM of MDA/mg of protein)

The mean MDA (µM/mg) values of kidneys in the group 4 rats (7.09±0.22) were significantly (p<0.05) increased as compared to groups 1, 2 and 3 rats (4.33±0.16, 5.51±0.15 and 6.26±0.18), respectively (Table 3).

The increased TBARS in kidneys could be due to increased LPO leading to an increased intracellular accumulation of ROS thereby leading to irreparable injury of Kidneys [18].

Reduced Glutathione Concentration (GSH-µM/mg protein)

The mean GSH (µM/mg) values of kidneys in the group 4 rats (7.16±0.11) were significantly (p<0.05) decreased as compared to groups 1, 2 and 3 rats (9.49±0.11, 8.35±0.18 and 8.10±0.10), respectively (Table 3). This can be explained by an excess production of Reactice Oxygen Species, responsible for kidney enzymes leakage [19]. Decreased antioxidant enzyme activities might be due to cellular injury and death of healthy tissue that are able to respond to the oxidative insult. On the other hand, it may be due to the insufficient detoxification capacity of CPF and damage caused by ROS [20].

Superoxide Dismutase (SOD-U/mg protein)

The mean SOD (U/mg) values of kidneys in group 4 (3.75±0.20) were significantly (p<0.05) decreased as compared to groups 1, 2 and 3 (6.01±0.16, 5.21±0.12 and 4.94±0.23), respectively (Table 3). The increase in lipid peroxidation and oxidative stress might be due to changes in the antioxidant defence systems. Co-exposure of rats to IMI and CPF might have generated excessive free radicals resulting in the depletion of antioxidants [21].

Table 3: Antioxidant Profile in different groups of rats- Day 28

Group	Treatment	TBARS (µM MDA/mg of protein)	GSH (µM/ mg protein)	SOD activity (U/mg protein)
Group 1	Sham	4.33^{a}±0.16	9.49^{a}±0.11	6.01^{a}±0.16
Group 2	IMI@10 mg/kg b.wt.	5.51^{b}±0.15	8.35^{b}±0.18	5.21^{b}±0.12
Group 3	CPF@7.5 mg/kg b.wt.	6.26^{c}±0.18	8.10^{b}±0.10	4.94^{b}±0.23
Group 4	IMI@10 mg/kg b.wt. + CPF@7.5 mg/kg b.wt.	7.09^{d}±0.22	7.16^{c}±0.11	3.75^{c}±0.20

Values are Mean ± SE (N=6), One way ANOVA

Means with different superscripts in a column differ significantly at p<0.05

Gross Changes

Kidneys from group 1 rats showed normal appearance (Fig. 1), group 2 showed mild congestion (Fig. 2), group 3 showed abscess and congestion (Fig. 3) and group 4 showed severe congestion (Fig 4).

The congestion may be due to vascular changes caused by toxins and severe atrophy of the brain observed in the combined toxicity group may be due to degenerative and oxidative damage caused by toxins. These changes clearly suggest the toxic effects of IMI and CPF on different organs. These observations are in accordance with the observations made by [22].

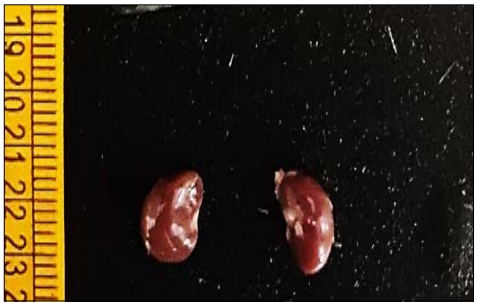

Fig 1: Photograph showing normal appearance of kidneys (Group 1)

Fig 2: Photograph showing mild congestion of kidneys (Group 2)

Fig 3: Photograph showing abscess and congestion of kidneys (Group 3)

Fig 4: Photograph showing severe congestion of kidneys (Group 4)

Histopathology

Sections of group 1 kidneys showed normal glomerulus and tubules architecture (Fig. 5). Sections of group 2 showed moderate tubular dilation and epithelial degeneration (Fig. 6), marked tubular epithelium degeneration (Fig. 7), moderate mononuclear cell infiltration, tubular epithelial degeneration, inflammation in glomerulus and tubular dilation (Fig.8). Group 3 sections showed tubular degeneration and moderate increase in inter tubular space, shrunken to enlarged glomeruli and few vacant glomeruli without Bowman's capsule (Fig. 9), moderate tubular congestion, tubular epithelial degeneration and loss of architectural details in tubule (Fig. 10), moderate

tubular degenerations and discontinuity in tubular epithelium (Fig. 11). Group 4 sections showed increase in intertubular space, dilation of tubules and few vacant glomeruli (Fig. 12), marked inter and intra tubular vacuolation, infiltration of mononuclear cells and nuclear degenerations (Fig.14), marked intertubular edema, congestion and distortion in tubular structure (Fig.14).

These changes might be due to oxidative stress, lipid peroxidation, increased cell apoptosis and DNA damage along with altered activities of tissue antioxidant enzymes. Degeneration of the glomeruli and tubules may be due to collection of albuminous material during the excretion of high concentrations of the toxin in the urine. Similar observations were reported by [23, 24, 26, 26].

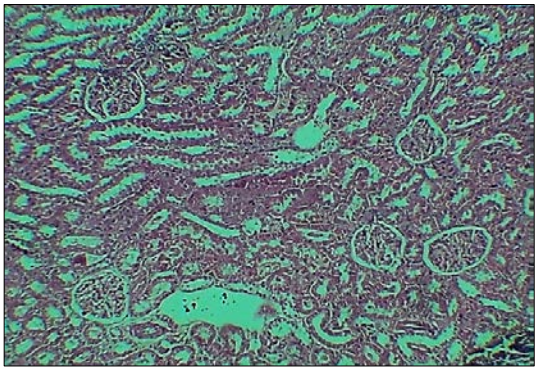

Fig 5: Photomicrograph showing normal glomerulus and tubules in the kidney (Group 1) H & E X 100

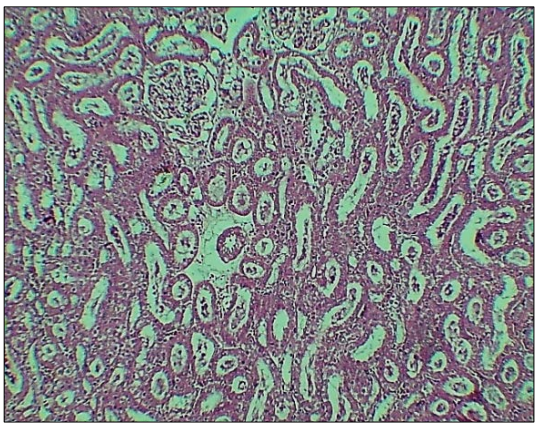

Fig 6: Photomicrograph showing moderate tubular dilation and epithelial degeneration in kidney (Group 2) H & E X 100

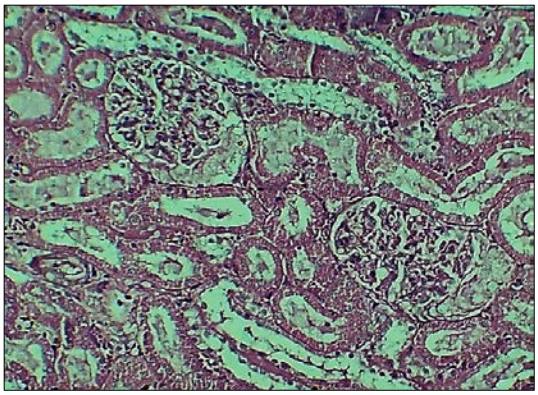

Fig 7: Photomicrograph of kidney showing tubular epithelium degeneration (Group 2) H & E X 200

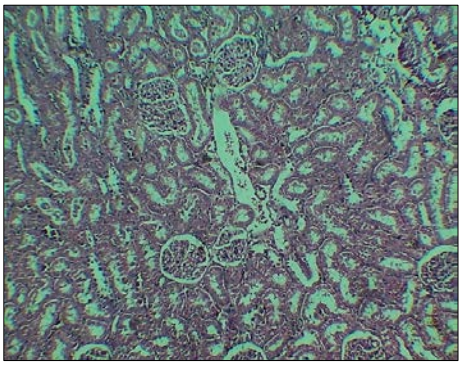

Fig 8: Photomicrograph of a kidney showing moderate round cell infiltration, tubular epithelial degeneration, inflammation in glomerulus and tubular dilation (Group 2) H&E X 100

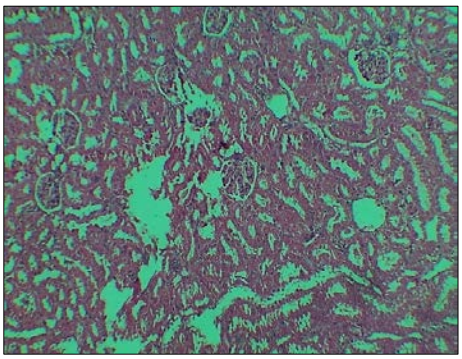

Fig 9: Photomicrograph showing moderate increase in intertubular spaces, shrunken to enlarged glomeruli, few vacant glomeruli without Bowman's capsule in kidney (Group 3) H&E X 100

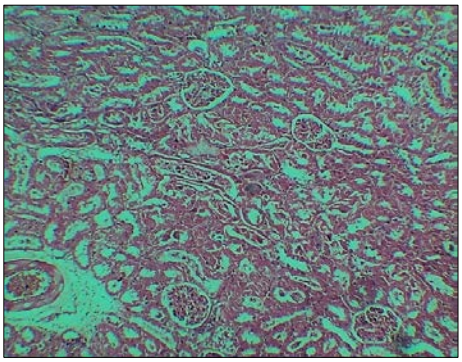

Fig 4.10: Photomicrograph showing moderate tubular congestion, tubular epithelial degeneration, loss of architectural details in tubule in kidney (Group 3) H&E X 100

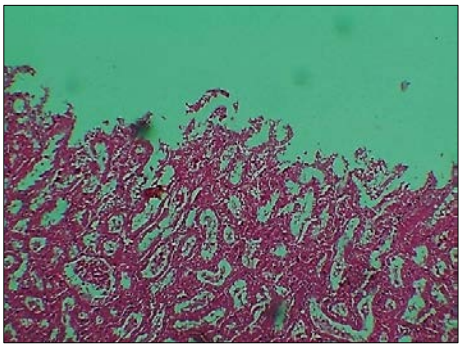

Fig 11: Photomicrograph of a kidney showing moderate tubular degenerations and discontinuity in tubular epithelium (Group 3) H & E X 100

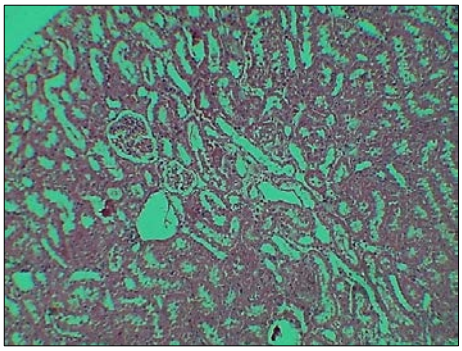

Fig 12: Photomicrograph of kidney showing a marked increase in intertubular space, dilation of tubules and few vacant glomeruli (Group 4) H & E X 100

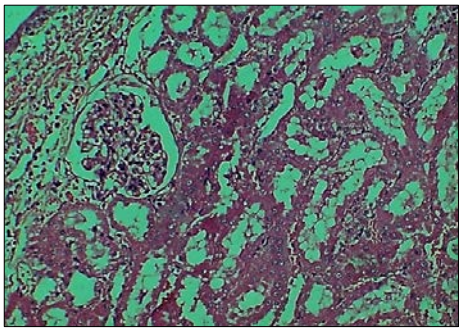

Fig 13: Photomicrograph of kidney showing marked inter and intra-tubular vacuolation, infiltration of round cells, nuclear degenerations (Group 4) H & E X 200

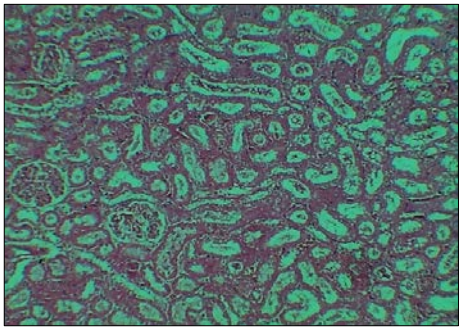

Fig 14: Photomicrograph of kidney showing marked intertubular edema, congestion and distortion in tubular structure (Group 4) H & E X 100

Conclusion

Based on the results obtained in this study it can be concluded that:

1. The changes in oxidative parameters revealed IMI and CPF induced oxidative stress due to overproduction of free radicals.
2. IMI and CPF were responsible for marked changes in Kidney which were evidenced biochemically and histopathologically.
3. Overall, the present study suggests IMI (10 mg/Kg body weight), CPF (7.5 mg/ Kg body weight) and combination of IMI (10 mg/ Kg body weight) + CPF (7.5 mg/ Kg body weight) exposure can cause severe damage to kidney.
4. The usage of IMI and CPF should be gradually curtailed for crop protection to prevent its toxic effects on the non-target organisms and human beings.
5. There is a lot of scope for studying molecular mechanisms of IMI and CPF-induced nephrotoxicity in rats and also ameliorative agents or combinations of different agents that can neutralize the toxic action of IMI and CPF.

Acknowledgement

Authors thank College of Veterinary Science, P V Narasimha Rao Telangana Veterinary University, Rajendranagar, Hyderabad for funding the research. I am also thankful to the subjects of my research for sacrificing their lives for a noble cause.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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