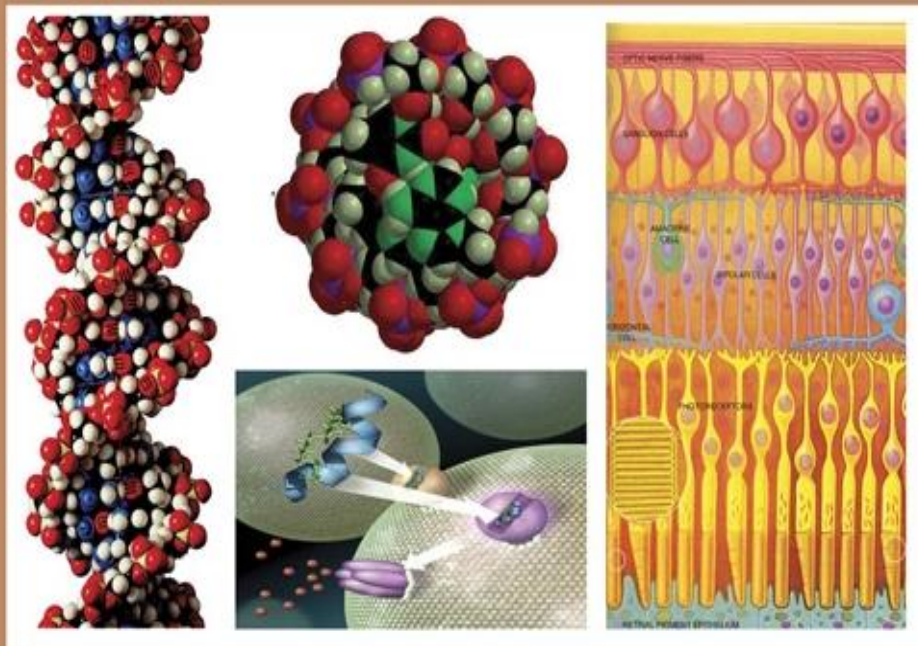




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Toxicity Induced by Indoxacarb Exposure in Male Albino Rats and The Possible Protective Effects of Vitamin C and Zinc

Hanaa F. Hassan^{1*}; Nisreen. D. M. Toni² and Noha M. Meligi¹

1- Zoology Department, Faculty of Science, Minia University 61519, Minia, Egypt

2- Pathology Department, Faculty of Medicine, Minia University 61519, Minia, Egypt

*E. Mail : hanaafawzy78@mu.edu.eg - Nisreen_dahi@yahoo.com

noha.melege@mu.edu.eg

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ABSTRACT

Accidental exposure or overexposure to pesticides can have considerable consequences. A pesticide poisoning happens after chemicals proposed to control a pest affect non-target organisms such as humans. The present work aimed to evaluate the potential adverse effects of the insecticide, indoxacarb, and the protective effects of supplementation with vitamin C (200 mg/kg), and zinc (Zn) (100 mg/kg) (separately or together) on the resulted toxicity. Thirty male Sprague-Dawley rats were randomly divided into five groups (n=6): group I served as the control group, group II received orally a sublethal dose of indoxacarb (100 mg/kg), group III received orally indoxacarb+vitamin C, group IV received orally indoxacarb+Zn and group V received orally indoxacarb+vitamin C+Zn, three times a week for 21 days. Exposure of rats to indoxacarb caused significant changes of some oxidative stress parameters (lipid peroxide (LPx) level, superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activity) besides a substantial increase in the activities of hepatic enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)). In addition, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total protein, albumin, and kidney functions (levels of creatinine, urea, uric acid, and aldosterone hormone activity) in indoxacarb-treated rats exhibited remarkable changes compared with the control rats. Also, the histological changes in the liver and kidney of all groups were investigated. Furthermore, the mixture of vitamin C and zinc ameliorated the biochemical parameters and the histological changes in the liver and kidney of indoxacarb-treated rats. In conclusion, the mixture of vitamin C and Zn may display beneficial effects against indoxcarb-toxicity through improvement in the examined biochemical parameters imbalance and histological alterations in liver and kidney over the use of either of them alone.

INTRODUCTION

Different groups of pesticides have harmful effects on habitats and are not limited to an ecological niche but often disturb human health through the accumulation of toxic residues in the food chain (Sandeep *et al.*, 2016).

Indoxacarb is a non-systemic insecticide that is used to control sucking and chewing insects and was found to be more effective in lepidopteran pests of rice, pears, apples, cotton, and fruiting vegetables. Indoxacarb has shown good effectiveness in the control of bees, cockroaches, fleas, termites, and houseflies. This insecticidal action occurs by blocking the channels of sodium in the target pest nervous system and communication routes leading to impaired nerve function, paralysis, stoppage of feeding and even death (US EPA, 2000).

Pesticides cause oxidative stress because of an increase in reactive oxygen species (ROS), a disturbance of antioxidant defense systems, or an inadequate capability to restore oxidative injury. Potential damage induced by oxidative stress exerts alterations of cellular membrane lipids, proteins, and DNA. Previous studies have reported that indoxacarb induces oxidative stress in different organs like the kidney, testes, and ovary and induces changes in biochemical constituents of the liver in albino mice (Mudaraddi *et al.*, 2012). Moreover, mice that have been treated with many doses of indoxacarb for 30 days showed distinguished adverse effects on protein and lipid contents and some antioxidant enzyme activities as catalase and superoxide dismutase (Abdelrasoul, 2018).

Vitamin C (VC) shows structural similarity to glucose and can be considered as its alternative in many chemical processes. The anticarcinogenic and antimutagenic roles of VC have been explored in a range of *in vivo* and *in vitro* systems subjected to pesticides (Durak *et al.*, 2009). VC is an influential antioxidant; it readily scavenges ROS such as superoxide, hydroxyl, and aqueous peroxy radicals. Moreover, it collaborates with Vitamin E in membranes and lipoproteins to redevelop α -tocopherol from α -tocopherol radicals (Kojo, 2004). Subjected to certain conditions, VC can

avert the vulnerability of lower respiratory tract infection (Hemila, 1997). Recently, it was demonstrated that a moderate amount of VC supplementation can be utilized as a possible route to prevent COVID-19, as it may lead to lower respiratory tract infection (Matthay *et al.*, 2020 and Wang *et al.*, 2020).

Zinc (Zn) is a trace element that is important for the standard growing and the growth of all animal species and is vital for the integrity of the immune system. The lack of Zn results in a reduction in the growth rate (Prasad *et al.*, 1996). Zn is vital for enzymes involved in DNA synthesis, mitosis, and protein synthesis (Bettger and O'Dell, 1993). Moreover, it shows ameliorative effects against oxidative liver damage induced by organophosphate intoxication (Goel *et al.*, 2005). Recently, Zhang and Liu (2020), reported that Zn supplement showed a potential efficacy on symptoms related to COVID-19 such as diarrhea and lower respiratory tract infection, as well as on the new COVID-19 coronavirus itself.

Some studies have been attempted to evaluate the amelioration role of VC and Zn on some biochemical alterations induced by insecticides such as chlorpyrifos (Uchendu *et al.*, 2012). However, to the best of our knowledge, there are no studies on indoxacarb with respect to co-administration with VC and Zn. Hence, the main objective of our investigation is to assess the degree of hazard posed by the exposure to a sub-lethal dose of indoxacarb on the antioxidant enzymes and hepatic and renal function in male albino rats. Furthermore, the present study evaluates the efficiency of VC and Zn in amending the damage produced by the examined insecticide. In addition, due to the documented synergistic activities between vitamins E and C (Hamilton *et al.*, 2000), vitamin E and Zn (Zago and Oteiza, 2001), a significant query arose whether or not a mixture of VC and Zn would provide

better protection against toxicity to indoxacarb.

MATERIALS AND METHODS

Animal Ethics:

The authors declare that all experimentation, transportation, and care of the animals used in this study were carried out in compliance with the approval of the Faculty of Science, Minia University's policy on animal use and ethics. All mandatory laboratory health and safety measures have been adhered to while performing the experimental work of this study.

Experimental Animals:

Thirty adult male albino rats (Sprague-Dawley) were collected from the research laboratory's animal house in Egypt at the age of 6-8 weeks, weighing 150-200 g. The rats were housed in a well-ventilated room at $25\pm 3^{\circ}\text{C}$ in clean plastic

cages for 3 weeks before the commencement of the experiment as an acclimatization period. Animals had access to ordinary food and water *ad libitum*.

Chemicals:

Indoxacarb is an oxadiazine pesticide (**Fig. 1**). its IUPAC (International Union of Pure and Applied Chemistry) name is methyl (4*aS*)-7-chloro-2-[methoxycarbonyl]-[4-(trifluoromethoxy)phenyl]carbamoyl]-3,5-dihydroindeno[1,2-*e*][1,3,4]oxadiazine-4*a*-carboxylate. Indoxacarb was supplied from the Agrimatco Business (Minia, Egypt). The sublethal dose of indoxacarb (100 mg/kg body weight in 1.0 ml corn oil) was received orally, three times a week for 21 days. Vitamin C (200 mg/kg) and Zn (100 mg/kg) were bought from local pharmacy.

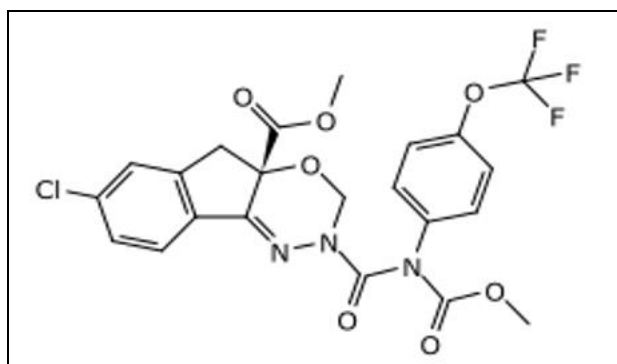


Fig. 1: General chemical structure of indoxacarb.

Experimental Design:

The rats were divided into five classes (n=6) as follows:

group 1 (control group) received orally 1.0 ml of corn oil, group 2 (indoxacarb-treated group) received orally indoxacarb (100 mg/kg body weight in 1.0 ml corn oil), group 3 (indoxacarb + VC- treated group) received orally a mixture of indoxacarb (100 mg/kg body weight in 1.0 ml corn oil) + VC (200 mg/kg), group 4 (indoxacarb + Zn- treated group) received a mixture of indoxacarb (100 mg/kg body weight in 1.0 ml corn oil)+ Zn (100 mg/kg), group 5 (indoxacarb + VC + Zn-treated group) received a mixture of

indoxacarb (100 mg/kg body weight in 1.0 ml corn oil) + VC (200 mg/kg) + Zn (100 mg/kg). All doses were given orally using a stainless-steel stomach tube three times per week for 21 consecutive days. Observation on animals was performed daily to record any sign of toxicity.

Recording of Body Weights:

At the start of the experiment and prior to dissection, rats were weighed to estimate the changes in the body weight.

Blood Sampling:

After 21 days, the animals were fasted overnight and then killed by cervical decapitation to collect the samples of blood. Blood samples collected on

ethylenediaminetetraacetic acid (EDTA) were used to separate plasma after centrifugation at 4000 rpm for 10 min. Other blood samples were left to coagulate at room temperature and centrifuged as described above to separate serum samples. For biochemical analysis, all samples were kept frozen at -80 °C.

Estimation of Biochemical Analysis:

Assays of both plasma and serum biochemicals were calorimetrically calculated using the commercial diagnostic kits. Plasma lipid peroxide (LPx) level and glutathione peroxidase (GPx) activity were measured according to the methods of Ohkawa *et al.* (1979) and Paglia and Valentine (1967) respectively. The activity of the plasma superoxide dismutase (SOD) was determined according to the method of Marklund and Marklund (1974). Determination of Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities was assessed using the method of Bergmeyer *et al.* (1986). Based on the methodology adopted by Badimon *et al.* (1990), serum high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) rates were assessed. Serum total protein and albumin levels were measured according to the methods reported by Leonard *et al.* (1971) and Young and Friedman (2001), respectively. Serum creatinine, urea and uric acid levels were determined according to the methods of Young and Friedman (2001), Patton and Crouch (1977) and Dumas *et al.* (1981) respectively. Plasma aldosterone hormone activity was measured using the method of Cartledge and Lawson (2000).

Histopathological Evaluation:

Histopathological examination of the liver and kidneys of the studied rats was done to assess the structural abnormalities and to correlate them to the biochemical findings. Multiple tissue sections were taken and fixed in 10% formaldehyde and embedded in paraffin blocks. Multiple 4 µm sections were stained with Hematoxylin and Eosin

(H&E) stain following the standard protocol. Sections were examined under a light microscope by a pathologist who was blind to the data of the groups. Images were taken by a digital camera (Nikon) connected to a light microscope (Olympus, Japan). Signs of liver injury are fatty change, cellular necrosis, hemorrhage, congestion, and inflammatory cells infiltration. Signs of renal injury are alteration of the architecture of the cortex and medulla and the glomerular structure in addition to both proximal and distal tubules.

Immunohistochemical Assessment of iNOS:

Deparaffinization then tissue rehydration was done by using xylene and descending grades of ethyl alcohol respectively for 10 min each. Blocking of peroxidase activity was done using hydrogen peroxide in methanol solution for 30 min. Antigen retrieval using citrate buffer (pH 6) was done. Samples were incubated with goat serum for 5 min followed by overnight incubation of samples at 4°C with an anti-iNOS polyclonal antibody. Incubation was done with secondary antibody for 10 min then with streptavidin-peroxidase for 5 min. Incubation was done with DAB for 3 min. Counterstaining of the slides using Meyer's haematoxylin was done. Dehydration in ascending grades of alcohol followed by clearance, mounting and finally covering with coverslips was done. With each run, both positive and negative controls were processed. Immunostaining was scored semi-quantitatively based on the assessment of both intensities of staining and the percentage of cells that stained positively. The staining score was calculated by multiplying the percentage of positive cells by staining intensity. The intensity of staining was classified as 0 (negative), 1+ (weak), 2+ (moderate) or 3+ (strong). The final score ranged from 0 to 300. Negative control slides were treated with PBS

instead of using the iNOS (Ali *et al.*, 2018).

Statistical Analysis:

The findings of the present analysis have been evaluated using statistical package of social sciences (SPSS) statistics software for Windows, Version 22 (IBM corp., Armonk, NY, USA). The significance among groups was evaluated by one-way analysis of variance (ANOVA), accompanied by Tukey's multiple comparison test. The data were expressed as the mean ± standard deviation, where P<0.05 was measured as the degree of significance. Graphs were plotted with Excel MS Office version 2016.

RESULTS

Toxicity signs and mortality

Throughout the study period, some treated rats with indoxacarb as represented in group 2 and its mix with VC and Zn as represented in groups 3 and 4, respectively, showed signs of toxicity such as circling, convulsions, weakness and muscle spasms in hind legs. In addition, the above groups reported a gradual decline in body weight. No death has been documented in all the experimental groups.

Evaluation of biochemical parameters

As shown in (Table 1), LPx level (Fig. 2) and SOD activity (Fig. 3) increased significantly after treatment with indoxacarb. However, GPx activity (Fig.

3) was decreased significantly in indoxacarb -treated group compared to the control group. These effects were recovered to a large extent by treatment with VC + Zn where there were significant changes in LPx level, GPx and SOD activation when compared to the indoxacarb-treated group. As shown in (Table 2), a substantial increase in liver enzyme activities (AST, ALT) (Fig. 4) and LDL-C levels (Fig. 5) and a significant decrease in total protein, albumin (Fig. 6) and HDL-C levels (Fig. 5) of rats treated with indoxacarb relative to control rats, demonstrating the incidence of hepatic toxicity. As indicated in the table (3), a significant rise in renal functions (creatinine, urea, and uric acid levels (Fig. 7) and aldosterone hormone activity (Fig. 8)) in an indoxacarb-treated group compared to the control group. While for rats treated with indoxacarb + VC and indoxacarb + Zn, there was a slight improvement of most of the tested biochemical parameters when compared to the indoxacarb-treated group. However, the administration of VC + Zn combined with indoxacarb amended the utmost of these parameters. We observed that the mixture of VC + Zn modulated significantly (P<0.05) all changes in biochemical parameters in indoxacarb-treated rats and returned approximately the values to the control values.

Table 1: Plasma LPx level and GPx and SOD enzyme activity in control and experimental rats.

Groups Parameters	Control group	Indoxacarb- treated group	Indoxacarb +VC treated group	Indoxacarb+Zn treated group	Indoxacarb +VC+Zn treated group
LPx (nmol/ml)	4.44 ± 0.11	12.00 ± 0.91 ^a	7.67 ± 0.31 ^a	7.67 ± 0.31 ^a	7.67 ± 0.31 ^a
GPx (U/L)	443.60± 4.15	210.80 ± 1.82 ^a	251.70 ± 5.63 ^a	251.70 ± 5.63 ^a	251.70 ± 5.63 ^a
SOD (U/ml)	219.20± 14.32	515.80 ± 6.77 ^a	303.20 ± 3.08 ^a	303.20 ± 3.08 ^a	303.20 ± 3.08 ^a

Values are means ± SD for six rats in each group. Significant at P < 0.05. ^a Comparison of the control with other groups. ^b Comparison of the indoxacarb-treated group with the indoxacarb+VC+Zn -treated groups.

Table 2: Serum AST and ALT enzyme activity and HDL-C, LDL-C, total protein, and albumin levels in control and experimental rats.

Groups Parameters	Control group	Indoxacarb-treated group	Indoxacarb +VC treated group	Indoxacarb+Zn treated group	Indoxacarb +VC+Zn treated group
AST (UL)	39.27 ± 0.64	181.30 ± 0.82 ^a	95.50 ± 0.53 ^a	120.50 ± 0.71 ^a	54.10 ± 1.52 ^{a, b}
ALT (UL)	32.10 ± 1.10	101.90 ± 2.1 ^a	85.30 ± 4.21 ^a	92.30 ± 0.83 ^a	44.20 ± 0.78 ^b
HDL-C (mg/dl)	39.18 ± 0.77	23.90 ± 0.74 ^a	28.60 ± 0.68 ^a	21.65 ± 0.45 ^a	40.88 ± 1.62 ^b
LDL-C (mg/dl)	28.48 ± 0.82	44.70 ± 0.95 ^a	39.60 ± 0.52 ^a	45.70 ± 0.43 ^a	34.70 ± 0.67 ^{a, b}
Total protein(g/dl)	8.30 ± 0.132	7.23 ± 0.08 ^a	7.71 ± 0.09 ^a	7.27 ± 0.08 ^a	8.98 ± 0.12 ^b
Albumin (g/dl)	6.12 ± 0.12	3.11 ± 0.08 ^a	3.43 ± 1.08 ^a	3.51 ± 0.09 ^a	4.36 ± 0.23 ^b

Values are means ± SD for six rats in each group. Significant at P < 0.05. ^a Comparison of the control with other groups. ^b Comparison of the indoxacarb-treated group with the indoxacarb +VC+Zn -treated groups.

Table 3: Serum creatinine, urea, uric acid levels, and plasma aldosterone hormone activity in control and experimental rats

Groups Parameters	Control group	Indoxacarb-treated group	Indoxacarb +VC treated group	Indoxacarb+Zn treated group	Indoxacarb +VC+Zn treated group
Creatinine	0.78 ± 0.83	0.96 ± 0.057 ^a	0.80 ± 0.10	0.96 ± 0.057 ^a	0.766 ± 0.057 ^b
Urea	37.40 ± 1.82	62.66 ± 1.15 ^a	50.33 ± 0.57 ^{a, b}	42.66 ± 4.04 ^b	38.66 ± 3.21 ^b
Uric acid	2.08 ± 0.83	6.66 ± 0.152 ^a	3.26 ± 0.30 ^{a, b}	5.30 ± 0.30 ^{a, b}	2.30 ± 0.52 ^b
Aldosterone hormone	187.64 ± 32.5	623.4 ± 122.2 ^a	476.4 ± 62.6 ^a	512.4 ± 8.8 ^a	304.6 ± 37.2 ^b

Values are means ± SD for six rats in each group. Significant at P < 0.05. ^a Comparison of the control with other groups. ^b Comparison of the indoxacarb-treated group with the indoxacarb+VC+Zn -treated groups.

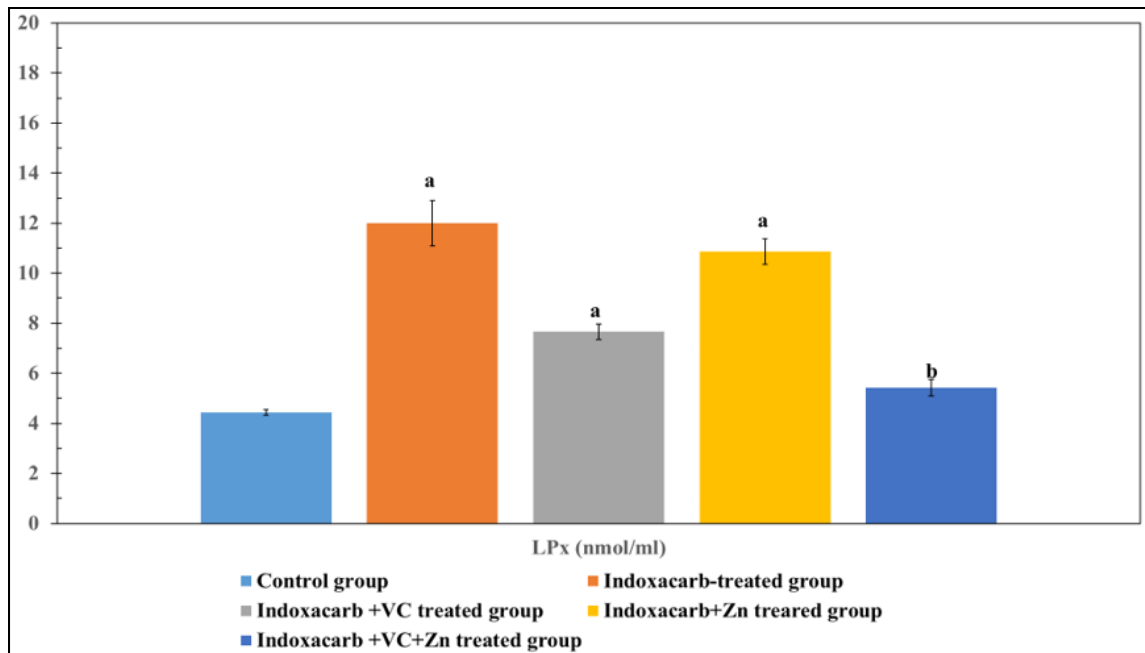


Fig. 2: Plasma LPx level in control and experimental rats. Values are means ± SD for six rats in each group. Significant at P < 0.05. ^a Comparison of the control with other groups. ^b Comparison of the Indoxacarb-treated group with the Indoxacarb +VC+Zn -treated groups.

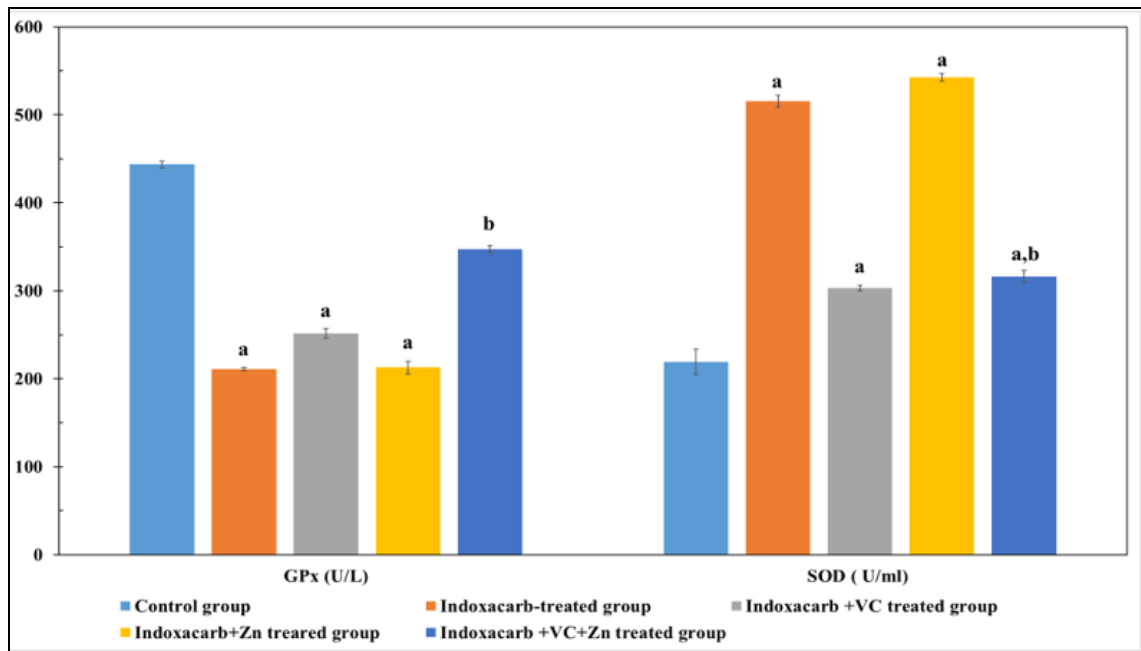


Fig 3: P.lasma GPx and SOD enzyme activity in control and experimental. Values are means \pm SD for six rats in each group. Significant at $P < 0.05$. ^a Comparison of the control with other groups. ^b Comparison of the Indoxacarb-treated group with the Indoxacarb +VC+Zn -treated groups.

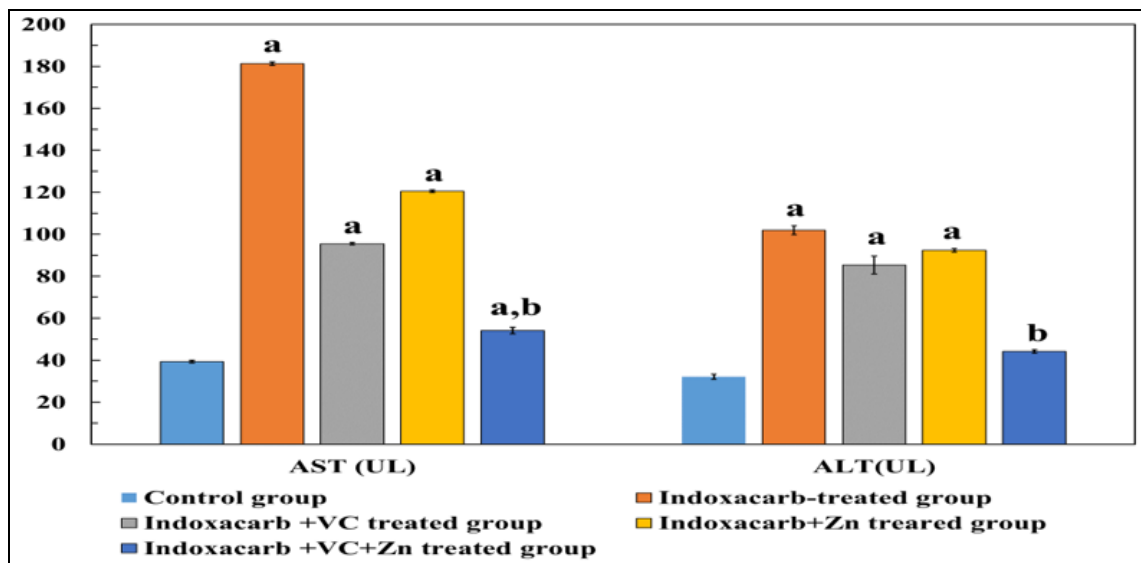


Fig. 4: Serum AST and ALT enzyme activity in control and experimental rats. Values are means \pm SD for six rats in each group. Significant at $P < 0.05$. ^a Comparison of the control with other groups. ^b Comparison of the Indoxacarb-treated group with the Indoxacarb +VC+Zn -treated groups.

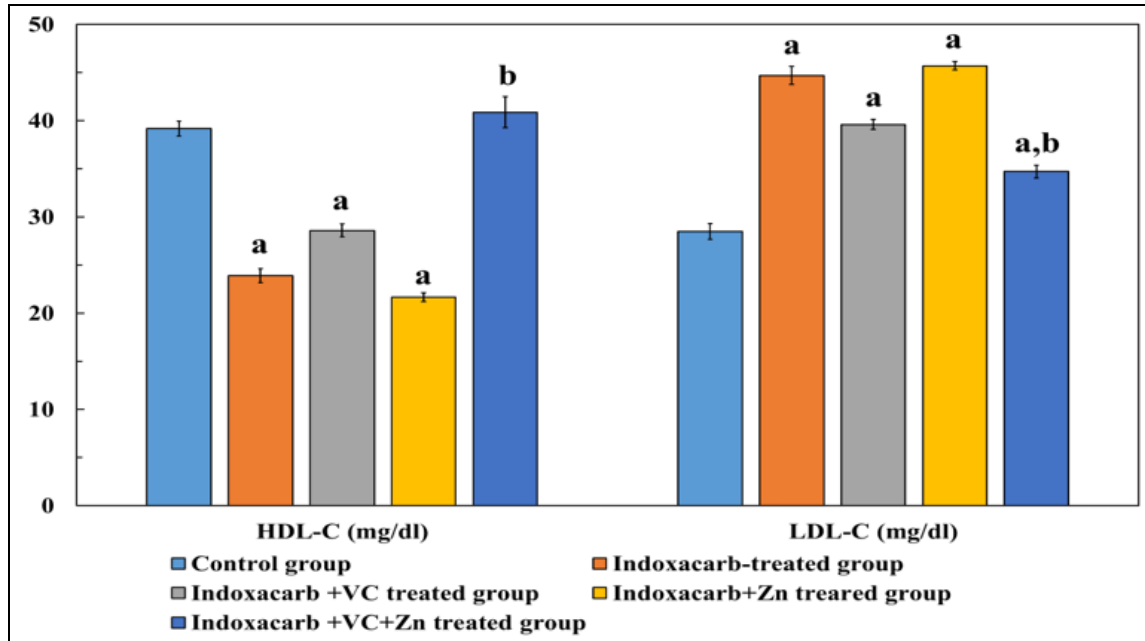


Fig. 5: Serum HDL-C and LDL-C levels in control and experimental rats. Values are means \pm SD for six rats in each group. Significant at $P < 0.05$. ^a Comparison of the control with other groups. ^b Comparison of the Indoxacarb-treated group with the Indoxacarb +VC+Zn -treated groups.

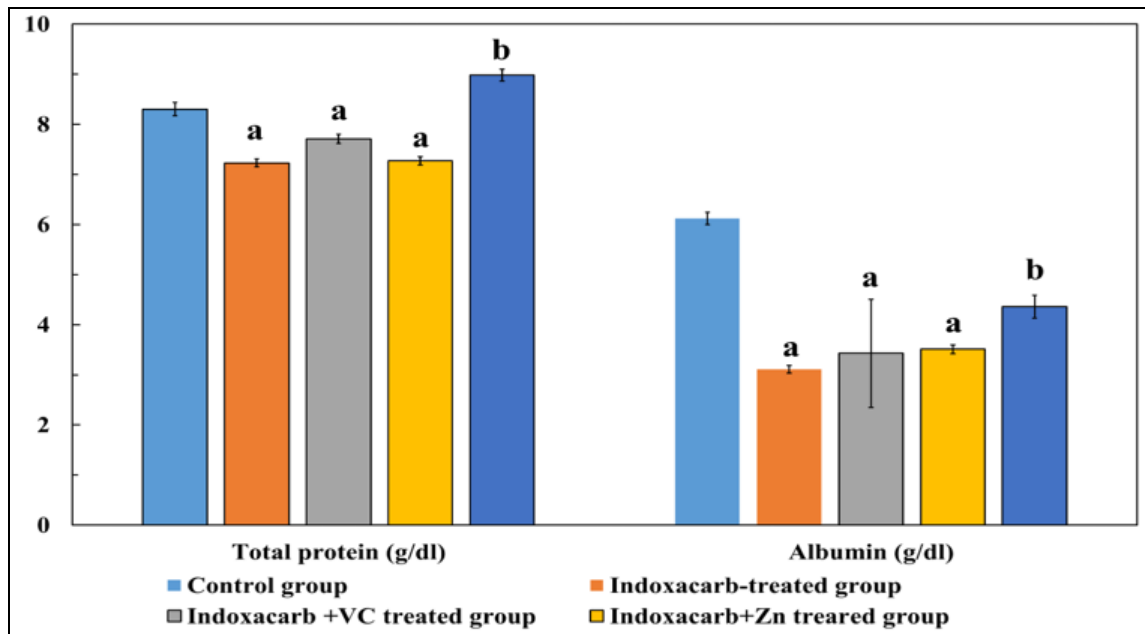


Fig. 6: Serum total protein and albumin levels in control and experimental rats. Values are means \pm SD for six rats in each group. Significant at $P < 0.05$. ^a Comparison of the control with other groups. ^b Comparison of the Indoxacarb-treated group with the Indoxacarb +VC+Zn -treated groups.

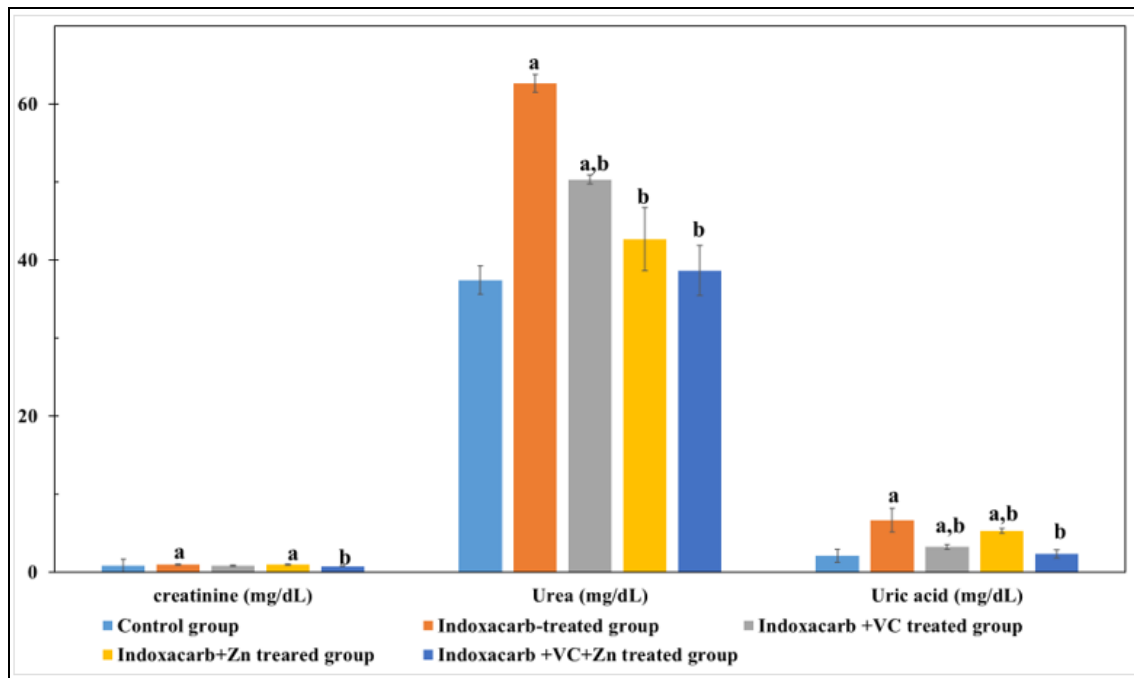


Fig. 7: Serum creatinine, urea, and uric acid levels in control and experimental rats. Values are means \pm SD for six rats in each group. Significant at $P < 0.05$. ^a Comparison of the control with other groups. ^b Comparison of the Indoxacarb-treated group with the Indoxacarb +VC+Zn -treated groups.

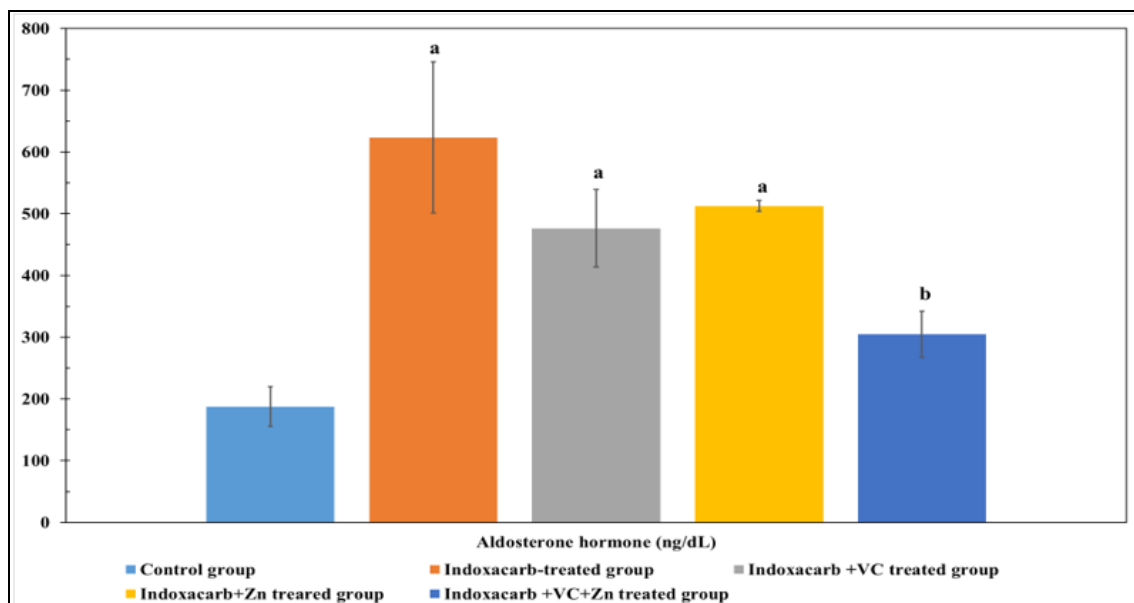


Fig. 8: Serum aldosterone activity in control and experimental rats. Values are means \pm SD for six rats in each group. Significant at $P < 0.05$. ^a Comparison of the control with other groups. ^b Comparison of the Indoxacarb-treated group with the Indoxacarb +VC+Zn -treated groups.

Histological examination of liver and kidney

1-Histological changes of the liver:

Microscopic examination of liver H&E-stained sections of the liver of

the (control) group showed normally arranged hepatocytes in cords (Fig. 9A). Sections of the Indoxacarb treated group of rats revealed severe damage in the form of severe necrosis, cell ballooning and

fatty changes. Inflammatory cell infiltration was marked in addition to the hemorrhagic infiltration of sinusoids (Fig. 9B). The hepatic tissue examined of both groups (Indoxacarb+Zn) (Fig. 9C) and (Indoxacarb+VC) (Fig. 9D) showed nearly similar results with marked protection against the toxic effect of the Indoxacarb.

Hepatocellular damage was minimal among rats treated with combined Indoxacarb+ Zn+VC (Fig. 9E). Microscopic examination of the liver of animals treated with (Indoxacarb+ VC+ Zn) showed mild toxic effects indicating an effective protective role.

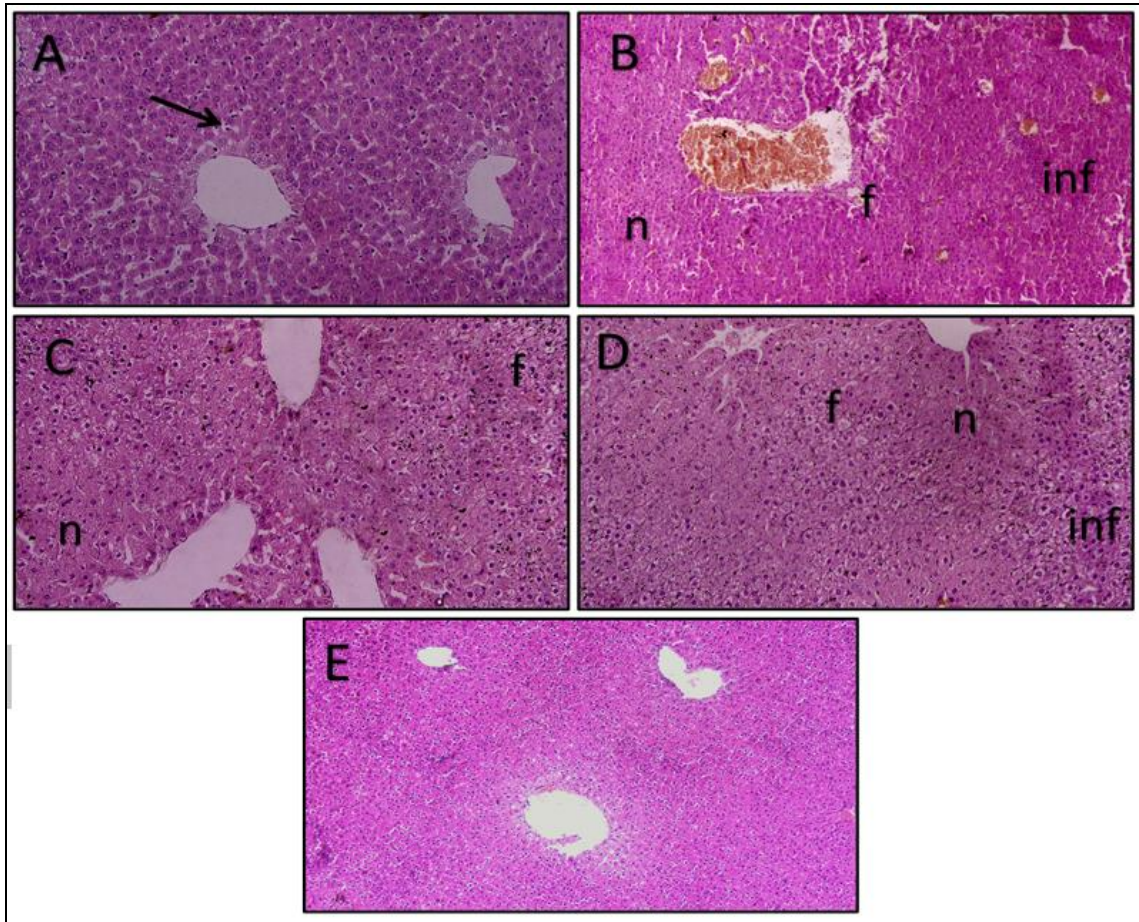


Fig. 9: Photomicrograph of different liver sections stained with H&E. (A) liver section from control rats showing normal hepatic architecture with normal hepatocytes (arrow). (B) Liver sections of the Indoxacarb treated rats show multiple large-sized areas of necrotic hepatocytes (n) with vacuolated cytoplasm and fatty change (f) and wide areas of inflammation (inf) with the loss of hepatic lobular architecture. Liver section from rats of both groups that received (C) (Indoxacarb+Zn) and (D) (Indoxacarb+VC) showed mild improvement of liver degeneration with moderate degeneration. (E) (Indoxacarb+ Zn+ VC) treatment markedly decreased hepatic damage and inflammatory infiltration which are restricted to few hepatocytes with mild vacuolation of their cytoplasm while the nuclei appear normal with the restoration of normal lobular architecture mimic to control.

2-Histological changes of the kidney:

Microscopic examination of kidneys sections stained by H&E of the (control) group showed normal renal

architecture of the cortex and medulla and the glomeruli structure in addition to both proximal and distal tubules (Fig. 10A). Sections of the Indoxacarb treated group

of rats revealed severe damage in the form of severe necrosis of the glomeruli and tubules, cell ballooning and cloudy swelling. Inflammatory cell infiltration was marked in addition to a marked loss of the normal renal architecture (Fig. 10B). The renal tissues examined of both groups (Indoxacarb+Zn) (Fig. 10C) and (Indoxacarb+VC) (Fig. 10D) showed

nearly similar results with valuable protection against the toxic effect of the Indoxacarb. Renal damage was minimal among rats treated with combined Indoxacarb+ Zn+VC (Fig. 10E). Microscopic examination of the kidneys of animals treated with (Indoxacarb+ VC+ Zn) showed mild toxic effects indicating an effective protective role.

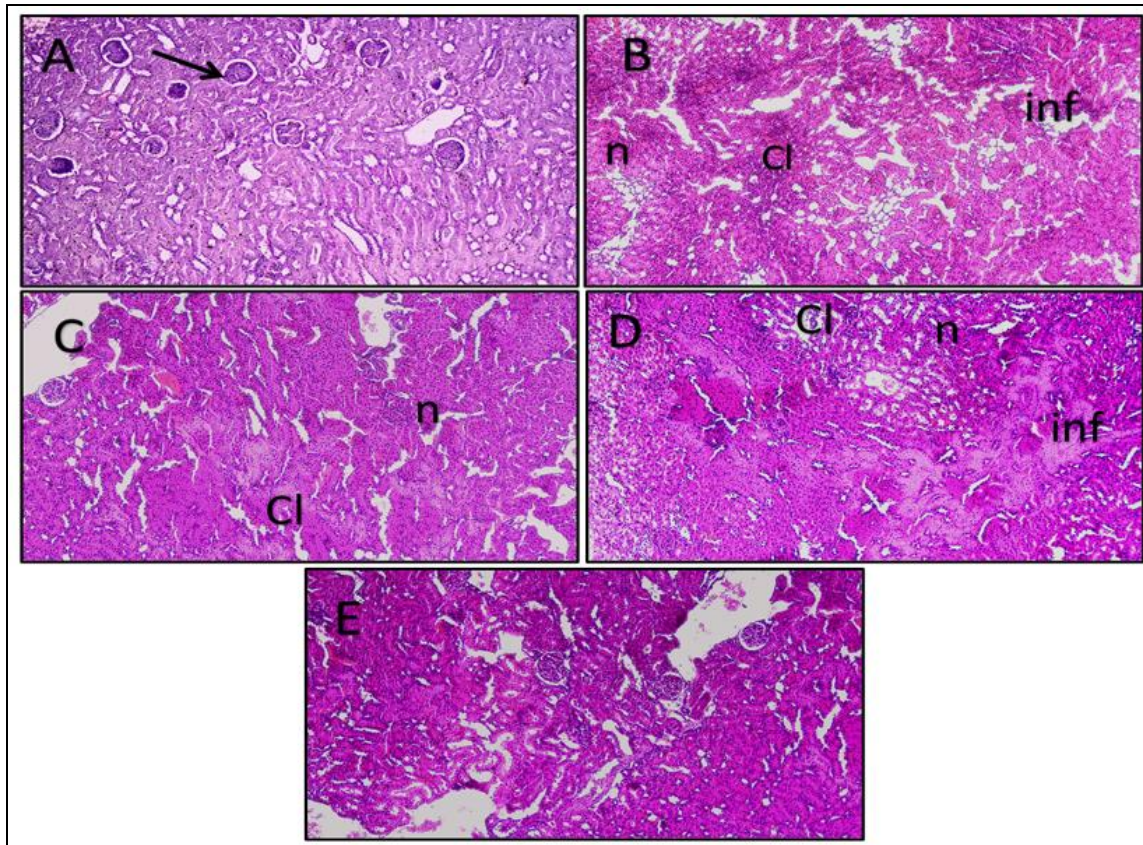


Fig. 10: Photomicrograph of different kidney sections stained with H&E. (A) kidney section from control rats showing normal renal architecture with normal glomeruli (arrow). (B) Kidney sections of the Indoxacarb treated rats show multiple large-sized areas of necrotic cells (n) with vacuolated cytoplasm and cloudy swelling (Cl) and wide areas of inflammation (inf) with the loss of normal renal architecture. Sections from kidney of rats of both groups that received (C) (Indoxacarb+Zn) and (D) (Indoxacarb+VC) showed mild protection of renal tissue with moderate degeneration. (E) (Indoxacarb+ Zn+ VC) treatment markedly decreased renal damage and inflammatory infiltration which are restricted to few foci with mild vacuolation of their cytoplasm while the nuclei appear normal with the restoration of normal architecture mimic to control.

Immunohistochemical assessment of iNOS:

1-Immunohistochemical staining of the liver by iNOS:

Liver sections that Immunostained

with the anti-iNOS primary antibody show different expression intensities among different groups of studied rats (Fig. 11). Liver sections from the normal control group show negative immune reactivity

(Fig. 11A). Liver sections from rats received Indoxacarb show a marked increase of the strongly positive immunostained hepatocytes between a few unstained hepatocytes (Fig. 11B). Liver sections from rats received (Indoxacarb+ Zn) (Fig. 11C) show moderate immuno-positive hepatocytes between many unstained normal cells. Also, Liver

sections from rats received (Indoxacarb+ VC) (Fig. 11D) show moderate immuno-positive hepatocytes between many unstained normal cells, Liver sections from rats received combined (Indoxacarb+ Zn+ VC) show mild expression as the majority of hepatocytes show negative reactions while positive cells are fewer with weak positivity (Fig. 11E).

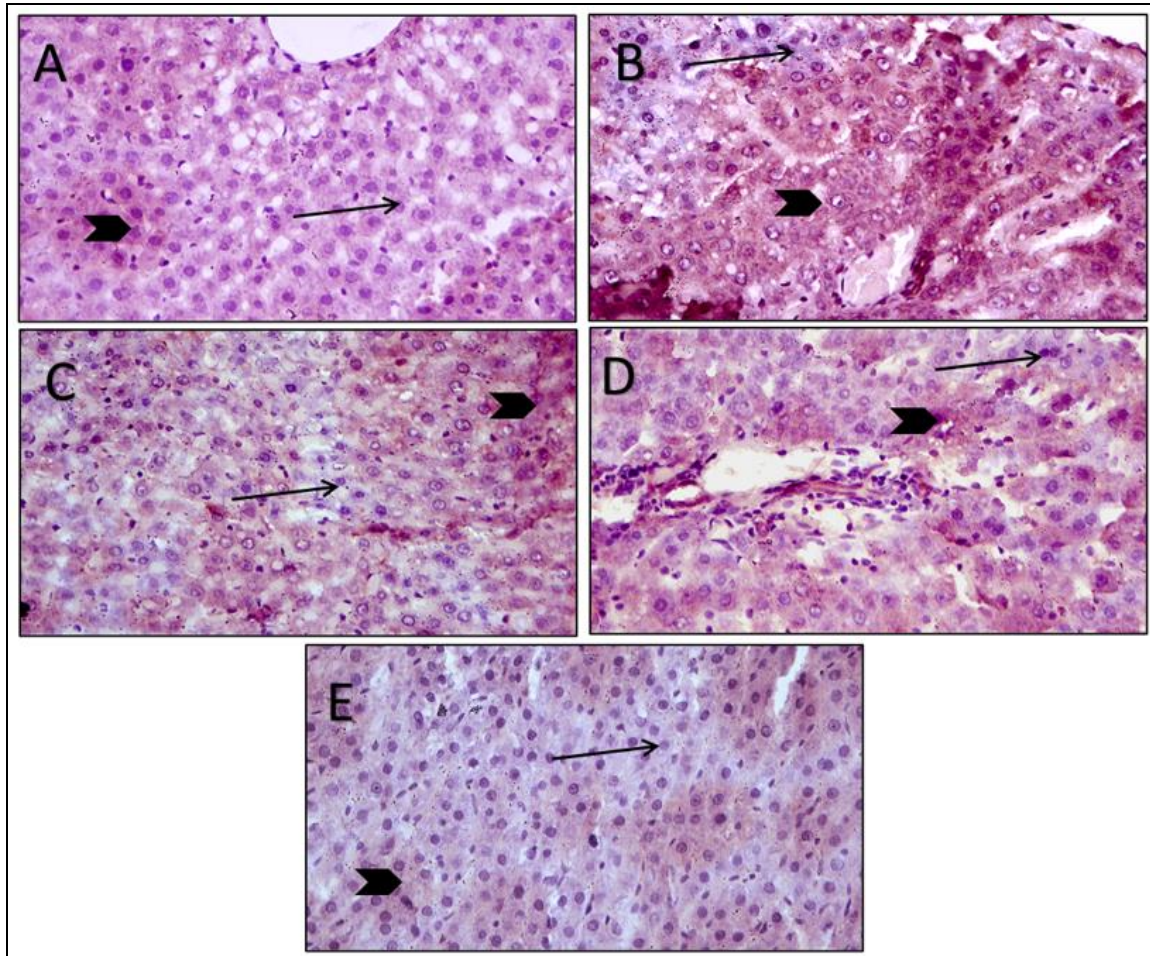


Fig. 11: Photomicrograph of liver sections Immuno-stained with the anti iNOS primary antibody. Immunohistochemistry assessment of iNOS in liver sections showed (A) Liver sections from normal control group shows negative immune reactivity (arrow). (B) shows a marked increase of the strongly positive immunostained hepatocytes (arrowheads) between few unstained hepatocytes (arrow). (C) Liver section from rat received (Indoxacarb+ Zn) shows moderate immuno-positive hepatocytes (arrowhead) between many unstained normal cells (arrow), (D) Liver section from rat received (Indoxacarb+ VC) shows moderate immuno-positive hepatocytes (arrowhead) between many unstained normal cells (arrow), (E) Liver section from rat received (Indoxacarb+ Zn+ VC) shows mild immune-expression as many hepatocytes with immuno-negative reaction (arrow) while immune positive cells are less with weak positivity (arrowhead).

2- Immunohistochemical Staining of the kidney by iNOS:

Kidneys sections that Immunostained with the anti-iNOS primary antibody show variable expression intensities among studied rats of different groups (Fig. 12). Kidney sections from the normal control group show negative immune reactivity (Fig. 12A). kidney section from rats received Indoxacarb shows a marked increase of the strongly positive immunostained cells between a few other unstained cells (Fig. 12B).

Kidney sections from rats received (Indoxacarb+ Zn) (Fig. 12C) show moderate immuno-positive cells between many unstained normal cells. Similarly, sections from rats received (Indoxacarb+ VC) (Fig. 12D) show moderate immuno-positive cells between many unstained normal cells. Kidneys sections from rats received combined (Indoxacarb+ Zn+ VC) show mild expression as most of the cells with a negative reaction while positive cells are fewer with weak positivity (Fig. 12E).

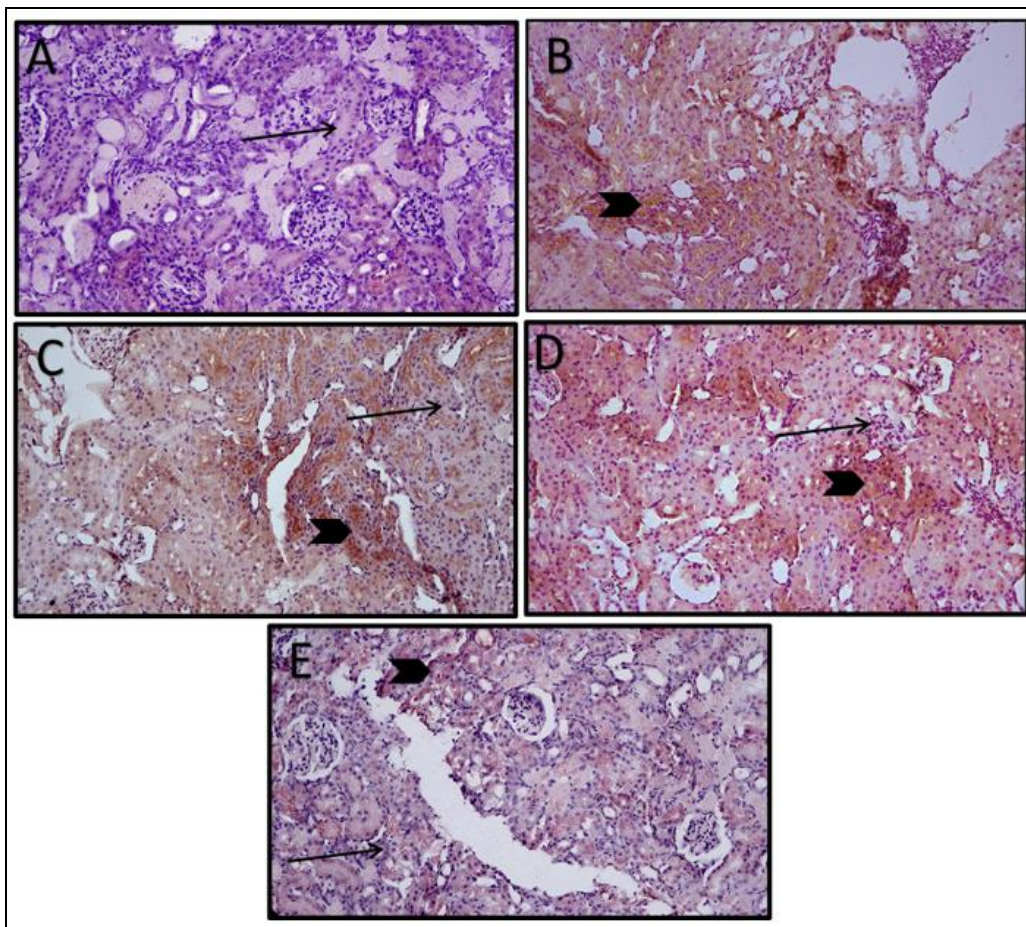


Fig. 12: Photomicrograph of kidney sections Immunostained with the anti iNOS primary antibody. Immunohistochemical assessment of iNOS in kidney sections showed (A) kidney sections from normal control group shows negative immune reactivity (arrow). (B) shows a marked increase of the strongly positive immunostained renal cells (arrowheads) between few unstained cells (arrow). (C) kidney section from rat received (Indoxacarb+ Zn) shows moderate immuno-positive cells (arrowhead) between many unstained normal cells (arrow), (D) kidney section from rat received (Indoxacarb+ VC) shows moderate immuno-positive cells (arrowhead) between many unstained normal cells (arrow), (E) kidney section from rat received (Indoxacarb+ Zn+ VC) shows mild immune-expression as many cells with immuno-negative reaction (arrow) while immune positive cells are less with weak positivity (arrowhead).

DISCUSSION

Indoxacarb is considered an organophosphate replacement, it was initially classified as a “moderately to very highly toxic” compound for aquatic organisms (Ghelichpour *et al.*, 2019). As previously reported, Swiss albino mice were given different doses of indoxacarb with the sub-acute level of toxicity for 30 days that induced ROS production and caused oxidative stress (Mudaraddi *et al.*, 2012). Our current research showed the sublethal dose of indoxacarb (100 mg/kg) induced a significant rise in LPx level (Fig. 2) and significant changes in antioxidant enzymes (GPx and SOD) activity (Fig. 3). Mudaraddi *et al.* (2012) indicated that indoxacarb induces oxidative stress as evidenced by the high level of LPx which is an indication of the generation of free radicals as the antioxidant mechanism failed to regulate them. It is well-known that antioxidant enzymes are the first-line defense against ROS (Martínez-Alvarez *et al.*, 2005). The increased SOD activity detected in this study could indicate the need to encounter the increase in ROS production by the indoxacarb as SOD comprises an important ROS-scavenging enzyme. An antioxidant enzyme (SOD) catalyzes the alteration of superoxide anions to hydrogen peroxide (H₂O₂). Therefore, the decreased GPx activity observed in this study led to the continuous generation and accumulation of ROS such as H₂O₂ under the influence of exposure to indoxacarb toxicity thus increasing oxidative stress and disturbance in the antioxidant mechanism (Monteiro *et al.*, 2019).

In general, according to the present study, the improvement induced by the mixture of VC and Zn (the examined doses) against indoxacarb toxicity was greater than that recorded for each of them individually and VC seems to be more effective than Zn to a small extent. A combination of VC and Zn can reduce lipid peroxidation caused by indoxacarb

and act as antioxidant scavenging free radicals. There are several publications that reported the ameliorative role of the antioxidants that used in the present study against different types of toxicants. Hamden *et al.* (2009) reported that VC is a very important and powerful antioxidant; It could minimize cell damage caused by free radicals by increasing activities of SOD and GPx and improving the antioxidant defense mechanism. In the same line, Mossa *et al.* (2011) suggested that the ameliorating effects of VC might be due to the inhibition of the generation of free radicals along with the free radical scavenging activity. Moreover, Banerjee *et al.* (2009) showed that VC opposed arsenic (As) induced oxidative stress in mice livers. Furthermore, El-Demerdash *et al.* (2005) showed the ameliorative role of VC against toxicity induced by stannous chloride in the liver of treated rats, as it decreased levels of free radicals and improved liver architecture. VC also has been shown to overwhelm the oxidative stress caused by acute exposure to chlorpyrifos in Wistar rats (Ambali and Ayo, 2011). Powell (2000) reported that zinc, the second most abundant trace element in the body, tends to protect against oxidative stress by several ways, such as preventing or minimizing ROS formation and minimizing the primary effect of such stress on the cell. Additionally, earlier studies have shown the ameliorative effects of Zn against oxidative stress induced by chlorpyrifos toxicity (Ambali *et al.*, 2011). Besides, the experiments done by Hfaiedh *et al.* (2012) confirmed that administration of Zn + Mg to Wister rats decreases hepatotoxicity and neurotoxicity caused by lindane toxicity. From our current study, VC and Zn acted together to overcome the oxidation stress and protect the rats from indoxacarb-evoked lipid peroxidation and free radicals' generation.

The liver is an essential organ in purifying the body from toxins.

Consequently, any disruption or impairment of its function led to hepatotoxicity and hazardous effects on health. AST and ALT (Fig. 4) are liver enzymes that detect hepatic dysfunction (Khalidoun-Oularbi *et al.*, 2013). In this work, a significant increase was observed in serum AST and ALT enzymes activity in indoxacarb-treated rats compared to the normal rats. Previous reports demonstrated that the disturbance in hepatic enzymes activities confirms the destruction of the hepatocyte membrane and allows these enzymes to leak into the circulation (Abdelrasoul, 2018). The high liver enzymes activity revealed in this study is consistent with the hepatic toxicity induced by indoxacarb toxicity.

Our present study indicated a significant decrease in serum level of HDL-C and a significant increase in serum LDL-C level in rats treated with indoxacarb compared to the control rats (Fig. 5). Moreover, comparable results were obtained from the previous study conducted on rats exposed to many pesticides as Coragen (Meligi *et al.*, 2019) and Chlorpyrifos (Ambali *et al.*, 2011). Hepatocytes and intestines are the principal sites for HDL-C synthesis. The decreased HDL-C level can be a sign of liver damage leading to obstruction of bile ducts causing a decrease or a closure of the liver duodenum secretion. HDL-C plays a significant function in the efflux of cholesterol from the cells and returns it to the liver for bile acid removal (Shakoori *et al.*, 1988). On the other side, the elevated level of LDL-C may be due to indoxacarb treatment that suppresses the formation of new LDL receptors and thus reduce the LDL-C intake by the cells. Also, the increase in LDL-C level as previously reported in Chlorpyrifos - treated rats related to the decrease in VLDL-C level due to stimulation of the harmful alteration of some VLDL-C to LDL-C (Ambali *et al.*, 2011).

Our results reflected the obvious substantial decrease in serum level of total

proteins besides albumin in the indoxacarb-treated group in comparison with the control group (Fig. 6). Veeraiah *et al.* (2013) reported a decrease in protein content in different tissues (liver, kidney, muscles, gills, and brain) in freshwater fishes exposed to different pesticides as indoxacarb. The liver is also a major organ for the synthesis of different proteins besides being the center of metabolism regulation. Exposure to pesticides can cause damage to hepatocytes leading to disorders of liver function with reduced protein synthesis and increased wasting by catabolism (El-Shafey *et al.*, 2011).

The kidney excretes creatinine, urea, and uric acid is the end products of protein catabolism (Refaie *et al.*, 2014). Our results indicated a significant increase in serum creatinine, urea and uric acid levels in rats treated with indoxacarb compared to the control rats (Fig. 7). These latter results are correlated with increased protein catabolism and may be confirmed a disturbance in glomerular filtration and functional damage in the kidney (Walmsley and White, 1994 and Abdelrasoul, 2018).

The adrenal glands secrete an aldosterone hormone that regulates the reabsorption of sodium and excretion of potassium of the kidney, fluids preservation or loss and blood pressure control (Ponda and Hostetter 2006). These current results demonstrated a significant rise in the activity of plasma aldosterone hormone after indoxacarb administration compared to the control group (Fig. 8). A significant increase in aldosterone hormone activity may be due to the surplus secretion frequently from a small benign adrenal tumor (hyperaldosteronism). It leads to high blood pressure (arterial hypertension) associated with low blood levels of potassium (hypokalemia) (Schaefer and Wolford 2005). Disturbance of aldosterone hormone activity is pathogenic and associated with the progression and development of cardiovascular

diseases and kidney dysfunction (Gajjala *et al.*, 2015). The clinical application indicated certain hypokalemia complications that may cause muscle weakness and cramps. This is supported by our results in which indoxacarb was noted to induce muscle cramps in indoxacarb-treated rats.

Our results showed that a mixture of VC and Zn improved the liver and kidney damage induced by indoxacarb. The improvement involves a significant decrease in AST, ALT activities (Fig. 4), LDL-C level and a significant increase in HDL-C (Fig. 5), total protein, and albumin levels (Fig. 6) in the blood as compared to indoxacarb-treated rats. In addition, there was a significant decrease in serum creatinine, urea, uric acid levels (Fig. 7), and plasma aldosterone hormone activity (Fig. 8); it should also be noted that a mixture of VC and Zn administration offered substantial defense against renal dysfunction.

These results may be attributable to reinstate antioxidant defense mechanisms that suppress free radicals' development. In this regard, as our findings show, supplementation of VC along with Zn to indoxacarb-treated rats is preventing the rise in the levels of hepatic transaminases enzymes (Fig. 4) suggestive of the main sign of their hepatoprotective action. Bashandy and Alwasel (2011) reported that VC was able to preserve the integrity of the cell and normalized levels of AST and ALT in the blood of carbon tetrachloride intoxication rats. In the same line, Attar (2011) showed that the hepatoprotective effect of VC on thioacetamide-induced liver cirrhosis in rats. Bray and Bettger (1990) indicated that the ameliorative properties of Zn ions may involve cell membranes stabilization and the increased antioxidant enzymes expression, indicative of the regeneration of hepatocytes and healing of hepatic parenchyma (Thabrew *et al.*, 1987).

Our analysis also showed the apparent substantial decrease in serum

LDL-C and the rise in HDL-C levels (Fig. 5) in the group treated with both VC and Zn when compared to the indoxacarb-treated group. Paolisso *et al.* (1995) reported that supplementation of VC is vital to hepatic metabolism and control of plasma lipids. Farvid *et al.* (2004) concluded that nutritive supplementation with Zn, vitamins C and E exhibited efficacy in lowering the levels of blood cholesterol and triglyceride and may improve HDL-C in diabetic patients by 24 percent. In the same line, Afkhami-Ardekani and Shojaoddiny-Ardekani (2007) stated that a daily intake of 1gm of VC in diabetic patients (type 2) can be beneficial in decreasing blood lipid levels. The particles of LDL are dense and small in diabetes (type 2) and so vulnerable to oxidation, while VC is needed for restoration of α -tocopherol and may therefore prevent LDL oxidation (Mullan *et al.*, (2002).

In this study, we observed a substantial increase in both serum total proteins and albumin (Fig. 6) in VC + Zn treated group compared to the indoxacarb-treated group. Bader *et al.* (2016), showed that treatment with VC (100 or 200 mg/kg diet) plus lead significantly increased serum total proteins and albumin, suggesting that VC can reduce liver toxicity induced by lead. Sandstead *et al.* (2000) suggested that Zn is important for coenzyme synthesis which catalyzes the synthesis and metabolism of biogenic amine. In view of the abovementioned points, it is considered that VC and Zn could increase the protein synthesis in the injured liver and improve the function of its hepatocytes.

From our study, a mixture of VC and Zn significantly decrease serum creatinine, urea, uric acid levels (Fig. 7) and plasma aldosterone hormone activity (Fig. 8). El Mashad *et al.* (2016) stated that administration of VC significantly reduced serum creatinine, urea, and uric acid level in STZ- diabetic rats in comparison with the control group. The

mechanisms by which VC decreased serum creatinine and urea levels may inhibit the development of diabetic nephropathy by suppressing apoptosis and protecting all groups of lipids from oxidation under several related oxidant stress forms. Treatment with VC decreased serum uric acid may be attributed to increased glomerular filtration and/or renal reabsorption competition, i.e., both VC and uric acid are reabsorbed at the proximal tubules by anion exchange transport (Sutton *et al.*, 1983). Possible explanations for increased glomerular filtration include an antioxidant effect in glomeruli that decreases microvascular ischemia and results in increased blood flow at the site, dilation of afferent arterioles, and competition for reabsorption with ions such as sodium and potassium that exerts osmotic effects. Vitamin C helps in the regulation or removal of renal injury progression in diabetic rats (El Mashad *et al.*, 2016). Zinc is a micronutrient that plays a significant role in human physiology from its involvement in the proper functioning of the immune system to its role in cell development, cell proliferation, cell apoptosis, and in the action of various zinc-binding proteins. In turn, zinc is an anti-inflammatory antioxidant, and controls innate and adaptive immune reactions, making it essential to infection resistance (Escobedo-Monge *et al.*, 2019). Mousavi *et al.* (2018) displayed a significant improvement in kidney functions after supplementation of Zn (50 mg/day) in adult's patients with renal insufficiency relative to healthy subjects. Escobedo-Monge *et al.*, (2019) showed that Zn supplementation could be useful for nutritional status in children and adolescents with chronic kidney disease as participants could have improved their nutritional status by substantially raising their body mass.

Compliance With Ethical Standards

All experimentation, transportation, and care of the animals used in this study

were following the Faculty of Science, Minia University's policy on animal use and ethics. All mandatory laboratory health and safety measures have been adhered to while performing the experimental work of this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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ARABIC SUMMARY

السُّمية الناتجة عن تعرض ذكور الجرذان البيضاء الى الأندوكساكارب
والتأثيرات الوقائية المحتملة لفيتامين "ج" والزنك

*هناة فوزي حسن¹, نسرين ضاحي توني², نهي محمد مليجي¹

¹قسم علم الحيوان، كلية العلوم، جامعة المنيا 61519، المنيا، جمهورية مصر العربية

²قسم الباثولوجي، كلية الطب، جامعة المنيا 61519، المنيا، جمهورية مصر العربية

يمكن أن يكون للتعرض بشكل غير مباشر أو الإفراط في التعرض لمبيدات الآفات عواقب وخيمة. يحدث التسمم بمبيدات الآفات بعد أن تؤثر المواد الكيميائية المخصصة لمكافحة الآفات على الكائنات الحية غير المستهدفة مثل الإنسان. يهدف العمل الحالي إلى تقييم الآثار الضارة المحتملة للمبيد الحشري "الأندوكساكارب" والتأثيرات الوقائية لفيتامين "ج" (200 ملليجرام/كجم)، والزنك (100 ملليجرام/كجم) (بشكل منفصل أو معاً) على السُّمية الناتجة من المبيد. تم تقسيم 30 جرذ من ذكور الجرذان المهقاء من سلالة Sprague-Dawley بشكل عشوائي إلى خمس مجموعات كل مجموعة بها ستة حيوانات: المجموعة الأولى تمثل المجموعة الضابطة، تلقت المجموعة الثانية عن طريق الفم جرعة تحت المميّنة من اندوكساكارب (100 ملليجرام/كجم)، تلقت المجموعة الثالثة عن طريق الفم "فيتامين ج+اندوكساكارب"، تلقت المجموعة الرابعة عن طريق الفم "زنك+اندوكساكارب" بينما تلقت المجموعة الخامسة فيتامين "ج + زنك + اندوكساكارب" عن طريق الفم، ثلاث مرات في الاسبوع لمدة 21 يوماً. وقد تسبب تعرض الجرذان للأندوكساكارب الى حدوث تغييرات كبيرة في بعض قياسات الإجهاد التأكسدي (مستويات بيروكسيد الدهون ونشاط انزيم SOD و GPx) إلى جانب زيادة كبيرة في أنشطة الإنزيمات الكبدية (AST, ALT). بالإضافة الى ذلك، أظهر الكوليستيرول عالي الكثافة ومنخفض الكثافة والبروتين الكلى والألبومين وظائف الكلى (مستويات الكرياتينين واليوريا وحمض اليوريك ونشاط هرمون الاللدوستيرون) تغييرات واضحة في الجرذان المعالجة بالأندوكساكارب مقارنة بجرذان المجموعة الضابطة. كما تم فحص التغييرات النسيجية في الكبد والكلى لجميع المجموعات. علاوة على ذلك، أدى مزيج فيتامين "ج" والزنك الى تحسين القياسات الكيميائية الحيوية والتغييرات النسيجية في الكبد والكلى للجرذان المعالجة بالاندوكساكارب. الخلاصة، قد يظهر مزيج فيتامين "ج" والزنك تأثيرات مفيدة ضد سمية الاندوكساكارب من خلال التحسن في اختلال القياسات الكيميائية الحيوية التي تم فحصها والتغييرات النسيجية في الكبد والكلى على استخدام أي منهما بمفرده.