

Diclofenac-Induced Kidney Damage in Wistar Rats: Involvement of Antioxidant Mechanism

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How to cite this paper: Abiola, Ti.S., Adebayo, O.C. and Babalola, O.O. (2019) Diclofenac-Induced Kidney Damage in Wistar Rats: Involvement of Antioxidant Mechanism. *Journal of Biosciences and Medicines*, 7, 44-57.

<https://doi.org/10.4236/jbm.2019.712005>

Received: October 25, 2019

Accepted: November 28, 2019

Published: December 2, 2019

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Abstract

Kidney damage has been associated with administration diclofenac, a phenylacetic acid derivative belonging to the nonsteroidal anti-inflammatory drugs (NSAIDs), which is commonly used for the treatment of various diseases such as rheumatoid arthritis, ankylosing spondylitis, acute muscle pain conditions and osteoarthritis. This study investigated the exact mechanism of diclofenac in renal toxicity by determining the involvement of oxidative stress in rats. Adult male Wistar rats were divided into two groups of eight rats in each group and orogastrically treated for three days. Group 1 served as the normal control and received normal saline (0.9% w/v) and group 2 received 40 mg/kg body weight of diclofenac for three days. Administration of diclofenac caused degeneration of the kidney of rats as evidenced by significant elevation in the serum levels of creatinine, urea, albumin, uric acid, protein and electrolytes and the activities of renal-5'-nucleotidase and glucose-6-phosphate-dehydrogenase (G6PDH) compared with control. Furthermore, administration of diclofenac decreased the activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione-S-transferase (GST) and the level of glutathione with concomitant increase in hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) levels in the kidney of the diclofenac treated groups compared with control. These findings reveal that administration of diclofenac may impair kidney functions through induction of oxidative stress.

Keywords

Orogastrically, Oxidative Stress, Toxicity, Diclofenac, Kidney Damage

1. Introduction

The kidney is highly susceptible to chemical damage, compared to other organs,

partly owing to its unequally high blood flow, and because of its complexity both anatomically and functionally. The kidney regulates various activities in the body such as maintenance of water balance, electrolyte balance acid-base balance, secretion of hormones and hemopoietic function as well as long term regulation of arterial blood pressure via renin-angiotensin mechanism and by regulation of extracellular fluid [1].

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are one of the most commonly prescribed drugs for inflammation and pain and their nephrotoxic effects are well known [2] [3]. NSAIDs exert anti-inflammatory, analgesic and antipyretic effects through the suppression of prostaglandin (PG) synthesis, by inhibiting the enzyme cyclooxygenase (COX). Cyclooxygenase exists in two isoforms: COX1 expressed constitutively in the kidney and functions mainly in the control of renal hemodynamic and glomerular filtration rate (GFR) and COX2 which is inducible in most tissues in response to injury or inflammation, but also present at detectable levels in normal adult mammalian kidneys and its functions primarily affect salt and water excretion [4] and is regulated in response to intravascular volume. Obstruction of any or both of these enzymes can therefore have diverse effects on renal function. Prostaglandins control a wide range of renal functions. PGE2 is considered to be primarily a tubular PG and regulates sodium and chloride transport in the loop of Henle and moderates water transport and renal medullary blood flow [4] while PGI2 a vascular PG controls renal vascular tone, GFR and renin release COX-2 stimulates the renin-angiotensin system, while an increased activity of the renin-angiotensin system inhibits COX-2. PGI2 and PGE2 increase potassium secretion primarily by stimulating the secretion of renin and activating the renin-angiotensin-aldosterone system [5].

The kidneys and the gastrointestinal tract are main foci for untoward clinical events associated with the use of NSAIDs [6]. Diclofenac is one of the most widely NSAID used to decrease inflammation and arthritis-associated pain [7] [8]. It is rapidly and completely absorbed after consumption and attaches to albumin in plasma [9]. Its side effects include nephrotoxicity and liver toxicity [10] [11] amongst others. Some serum parameters are used to evaluate organ damage. Increased serum urea and creatinine concentrations are accepted as indicators of kidney damage.

Normally, combinations of biochemical and physiological phenomena play a key role in kidney vulnerability and renal toxicity [12]. Diclofenac toxicity has been associated with oxidative stress but there is paucity of information on the involvement of antioxidant mechanism. This study therefore investigated the detailed mechanisms of action of diclofenac on kidney damage.

2. Materials and Methods

2.1. Chemicals and Reagents

Glucose-6-phosphate, adenosine monophosphate (AMP), trichloroacetic acid

(TCA), bovine serum albumin (BSA), Folin-Ciocalteu reagent, methanol, epinephrine, reduced glutathione, Ellman's Reagent [5'-5'-dithiobis-(2-dinitrobenzoic acid), DNTB], sulphosalicylic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), reduced glutathione, 1-chloro-2, 4-dinitrobenzene were supplied by Sigma-Aldrich® (USA), were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Albumin kit, urea kit, creatinine kit and Uric acid kit from Randox Laboratories Limited. Other chemicals and reagents used were of analytical grade.

2.2. Experimental Animals

Forty male Wistar strain albino rats (150 - 170 ± 5 g) were procured from Central Animal House, Faculty of Basic Medical Science, College of Medicine, University of Ibadan, Ibadan, Nigeria. To avoid coprophagy, rats were kept in polyethylene-walled cages in a temperature-controlled room (25°C ± 2°C) with 12 h light and 12 h dark cycle prior to the experiments and were fed with standard rat's chow (Ladokun Feeds, Nigeria) with fresh water *ad libitum*. All procedures in this study conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding principles in the care and use of animals and as approved by the Research Ethical Committee, Bowen University, Nigeria. The rats were deprived of food for 18 hours but had free access to clean water prior to the commencement of the experiment.

2.3. Drug

Diclofenac (Novartis Pharmaceutical Limited, Malaysia) was purchased from the Pharmacy unit of Bowen University Teaching Hospital (BUTH), Iwo, Osun State. The tablets were dissolved in normal saline (0.9% v/v).

2.4. Experimental Design

Sixteen adult male Wistar rats weighing (180.5 ± 20.0 g) were purchased from the Central Animal House, Faculty of Basic Medical Science, College of Medicine, University of Ibadan, Nigeria. Animals were kept in a temperature-controlled room (25°C ± 2°C) with 12 h light and 12 h dark cycle. The rats were kept in polypropylene cages under standard laboratory conditions and were fed with standard rat's pellet (Ladokun Feeds, Nigeria) with fresh water *ad libitum*. They were acclimatized for 14 days. All the animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institute of Health [13]. The experiment was performed according to the guidelines and approval of institutional animal ethics committee.

Rats were divided into two groups of eight rats per groups and treated orally once daily for three days by oral gavage. Rats in group 1 received 1 ml/kg body weight of normal saline and group 2 received 40 mg/kg for three days. Twenty four hours after the last dose, all animals were humane sacrificed by cervical dislocation.

2.5. Serum Preparation and Post Mitochondrial Fraction Collection

After sacrifice, blood from each rat was collected separately by cardiac puncture technique using sterilized needles and syringes into clean anticoagulant-free centrifuge tubes. Then, the blood was allowed to coagulate by standing for about 30 minutes, and the serum in each tube was separated by centrifugation at 4000 g for 10 min with a table centrifuge. Subsequently, the kidneys of each rat were quickly removed, rinsed in ice-cold 1.15% KCl. The portions of the kidneys were then homogenized in 0.1 M phosphate buffer pH 7.4 with the aid of Potter-Elvehgen homogenizer. The homogenates were then centrifuged at 10,000g for 10 min in a cold centrifuge at 4°C, and the supernatants (post mitochondrial fractions) were used for the determination of the oxidative stress and antioxidant markers.

2.6. Serum Biochemistry

The levels of serum urea, creatinine, albumin and uric acid were estimated by Fawcett and Scott [14] and Henry [15], Tietz [16], Fossati *et al.* [17] and Glucose-6-phosphate dehydrogenase activity by method of Webster [18] respectively were evaluated spectrophotometrically by an Ultrospec2000® spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) in accordance with the methods provided by the diagnostic kits (Randox Laboratories Limited, UK) respectively. Serum electrolytes (sodium, potassium, calcium, chloride, phosphate and bicarbonate ions) were determined by flame photometry.

2.7. Biochemical Studies

2.7.1. Determination of Kidney Renal-5'-Nucleotidase Activity

The activity of Renal-5'-Nucleotidase carried out according to the method described by George *et al.* [19]. Briefly, 0.15 ml of AMP was incubated with 0.1 ml of 5 mM Tris-KCl (pH 7.6) followed by 0.1 ml of 10 mM MgCl₂ and 0.15 ml aliquot kidney homogenate (PMF) at 37°C for 20 minutes. The reaction was stopped by addition of 0.5 ml 10% (w/v) trichloroacetic acid and the protein precipitate was removed by centrifugation. 0.5 ml of supernatant was added to 0.5 ml of ammonium molybdate, then 0.8 ml of ferrous sulphate solution. The optical density was measured at 700 nm after shaking. The kidney Renal-5'-Nucleotidase activity was expressed as $\mu\text{mol}/\text{mg}$ protein.

2.7.2. Determination of Kidney Superoxide Dismutase (SOD) Activity

The SOD activity was determined based on the principle of inhibition of autoxidation of epinephrine (pH 10.2) at 30°C [20]. The assay mixture contained 20 μL of the kidney supernatant sample, and 2.5 mL of carbonate buffer (0.05 M, pH 10.2). Then, 0.3 mL of freshly prepared solution of adrenaline (0.3 mM) was added after equilibration in the spectrophotometer and mixed by inversion. Thereafter, the increase in absorbance was monitored in a spectrophotometer at 480 nm for 150 s at 30 s intervals. The kidney SOD activity was expressed in

Units/mg of protein.

2.7.3. Determination of the Activity of Catalase (CAT) in the Kidney

The activity of catalase was evaluated by monitoring H₂O₂ clearance at 240 nm for 2 min at 10 s intervals using a UV-vis spectrophotometer [21]. The reaction assay contained 1800 µL of phosphate buffer (50 mM, pH 7.0), 180 µL of H₂O₂ (300 mM), and 20 µL of the kidney supernatant sample. The activity of catalase was expressed as µmol H₂O₂ consumed/min/mg protein.

2.7.4. Determination of the Activity of Kidney Glutathione Peroxidase (GPx) Activity

The GPx activity was determined using the method of Rotruck *et al.* [22]. The reaction mixture which contained 500 µL sodium phosphate buffer, 100 µL of 10.0 mM sodium azide, 200 µL of 4.0 mM GSH, 100 µL of 2.5 mM H₂O₂, and 50 µL kidney sample was made up to 2.0 mL with distilled water. This was followed by incubation for 3 min at 37°C, and termination of the reaction by the addition of 0.5 mL of 10% trichloroacetic acid. Subsequently, the mixture was centrifuged and the supernatant obtained was used to evaluate residual level of GSH by the addition of 4.0 mL of disodium hydrogen phosphate (0.3 M), and 1 mL of DTNB reagent. The absorbance was then read at 412 nm in a spectrophotometer and the activity of GPx was expressed as units/mg protein.

2.7.5. Determination of Glutathione S-Transferase (GST) Activity

The activity of GST was determined in the kidney of rats using the method of Habig *et al.* [23]. This assay involves the pre-incubation of a mixture containing 1.7 mL of 100 mmol/L of phosphate buffer (pH 6.5) and 0.1 mL of 30 mmol/L of CDNB at 37°C for 5 min. After the addition of 20 µL of the sample, the absorbance of the solution was monitored at a wavelength of 340 nm in a spectrophotometer for 5 min. Subsequently, GST activity was expressed in units/min/mg protein using an extinction coefficient of 9.6 mM⁻¹·cm⁻¹.

2.7.6. Determination of Glutathione (GSH) Level

The determination of the GSH level was carried out using the method of Jollow *et al.* [24]. An aliquot of the kidney samples was deproteinized by the addition of an equal volume of sulphosalicylic acid (4%) and centrifuged at 10,000 g for 15 min at 4°C. Thereafter, 50 µL of the supernatants obtained was added to DTNB (10 mM, 4.5 mL), and the absorbance was read at 412 nm. The values were then expressed in µmol/mg protein.

2.7.7. The Determination of Malondialdehyde (MDA) Level in the Kidney

The level of malondialdehyde (MDA which is a marker of lipid peroxidation (LPO)) was determined using the method of Buege and Aust [25]. Each of the kidney supernatant (0.4 mL) was mixed with Tris-KCl buffer (1.6 mL) containing 0.5 mL of trichloroacetic acid (30%). This was followed by the addition of 0.5 mL of 0.75% thiobarbituric acid to each of the tubes. The tubes were then incubated at 80°C for 45 min in a water bath, cooled in ice, and centrifuged at 3000 g.

Subsequently, the absorbance of the clear supernatant in each tube was read in a spectrophotometer against a reference blank at 532 nm. The LPO status was then expressed in μmol MDA formed/mg protein using a molar extinction coefficient of $1.56 \times 10^5 \text{ m}^{-1}\cdot\text{cm}^{-1}$.

2.7.8. Determination of Hydrogen Peroxide (H_2O_2) Generated in the Kidney

Kidney hydrogen peroxide (H_2O_2) generated was determined based on the method of Wolff [26], based on oxidation of ferrous with xylenol orange. The kidney supernatant (50 μL) was added to a mixture containing 100 $\mu\text{M}/\text{L}$ of xylenol orange, 250 $\mu\text{M}/\text{L}$ of ammonium ferrous sulphate, 100 mmol/L of sorbitol, and 25 mmol/L of H_2SO_4 and vortexed. This was followed by incubation for 30 min at room temperature. The absorbance was then read spectrophotometrically at 560 nm and the values were expressed in nmol/mg protein.

2.7.9. Determination of Protein Concentration

The protein concentration in the kidney of the rats was determined by the method described by Gornall *et al.* [27] with some modifications. Potassium iodide was added to the reagent in order to prevent precipitation of Cu^{2+} ions as cuprous oxide.

2.8. Histological Assessment of the Kidney

Representative kidney sections (5 μm) from the control and diclofenac-treated rats were fixed in 10% formalin, and processed for histology using standard procedure [28]. Then, the slides were coded and examined under a light microscope (Olympus CH; Olympus, Tokyo, Japan) by pathologists who were blinded to control and diclofenac groups. Photomicrographs were taken with a Sony DSC-W 30 Cyber-shot (Sony, Tokyo, Japan).

2.9. Statistical Analysis

Data obtained were statistically analysed using Graph Pad Prism (Version 7.0), and the data were expressed as Mean \pm Standard error of mean. Statistical significant differences were determined using a one-way analysis of variance (ANOVA), followed by Duncan's multiple comparisons test using Statistical packages for social Scientists version 18.0 (SPSS version 18.0). Value of $p < 0.05$ was considered statistical significant.

3. Results

3.1. Diclofenac Induced Kidney Dysfunction

To examine the integrity of the kidney following administration of diclofenac to rats, the concentrations of biomarkers of renal dysfunction were determined. The effects of diclofenac on Na^+ , K^+ , Ca_2^+ , PO_4^{2-} , HCO_3^- , Cl^- , protein, urea, creatinine, albumin and uric acid levels in the kidneys of rats are shown in **Table 1** and **Figure 1** respectively. The result indicated that diclofenac administration

Table 1. Biomarkers of renal dysfunction in rats exposed to diclofenac.

Parameters	Na ⁺ (mmol/L)	K ⁺ (mmol/L)	Ca ²⁺ (mmol/L)	Cl ⁻ (mmol/L)	PO ₄ ³⁻ (mmol/L)	HCO ₃ ⁻ (mmol/L)	Protein (mg/dl)
Control	18.08 ± 0.21	3.16 ± 0.32	6.51 ± 0.23	14.20 ± 0.21	4.12 ± 0.25	2.05 ± 0.02	5.47 ± 0.17
Diclofenac	42.68 ± 1.05*	8.12 ± 0.87*	8.02 ± 0.13*	26.14 ± 1.03*	6.57 ± 0.11*	5.21 ± 0.26*	7.52 ± 0.19*

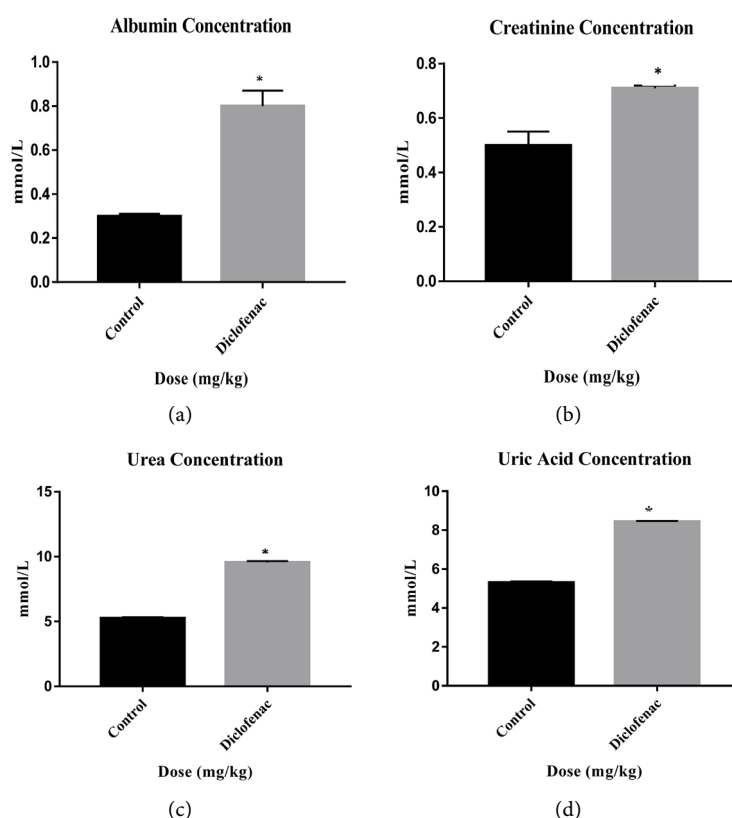


Figure 1. Concentrations of albumin, creatinine, urea and uric acid of control and diclofenac treated rats. Each bar represents mean ± standard error of mean. *p < 0.05 is significant when compared with the diclofenac group.

to rats caused a significant elevation ($p < 0.05$) in the concentration of serum albumin, creatinine, urea and uric acid and all the electrolytes levels when compared with the control.

Diclofenac provoked changes in the activities renal-5'-nucleotidase, G6PDH and antioxidant enzymes of kidney following administration of diclofenac to rats for 3 days. Renal-5'-nucleotidase and G6PDH activities in the diclofenac treated rats kidney were significantly elevated compared with control at $p < 0.05$ (**Figure 2(a)** and **Figure 2(b)**). The activities of antioxidant enzymes after the treatment of rats with diclofenac are also depicted in **Figures 2(c)-(f)**. There were significant ($p < 0.05$) decrease in the activities of SOD, CAT GPx and GST; a drug metabolizing enzyme.

The level of GSH is shown in **Figure 3(a)** following treatment of rats with diclofenac for 3 days. The GSH level was significantly lowered in the kidneys of rats administered with 40 mg/kg body weight of diclofenac when compared with

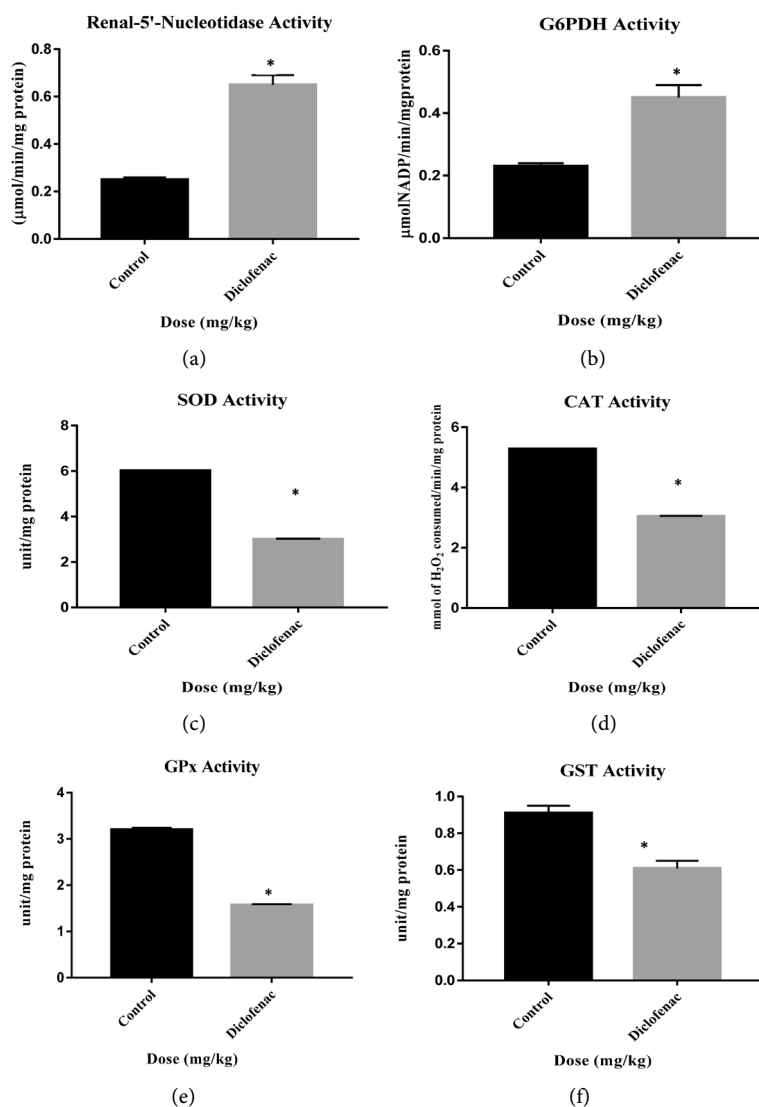


Figure 2. Activities of renal-5'-nucleotidase, G6PDH, SOD, CAT, GPx and GST of control and diclofenac treated rats. Each bar represents mean \pm standard error of mean. * $p < 0.05$ is significant when compared with the diclofenac group.

the control rats ($p < 0.05$). In addition, the data on the levels of MDA, an index of lipid peroxidation, and H_2O_2 generated following treatment of rats with diclofenac are depicted in **Figure 3(b)** and **Figure 3(c)** respectively. The MDA and H_2O_2 generated were elevated in the kidneys of rats that received 40 mg/kg body weight of diclofenac when compared with the control rats ($p < 0.05$).

3.2. Histopathological Findings

The histological examinations of kidney tissues of the control and diclofenac-treated group of rats revealed the kidney section of the control rats was normal (**Figure 4(a)**). Diclofenac-treated rat (**Figure 4(b)**), showed congestion of the blood vessels of the renal parenchyma with mononuclear cells filtration and dilation of the renal tubules (black arrow).

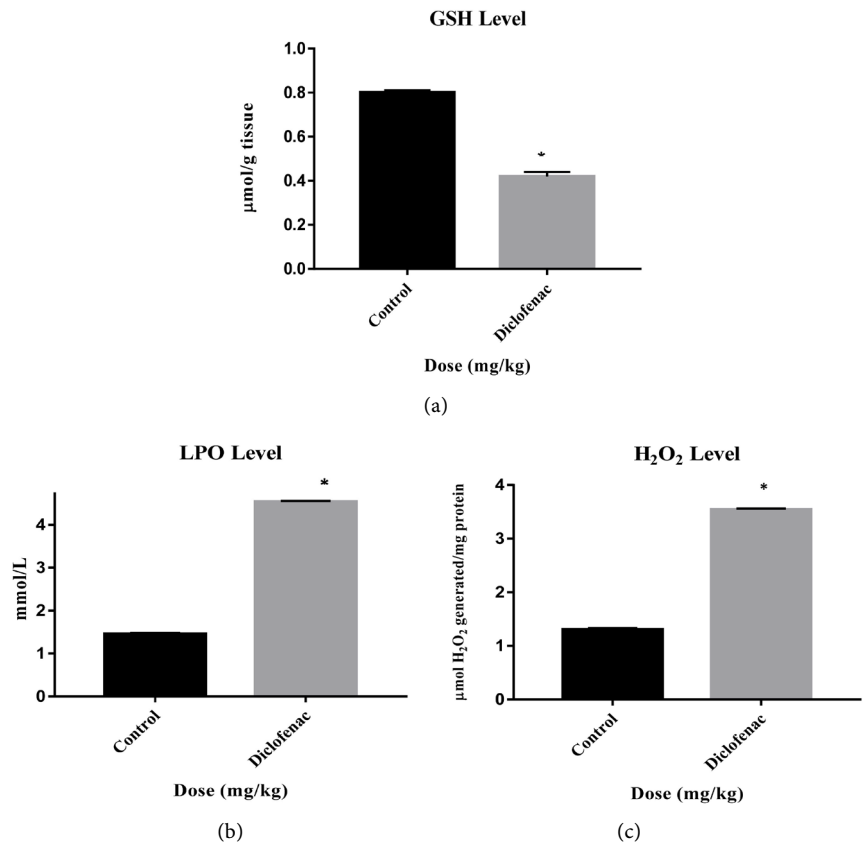


Figure 3. Levels of reduced glutathione, lipid peroxidation and activity of hydrogen peroxide generated of control and diclofenac treated rats. Each represents mean \pm standard error of mean. * $p < 0.05$ is significant when compared with the piroxicam alone group.

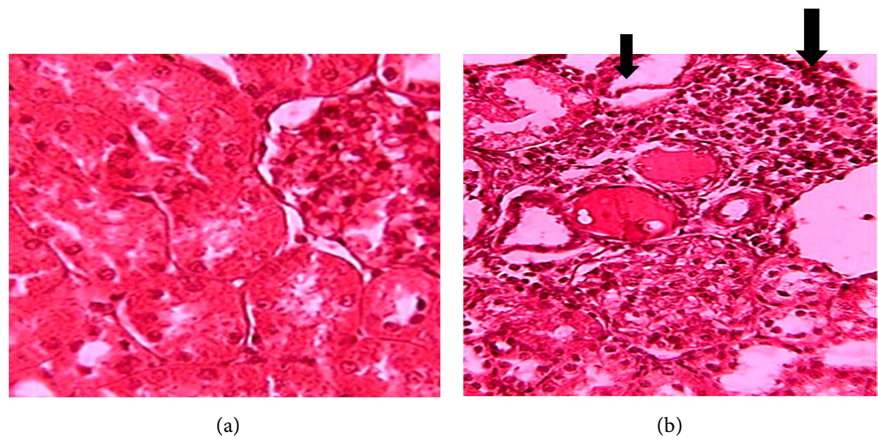


Figure 4. Effect of diclofenac on renal histopathology of rats. (a) Control rat: show normal renal architecture with healthy glomerulus and kidney tubule arrangement; (b) Diclofenac-treated rat: show inflammation and dilation of kidney tubules. $\times 100$.

4. Discussion

Diclofenac, a non-steroidal anti-inflammatory drug, have an undesirable consequence on renal functioning. Diclofenac inhibits renal endogenous prostaglandin production, decreases renal afferent vasodilation, raises afferent resistance;

all of this causes the glomerular capillary pressure to fall below normal values and decline in Glomerular Filtration Rate (GFR) [28]. These are displayed as acute renal dysfunction, fluid, and electrolyte disorders and pathologically reveal renal papillary necrosis, interstitial nephritis [28].

Serum creatinine, urea, uric acid and electrolytes levels are the most important clinical parameters for evaluating abnormalities in renal function. Serum investigation of creatinine, urea, uric acid, sodium, potassium was impaired and severe tubular damage was noticed in this study. Creatinine is an anhydride of creatine and is generated by spontaneous and irreversible reaction in skeletal muscle metabolism. Serum creatinine is one of the kidney variables that indicate renal toxicity. Creatinine may be suggestive of kidney-specific functional maladies [29]. An increase in serum creatinine is a biomarker for renal damage. Urea is formed by the liver and considered the main end product of protein catabolism in mammals especially humans. Plasma urea levels can be a dependable indicator of renal function [30]. Uric acid is produced by the breakdown of purines and by direct synthesis from 5-phosphoribosyl pyrophosphate (5-PRPP) and glutamine. Uric acid is excreted in the urine in humans, but in other mammals, uric acid is further metabolized to allantoin before excretion. Another probable explanation for the elevated serum uric acid level in diclofenac treated group may be because uric acid been an electron donor; donates electrons in defence against free radical-generated oxidative damage [31]. This may exacerbate the condition of renal damage resulted from uric acid. The results of these biomarkers in this study are in accordance with the reports of [32] [33] [34]. Diclofenac-induced nephrotoxicity was manifested by elevation in the serum levels of creatinine, urea, and uric acid, and was confirmed through oxidative stress disturbances that have been reported by many workers [11].

In normal conditions, the glomerulus restricts the migration of high molecular weight proteins from blood to nephron lumen by filtration [35]. In some pathological states, however, high molecular weight proteins can be detected in the urine because the selective penetration through glomerulus is not functioning properly [36]. High molecular weight proteins that can reveal kidney damage include albumin which can be used for early diagnosis of changed glomerular filtration and diabetes [32]. In this study, there was elevated levels serum of protein and albumin in the kidney of diclofenac treated rats.

Multiple factors, such as dose regulation based on renal function, hydration and avoidance of nephrotoxic agents, have been suggested to prevent or improve drug-induced nephrotoxicity [12] [37]. Nevertheless, drug induced toxicity remains a major problem for health care specialists. Therefore, outcome of impressive approaches to understand the mechanisms of action of drugs and potential injurious chemicals can help to reduce renal injuries.

Several lines of evidence have shown that exposure to drugs can result in a number of side effects that can lead to various disease states including kidney diseases. In the current study, we evaluated kidney toxicity of diclofenac administered orally to rats for three days. The results from this study showed that

diclofenac induced kidney injury via oxidative damage in rats. This was evidenced by the perturbation of the kidney antioxidant defence system.

Enzymatic antioxidants such as SOD and CAT inactivate superoxide anion and peroxide radicals by transforming them into water and oxygen [38]. These two enzymes serve as important line of defence against oxidative stress. Our results indicated decrease in the activities of SOD and CAT in the kidney of rats administered with diclofenac. This is in accordance with the report of [39] [40]. GPx an enzymatic antioxidant that aids the GSH in the scavenging of hydroxyl radical and singlet oxygen directly as well as in the detoxification of hydrogen peroxides and lipid hydroperoxides. GST functions in cell survival and protection against oxidative stress [41]. GST is concerned in the conjugation of electrophilic oxidants with GSH to form water soluble compound products that are readily excreted from the system [22] [42]. The depletion of these vital enzymes revealed a serious damage to the kidney through generation of ROS.

GSH depleted in rats administered with diclofenac. GSH is considered as a free radical scavenger that acts as a non-enzymatic antioxidant by directly interacting with ROS via its thiol group. It is a co-substrate of GST in the conjugation of electrophilic species, and also as a substrate for GPx [43] [44]. Thus, the decrease in the level of GSH in the kidney of rats after administration of diclofenac, might be due to its conjugation with diclofenac to form diclofenac-SG adduct and/or its rapid utilization by the glutathione-dependent enzymes or its overutilization by the cell possibly to combat ROS generation in the piroxicam-treated rats.

It is generally believed that the toxicity of diclofenac is strongly linked with oxidative stress leading to lipid peroxidation and damage to the cellular macromolecules [2] [10]. In this study, diclofenac increased the kidney MDA and H₂O₂ levels. The MDA, being a product of lipid peroxidation, is produced in response to the oxidative deterioration of polyunsaturated fatty acids (PUFAs) in kidney membranes. In this situation, lipid peroxides are produced in the membranes of the kidney tissues. H₂O₂ is a normal product of aerobic metabolism in animals; however, its level is enhanced during conditions of intracellular build-up of reactive oxygen species (ROS) concentration [45]. The result of this study agreed with the report of [40].

5. Conclusion

In conclusion, this research work affirmed that diclofenac induced kidney damage in experimental animals by exploring the cellular mechanisms of its action that leads to oxidative damage apart from the routine test biomarkers.

Conflicts of Interest

The authors declared no competing interests.

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