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Evaluation of Stress Enzymes Activities and Lipid Peroxidation in Heart Homogenates of Male Albino Rats Following the Administration of Diclofenac

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Authors' contributions

This work was carried out in collaboration among all authors. Author FNO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SA and MOM managed the analyses of the study. Author MOM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Nonsteroidal anti-inflammatory drugs are associated with an increase in cardiovascular events despite its uses in the therapeutic agent for the management of long- and short-term pain. Over the last years, evidence has accumulated showing that oxidative stress plays an important role in the pathogenesis of cardiovascular diseases. Oxidative stress is no longer considered as a simple imbalance between the production and scavenging of reactive oxygen species (ROS), but as a dysfunction of enzymes involved in ROS production. This study investigated the effect of diclofenac on the activity of oxidative stress enzymes as well as formation of lipid peroxidation. Male rats weighing about 100-120 g were divided into four groups: group one (control, feed+water) group two, group three and group four treated with different mg/kg/day of drugs (50 mg/kg/day, 100 mg/kg/day and 150 mg/kg/day) feed and water respectively for 7 days. Analysis on the effect of diclofenac on the activities of stress enzymes such as nicotine adenosine dinucleotide phosphate hydrogenase oxidase (NADPHoxidase), xanthine oxidase(XOD), catalase(CAT), superoxide dismutase(SOD) and Glutathione Peroxidase as well as evaluation of lipid peroxidation by measuring malondialdehyde (MDA) in the heart homogenate were carried out and the result showed a significant increase in

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each parameter given rise to the production of reactive oxygen species (ROS) if not moderated by the antioxidant defense can lead to cardiac impairment as a result of oxidative stress damage or injury. The result obtained implies that diclofenac (NSAIDs) affects the redox status of vascular tissues (heart tissues).

Keywords: Nonsteroidal anti-inflammatory drugs; diclofenac; cardiovascular events; oxidative stress; lipid peroxidation; stress enzymes; heart homogenate.

1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used therapeutic agent for the management of both long- and short-term pain as well as other inflammatory conditions. They are mainly classified into two main types namely: Selective-inhibitors and Non-selective - inhibitors. However, diclofenac belongs to the class of non-selective inhibitors of NSAIDs.

These drugs (NSAIDS) inhibit the activity of cyclooxygenase (Cox). Cox is the pacemaker enzyme in the synthesis of prostaglandins and thromboxane from arachidonic acid. Two isoforms of Cox have been identified, Cox-1 and Cox-2. Cox -1 is constitutively expressed in may tissues and generates prostanoids mediating normal physiological functions whereas Cox-2 may intensely increase in the tissue damage or inflammatory situations.

However, serious cardiovascular side effects have been reported for these drugs. The most widely accepted hypothesis to explain the greater numbers of cardiovascular events associated with them is that these agents cause an imbalance between vascular prostacyclin and platelet thromboxane [1]. Platelets are without cox-2 and produce thromboxane via Cox-1. Endothelial cells express cox-1, and the shear stress of the flowing blood stimulates Cox-2 is the dominant source of prostacyclin from endothelial cells in vivo. Thus, according to the prostanoids imbalance theory, cox-2 inhibition by diclofenac would markedly reduce prostacyclin production from endothelial cells without inhibiting cox-1 platelets, and thus lead to a thromboxane over production and promote platelet dependent thrombosis. However, there is an increasing body of evidence indicating that this paradigm may be too simplistic and cannot explain the available clinical data for example, addition of aspirin (a preferential cox-1 inhibitor) does not prevent the adverse cardiovascular effects of diclofenac moreover, also nonselective (NSAIDs), which primarily inhibit cox-1 have been found to be associated with an increase rate of cardiovascular event. Thus, prostanoid imbalance cannot explain all of the adverse cardiovascular effects coxibs observed in clinical studies, and other pathogenic mechanism have to be postulated.

Over the last years, evidence has accumulated showing that oxidative stress plays an important role in the pathogenesis of cardiovascular diseases [2,3]. Oxidative stress is a molecular deregulation in reactive oxygen species (ROS) metabolism involved in the pathogenesis of several diseases. Oxidative stress is no longer considered as a simple imbalance between the production and scavenging of reactive oxygen species (ROS), but as a dysfunction of enzymes involved in ROS production [4,5]. Research has shown that NSAIDs may affect the redox status of vascular tissues by affecting the activities of stress enzymes. For example, celecoxib has been shown to increase the susceptibility of human low-density lipoprotein and cell membrane lipids to oxidative modification [6]. Rofecoxib can also promote non-enzymatic formation of isoprostanes from biological lipids [7].

Oxidative stress plays an important role in the pathogenesis of cardiovascular diseases which is the major cause of death in the world today thus, this research is aimed at identifying effects of Diclofenac on oxidative parameters despite their benefits in the management of both long- and short-term pain.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Twenty adults male wistar rats weighing 150 g – 200 g obtained from appreciably healthy rats from the animal house, department of biochemistry, University of Port Harcourt were used to evaluate the activities of stress enzymes and lipid peroxidation in heart homogenates of male albino rats following the administration of Diclofenac. Acclimatization was carried out for one week prior to commencement of the study.

The rats were kept in well ventilated cages containing saw dust (for water absorption) with wire mesh top and fed with commercial growers mash, manufactured by Top Feeds Ltd, Sapele, Delta State, Nigeria. Water and feed were administered ad libitum.

2.2 Experimental Protocol

After acclimatization and grouping, each of the rats in group 2, 3 and 4 was administered 50mg/kg/day, 100mg/kg/day and 150mg/kg/day per body weight respectively according to the previous work of Taha et al [8]. using canular for daily oral administration of the drug for a period of 7 days while the group 1 rats (control) received distilled water only. Each group consisted of five rats each.

2.3 Drug Administration

Diclofenac sodium manufactured by laborate pharmaceuticals Ltd, India and marketed in Nigeria by TSK Global Pharmacy Alakahia, UPTH gate Port-Harcourt, with Batch number EDKF1-001 and NAFDAC Registration number A4-0035HP/DRUGS/MIS/04/87 was used each tablet contains a concentration of 100 mg diclofenac. The drugs were orally administered to the rats at varying doses (50-150 mg/kg body weight) once daily.

2.4 Sample Collection

At the end of experimental period, three rats from each group were sacrificed under chloroform anesthesia [9]. The heart samples were collected surgically and put into plain bottles for further sample preparation prior to biochemical analysis.

2.5 Tissue Homogenates

100 mg tissue was rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g, 2-8°C. The supernatant was removed and assayed immediately [10].

2.6 Biochemical Analysis

Determination of Stress enzymes and malondialdehyde (MDA) determination (index of lipid peroxidation). The homogenized heart tissue samples were used to evaluate activities of NADPH oxidase (NOX), Xanthine oxidase (XOD), superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GSH-Px) enzymes and the malondialdehyde (MDA) concentration (index of lipid peroxidation) using commercially available ELISA kits (Cusabio Biotechnology Company)) with the aid of ELISA plate reader as stated in the manufacturer's manual. We followed the manufacturer's instructions. 100µl of the homogenized sample was used for each enzyme analysis while 50µl of sample was used for determination of MDA level. All absorbance was read at 450 nm.

2.7 Protein Determination

The protein content of liver homogenates was determined by spectrophotometer according to the method of Bradford [11]. The aim is to relate malondialdehyde (MDA) concentrations as U/mg tissue protein, NOX, XOD, SOD, CAT, GSH-Px enzyme activities as unit/mg tissue protein.

2.8 Statistical Analysis

Data were analyzed by comparing values for different treatment groups with the values for control. All values were normally distributed and were expressed as the mean ± standard error of mean (SEM). To determine whether there was a statistically significant difference among experimental groups, the one-way analysis of variance (ANOVA) was used, followed by post hoc Tukey's test. A p value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, Chicago, IL, USA, version 20.0 for Windows).

3. RESULTS

3.1 Results of Biochemical Study

Biochemical tissue analyses of stress markers namely NOX, XOD, SOD, CAT, GSH-Px and MDA an index of lipid peroxidation in the control and different experimental rat groups treated with varying doses of diclofenac are shown in Table 1.

3.2 Evaluation of NADPH Oxidase Activity in Heart Homogenates of Male Albino Rats Following the Administration of Diclofenac

The activity of pro oxidant enzyme NADPH oxidase, in the control and different experimental

rat groups treated with varying dose of diclofenac are illustrated in Table 1. Daily administration of diclofenac for 7 days significantly ($p<0.05$) elevated NOX activity values in the heart of rats when group 2 (50mg/kg diclofenac), group 3 (50mg/kg diclofenac and group 4 (150mg/kg diclofenac) were compared with group 1 (control rats). The control (group 1) recorded NOX activity value of 833.84 ± 0.01 µg/mg while groups 2, 3 and 4 had NOX activity values of 895.59 ± 0.00, 972.0 ± 0.00 and 1048.58 ± 0.00 µg/mg respectively.

3.3 Evaluation of Xanthine Oxidase Activity in Heart Homogenates of Male Albino Rats Following the Administration of Diclofenac

Results in Table 1 revealed that there were differences in Xanthine oxidase activity among the different test groups. Diclofenac administration for 7 days significantly elevated XOD activity when groups 2, 3and 4 were compared with the control (group 1). The study also showed that the control (group 1) recorded a XOD activity value of 400.54 ± 0.01 µg/mg while groups 2, 3 and 4 had XOD activity values of 395.82 ± 0.03, 424.81 ± 0.04, 453.68. ±0.00 µg/mg respectively.

3.4 Evaluation of Superoxidase Dismutase Activity in Heart Homogenates of Male Albino Rats Following the Administration of Diclofenac

Results as presented in Table 1 showed that the control (group 1) recorded SOD activity values of 60.25 ± 0.01U/mg while the other groups recorded 195.54 ± 0.01, 131.16 ± 0.00 and 128.17 ± 0.00 U/mg for groups 2, 3 and 4 SOD activity values respectively. It was also observed that at the end of the study, SOD activity significantly increased in the heart of rats when groups 2, 3and 4 were compared with the control (group 1).

3.5 Evaluation of Catalase Activity in Heart Homogenates of Male Albino Rats Following the Administration of Diclofenac

There were marked variations recorded in Catalase enzyme activity as shown in Table 1. Daily diclofenac administration significantly ($p<0.05$) elevated the heart CAT activity when groups 2, 3 and 4 were compared with the control (group 1). The study also showed that at end of the experimental period, the control (group 1) recorded CAT activity of 48.47 ± 0.03 U/mg while 78.61 ± 0.00, 158.18 ± 0.00 and180.17 ± 0.00 U/mg were recorded for groups 2, 3 and 4 respectively.

3.6 Evaluation of Glutathione Peroxidase Activity in Heart Homogenates of Male Albino Rats Following the Administration of Diclofenac

There were significant differences recorded in glutathione peroxidase activity as shown in Table 1. Diclofenac administration significantly ($p<0.05$) elevated the heart GSH- Px activity when groups 2, 3 and 4 were compared with the control group. The study also showed that at end of the experimental period, the control (group 1) recorded GSH- Px activity of 42.62 ± 1.09 while groups 2, 3 and 4 had GSH- Px activities of 48.69 ± 1.13, 56.41 ± 1.54 and 60.69 ± 1.12 U/mg respectively.

Table 1. Result of catalase, SOD, NADPH oxidase, xanthine oxidase, MDA and GSH level on treatment with diclofenac

Treatment	NADPH oxidase (µg/mg)	Xanthine oxidase (µg/mg)	SOD (U/mg)	Catalase (U/mg)	MDA (U/mg)	GSH-Px (U/mg
Group 1 Control	833.84 ± 0.01^{a}	400.54 ± 0.01^{a}	60.25 ± 0.01^{a}	48.47 ± 0.03^{a}	1.33 ± 0.35^{a}	42.62 ± 1.09^{a}
Group 2 (50mg/kg/day)	895.59 ± 0.00^{d}	395.82 ± 0.03^{d}	195.54 ± 0.01^{d}	78.61 ± 0.00^{b}	2.58 ± 0.13^{b}	48.69 ± 1.13^{b}
Group 3 (100mg/kg/day)	972.0 ± 0.00^{c}	424.81 ± 0.04^{c}	131.16 ± 0.00^{C}	158.18 ± 0.00^{c}	2.87 ± 0.50^{b}	56.41 ± 1.54^{c}
Group 4 (150mg/kg/day)	1048.58±0.00^{b}	453.68. ±0.00^{b}	128.17 ± 0.00^{b}	180.17 ± 0.00^{d}	3.70 ± 0.42^{c}	60.69 ± 1.12^{c}

Values are presented as mean ± SEM, n=3 per group; Values on the same column with different superscript letters (a, b, c, d) differ significantly at p<0.05

3.7 Evaluation of Lipid Peroxidation in Heart Homogenates of Male Albino Rats Following the Administration of Diclofenac

Results in Table 1 revealed that there were differences in Malondialdehyde (MDA) activity among the different test groups. Diclofenac administration for 7 days significantly elevated XOD activity when groups 2, 3 and 4 were compared with the control (group 1). The study showed that the control (group 1) recorded MDA activity value of 1.33 ± 0.35 U/mg while groups 2, 3 and 4 had MDA values of 2.58 ± 0.13, 2.87 ± 0.50, 3.70 ± 0.42 U/mg respectively.

4. DISCUSSION

NOX is an enzymatic prooxidants that mediates the production of reaction oxygen species (ROS) [12]. XOD is also an enzymatic pro oxidant that mediates the production of reactive oxygen species [13]. The majority of prooxidants enzymes only produce ROS after they have been damaged by ROS, example is in the case XOD. In contrast, membrane-associated enzymes NOX that catalyze the one electron reduction of oxygen using NADH or NADPH as the electron donor produce ROS as their primary and sole function. The NOX family is composed of 7 catalytic subunits termed NOX 1-7. Both enzymatic prooxidants NOX and XOD are widely distributed throughout various organs including the heart. They were suggested to play important roles in multiple diseases associated with oxidative stress. Previous studies have also shown that NOX and XOD activity is regulated by a number of factors known to be involved in the pathogenesis of cardiovascular disease 10]. The result from this present study, shows a significant increase in the activities of NOX and XOD in a dose dependent manner These data demonstrate that non-selective NSAIDS (diclofenac) increases the production of ROS in the cardiovascular system. This is indicated by enhanced vascular superoxide content. The increase superoxide content is consistent with an enhanced expression of NOX and XOD respectively thus resulting to an increase or elevated vascular ROS content that can give rise to oxidative injury in the heart tissue. The result from this study agrees with the works of Huige et al. [14] and Li et al. [15], who in their separate work, confirmed that diclofenac is a potent inducer of NOX and XOD, thereby causing excessive ROS production. Also, this result agrees with the findings of Zhao et al. [16], who suggested that ROS specifically derived from NOX make a substantial contribution to several key processes underlying the development of cardiac contractile dysfunction and remodeling.

CAT and SOD are enzymatic antioxidant that plays an important role in scavenging reactive oxygen species (ROS), they are crucial in protecting the tissues from oxidative stress damage [17]. SOD dismutase's superoxide radicals to form hydrogen peroxide, which in turn is decomposed to water and oxygen by CAT and GSH-Px. If an increase in ROS is not prevented, this may initiate a chain reaction leading to cell death. The result of the present study reveals a significant ($p<0.05$) increase of SOD, CAT and GSH-Px activity in the cardiovascular system in the treatment groups when compared to the control. The effect of diclofenac on these antioxidants enzymes was dose dependent. However, animals in group 2 that received the lowest dose of diclofenac (50mg/kg diclofenac) had higher SOD activity than group 3 and group 4 rats that received higher doses of diclofenac :100 mg/kg and 150 mg/kg respectively. This may be as a result of initial response of the antioxidant enzymes to increased generation of ROS as evidenced by significant increase in the activities of pro oxidant enzymes NOX and XOD in heart tissues of diclofenac treated rats versus control rats (Table 1) which may have been overwhelmed by increased generation of ROS at higher doses of 100mg/kg and 150mg/kg. If an increase in ROS is not prevented, this may initiate a chain reaction leading to cell death. This finding agrees with the work of Forstermann and Munzel,[3] whose result revealed a significant increase in human low-density lipoprotein and cell membrane lipid on treatment with celecoxib. The result of this present study also agrees with the work of Abdulrauf, et al. [4], whose result showed increased serum levels of oxidative stress biomarkers SOD, CAT and GSH-Px activities in the restraint stress rats, an indication of enhanced oxidative stress from the chronic stress induction.

The oxidative stress in heart homogenates was assessed by measuring lipid peroxidation indicated by MDA level. MDA is one of many low molecular weight end-products of lipid hydro-peroxide decomposition and is the most often measured as an index of lipid peroxidation Lipid peroxidation involves oxidative deterioration of poly-unsaturated fatty acids associated with the abnormal membrane lipid bilayer arrangement

and enzymes deactivation during myocardial ischemia. This study reported a significant ($p<0.05$) increase in MDA level, as an end product of polyunsaturated fatty acid oxygenation in the treatment groups when compared to the control on administration of diclofenac. This result provides an indirect evidence of an increase in the level of oxygen free radicals. This could be as a result of increase in the activity of pro oxidant enzymes (NOX and XOD) following the administration of diclofenac. Increased ROS production due to enhanced activity of ROS producing entities is an underlying cause of oxidative stress in tissues [15]. This is in line with the finding of Ahmad et al. [18] and Abdulrauf et al. [4], whose results showed a significant increase in MDA level in celecoxib treated rats and restraint stress rats respectively.

5. CONCLUSION

This study shows that administration of non-steroidal anti-inflammatory drugs, diclofenac affects the redox status of the heart tissue, this results to the generation of reactive oxygen species (ROS) including hydroxyl radical, nitric oxide, hydrogen peroxide, superoxide etc. if not moderated from the source, this can lead to impairment of cardiac tissue as a result of oxidative stress damage giving rise to cardiovascular diseases.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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