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HISTOMORPHOLOGICAL AND BIOCHEMICAL ALTERATIONS ON THE LIVER OF WISTAR RATS FOLLOWING CO-ADMINISTRATION OF NSAIDS (PIROXICAM, DICLOFENAC AND IBUPROFEN)

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are an important therapeutic class of drugs widely used to suppress acute or chronic pains and inflammatory diseases such as in rheumatoid arthritis (RA), osteoarthritis (OS) etc. The aim of this study was to investigate the random combination and individual effect of COX-2 inhibitors (NSAIDs) on the liver. This study was conducted using a total of 40 adults Wistar rats. The rats were divided into eight groups of 5 rats each. Group one was the control group, group 2 was given piroxicam (0.29mg/kg) daily, group 3 was given ibuprofen (5.71mg/kg) daily, and group 4 received diclofenac (1.42mg/kg) daily. Group 5 received piroxicam (0.29mg/kg bodyweight) plus ibuprofen (0.58mg/kg), group 6 received piroxicam (0.29mg/kg) plus diclofenac (1.42mg/kg) and group 7 received ibuprofen (0.58mg/kg) plus diclofenac (1.42mg/kg) respectively daily. Group 8 was given Piroxicam (0.29mg/kg bodyweight) plus Ibuprofen (5.71mg/kg) plus diclofenac (1.42mg/kg) daily. Intervention was over a period of 3days. Animals were sacrificed after 24 hours and the liver tissues were excised, some were fixed in 10% neutral buffered formalin for histological studies while others were placed in normal saline for biochemical analysis. The results revealed that there was no significant statistical difference in total bilirubin, ALT and AST when compared to the control. This suggests that NSAIDs may have no significant effects on the liver for this regimen. Histological changes observed were congested portal vein, haemorrhagic bile ducts, distortion of portal triad and sinusoids, and degeneration of hepatocytes. In conclusion, NSAIDs may have harmful effect on the cytoarchitecture of the liver which can lead to liver damage especially when given in high doses and in combination, but however it had no effect on liver function.

Key words: Ibuprofen, Piroxicam, Diclofenac, Biochemical markers, Histomorphology, Liver
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INTRODUCTION

Polypharmacy is the concurrent ingestion of multiple medications and it is most common among older adults (23). Nonsteroidal anti-inflammatory drugs (NSAIDs) are important therapeutic class of drugs used to suppress pains, inflammatory reactions and treatment of inflammatory diseases such as rheumatoid arthritis (RA) and osteoarthritis (OS) (31). Besides being anti-inflammatory, they are analgesics and antipyretics (22). They produce their therapeutic effects by inhibition of prostaglandin synthesis (39). The commonly used NSAIDs are diclofenac, ibuprofen, piroxicam, aspirin, and paracetamol, which are mostly prescribed by physicians especially orthopaedic surgeons (76%) (28). More than 100 million NSAIDs are prescribed throughout the world yearly and they are associated with different systemic disorders (2).

Diclofenac sodium (voltaren) is a potent and widely used NSAIDs. It is classified as most powerful drug of its kind being one of the best tolerated (42). Diclofenac sodium was first introduced in the late 70's which on its long term use has shown hepatotoxic effects present in the form of hepatic injury ranging from mild to fatal liver injury (18). It acts by inhibiting cellular cyclooxygenase (cox-1 and cox-2) leading to a decrease in pro inflammatory prostaglandins which are potent mediators of pain and inflammation (22). Piroxicam is also a commonly used NSAIDs that is available only by prescription and is used in the treatment of chronic arthritis. It acts through inhibition of tissue cyclooxygenase (cox-1 and cox-2) leading to a decrease in pro-inflammatory prostaglandins which are potent mediators of pain and inflammations (15). Piroxicam is often used to relief non-specific fever conditions, it was first approved for use in the United States in 1982 (30).

Like other NSAIDs, Ibuprofen is potent inhibitor of cellular cyclooxygenases (cox-1 and cox-2) which blocks the formation of prostaglandins, prostacyclin and chromboxan products, which is an important mediator of inflammation and pain (15). It was approved for use by prescription in the United States in 1974 and was available over the counter in 1984 (44). Currently more than 20 million prescriptions are filled yearly; it has a vast over-the-counter use. Ibuprofen is used to treat mild-to-moderate forms of joints pains and arthritis from trauma, osteoarthritis or rheumatoid arthritis (36). It can also be used to treat other forms of pains like headache and dysmenorrhea (7). Although reports of serious liver toxicity with ibuprofen are rare, subacute hepatic failure requiring orthotropic liver transplant has been reported in a 59-year-old female taking 600mg of ibuprofen (32).

The liver is one of the most vital organs of the body that plays an important role in the metabolism of drugs, storage of food and nutrients and the detoxification of chemicals (1). It receives nutrients and poisonous substances which enters the digestive tract through the portal vein, and however, are subject to insults from infections, cancers and toxic chemicals like drugs (10, 5). The liver is an organ of drug metabolism and biotransformation (40), and so it is subject to injuries such as hepatocellular damage (19). Thus, this study was carried out to investigate the effects of combined NSAIDs ingestion on the cytoarchitecture and biochemical activities of the liver.

MATERIALS AND METHODS

Drugs and Chemicals

Piroxicam was obtained from Neimeth Pharmaceuticals PLC (Lagos, Nigeria). Ibuprofen was obtained from Ranbaxy Nigeria Limited (Ogun, Nigeria) while diclofenac was obtained from Pharmatex Nigeria Limited (Lagos, Nigeria). Liver function test kits were obtained from MonobindInc (Woodland Hills, California).

Animal Care and Use

A total of 40 adult Wistar rats weighing between 135-150g were obtained from the Animal House Unit, Faculty of Pharmacy, University of Uyo. The animals were randomly divided into eight (8) groups of five (5) rats each. The animals were fed with standard growers feeds and distilled water given ad libitum. The cages were properly maintained by regular changing of wood shavings and feed.

Ethical Consideration

Consent for the care and use of the animals was obtained from the Ethics committee, Faculty of Pharmacy, University of Uyo. All the recommendations and protocols were strictly adhered to in accordance with International Guidelines for care and use of Laboratory animals (24).

Drug Preparation and Administration

Experimental drugs were dissolved in distilled water and dosage administered according to standard dose regimens for each drug. The administration was done orally using an orogastric tube. All groups except group 1 were administered with therapeutic doses of the test drugs. This is shown in the table below:

Experimental Design

GROUPS	REGIMEN	DURATION
Group 1	Distilled water	3 days
Group 2	Piroxicam (0.29mg/kg)	Once daily for 3 days
Group 3	Ibuprofen (5.71mg/kg)	Once daily for 3 days
Group 4	Diclofenac (1.42mg/kg)	Once daily for 3 days
Group 5	Piroxicam (0.29mg/kg) + Ibuprofen (5.71mg/kg)	Once daily for 3 days
Group 6	Piroxicam (0.29mg/kg) + Diclofenac (1.42mg/kg)	Once daily for 3 days
Group 7	Ibuprofen (5.71mg/kg) + Diclofenac (1.42mg/kg)	Once daily for 3 days
Group 8	Piroxicam (0.29mg/kg)+ Ibuprofen (5.71mg/kg)+ Diclofenac(1.42mg/kg)	Once daily for 3 days

Termination of Experiment

The animals were sacrificed using chloroform inhalation method. The animals were put in a chamber filled with chloroform to anaesthetize them on day 4 after the experiment.

Morphometric Analysis

The weights of the animals were taken before and after the experiment, and the student unpaired T test was used to calculate the bodyweight. The weight of the liver was assessed using laboratory balance equipment.

Biochemical Analysis

3ml of blood was obtained by intracardiac puncture for biochemical analysis. The blood was centrifuged at 300rpm for 10 minutes to separate serum that was subjected to analyze the Liver Function Tests (LEFTs) so as to evaluate its hepatotoxicity.

Histological Analysis

Liver tissues were excised and fixed in 4 % paraformaldehyde for tissue processing and light microscopy. The paraffin wax blocked tissues were sectioned at 5 microns with the rotary microtome (Microtome Thermo Scientific – Microm HM 325, England) and stained with haematoxylin and eosin. The photomicrographs were blindly assessed by 3 independent histopathologists, and the images obtained via an Amscope digital camera (MU 1000, China) attached to a microscope (Olympus - CX31, Japan) (14).

Assay Procedure for liver enzymes and bilirubin

Estimation of alanine amino transaminase (ALT) activity using Agappe kit based on Huang *et al.* (16).

$$\text{A-oxoglutarate} + \text{L- alanine} \xrightarrow{\text{ALT}} \text{L-glutamate} + \text{pyruvate}$$

Pyruvate whose concentration depends on the amount of L-alanine transaminated and hence the activity of ALT is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine at 546nM. 100 µl of serum was added to 1 ml working reagent. After mixing tubes were incubated for 1 minute at 37°C. The change in absorbance per minute during 3 minute was recorded against blank at 340 nm.

Estimation of aspartate amino transaminase (AST) activity using Agappe kit based on Huang *et al.* (16).

α-oxoglutarate + L- aspartate $\xrightarrow{AST}$ L- glutamate + oxaloacetate

Oxaloacetate, with concentration in proportion to aspartate consumed by enzyme and hence its activity is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine. 100 µl serum was added to 1 ml of working reagent. The tubes were incubated for 1minute at 37°C after mixing. The change in absorbance per 20 second during 1 minute was recorded against blank at 340 nm. Distilled water was used as blank.

Determination of concentration of Total Bilirubin in serum using assay kits from Agappe Diagnostics Ltd (25)

Sulfanilic acid reacted with sodium nitrite to form diazotized sulfanilic acid. Total Bilirubin reacted with diazotized sulfanilic acid to form azobilirubin. 1000 µL of Total bilirubin reagent was added into 50 µL of serum and mixed well, incubated for 5 minutes at room temperature. The absorbance of sample and standard was measured against the reagent blank at 546 nm.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by bonferri post-hoc test for comparison between test groups.

RESULTS

Body Weight

All the groups administered with NSIADs showed significant increase @p<0.001 in body weight, respectively.

Table 1. Showing difference in body weight before and after administration

Groups	Before Administration (g)	After Administration (g)	P Value
Control	154.8 ± 6.6	168.3 ± 6.5 ***	<0.0001
Piroxicam	141.8 ± 6.4	157.8 ± 7.5 ***	<0.0001
Ibuprofen	142.0 ± 8.0	159.5 ± 9.5***	<0.0001
Diclofenac	148.8 ± 5.0	164.0 ± 1.5 ***	<0.0001
Piroxicam + Ibuprofen	136.8 ± 5.4	146.5 ± 5.7 ***	<0.0001
Piroxicam + Diclofenac	143.8 ± 5.7	151.8 ± 6.4 ***	<0.0001
Ibuprofen + Diclofenac	149.8 ± 10.8	162.0 ± 12.3 ***	<0.0001
Piroxicam + Ibuprofen + Diclofenac	139.5 ± 11.0	149.8 ± 11.6***	<0.0001

Values are expressed in mean ± standard error of mean. *** indicates significance from before administration @p<0.001 respectively.

Liver Weight

There was a significant (@p<0.05) decrease in Piroxicam group, Piroxicam and Ibuprofen group and Ibuprofen and Diclofenac group compared to control, respectively. Other groups of NSAIDS administration did not show significant difference regarding the weight of the liver.

Table 2. Showing effect on weight of Liver

Groups	Tissue weight (g)
Control	4.72 ± 0.32
Piroxicam	3.64 ± 0.09 *
Ibuprofen	3.97 ± 0.25
Diclofenac	3.82 ± 0.19
Piroxicam + Ibuprofen	3.53 ± 0.07 *
Piroxicam + Diclofenac	3.90 ± 0.18
Ibuprofen + Diclofenac	3.59 ± 0.09 *
Piroxicam + Ibuprofen + Diclofenac	3.75 ± 0.19
	P=0.8197, F=0.5075

Values are expressed in mean ± standard error of mean. * indicates significant increase from control @p<0.05

Liver Function Test

Total Bilirubin, ALT and AST respectively, showed no marked significant difference in all groups administered with NSAIDS

Histological observations

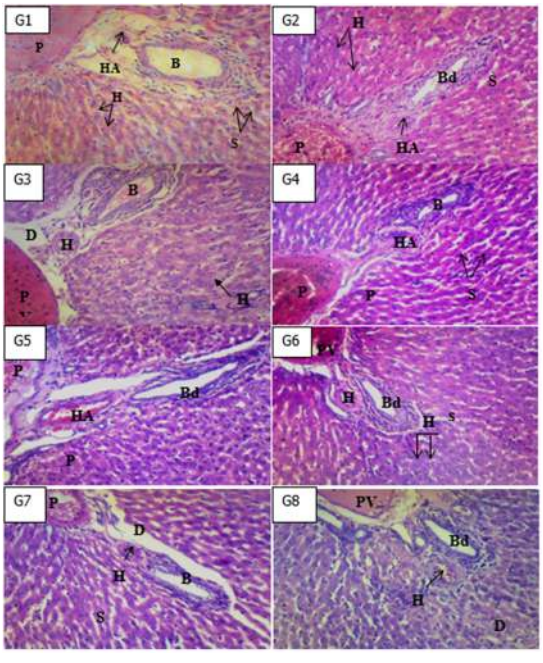

Figure 1. Photomicrographs of the Liver; Control group (G1) with normal histological features. Portal Vein (PV), Hepatic Artery (HA), Bile duct (Bd), Sinusoids (S), Hepatocytes (H). Piroxicam treated group (G2) with congested portal vein and other features as control. Ibuprofen treated group (G3) with Haemorrhage in Bile duct (Bd) and Dilatation of the portal triad (D). Diclofenac treated group (G4) with Distortion of the Portal triad/Area (PA) and Distorted and wavy Sinusoids (S). Piroxicam and Ibuprofen treated group (G5) with congested hepatic artery (HA), Dilatation of Bile duct (Bd) and Distortion of the Portal Area (PA). Piroxicam and Diclofenac treated group (G6) showing congested portal vein, elongated sinusoids (S) and distorted nucleus of hepatocytes (H). Ibuprofen and Diclofenac treated group (G7) with congested portal vein (PV), Dilatation of Portal Area (D), Distorted Sinusoids (S). Piroxicam, Ibuprofen and Diclofenac treated group (G8) with congested hepatic artery (HA) and degenerated hepatocytes (D). H&E; x100 mag.

Table 3: Showing effect of Total Bilirubin, AST and ALT

Groups	T.Bilirubin (mg/dL) Normal Range (0.1 to 1.2 mg/dL)	AST (U/L) Normal Range (8 to 48 U/L)	ALT (U/L) Normal Range (7 to 56 U/L)
Control	1.46 ± 0.03	141.6 ± 5.63	90.52 ± 9.07
Piroxicam	1.48 ± 0.02	121.7 ± 11.00	78.33 ± 1.08
Ibuprofen	1.47 ± 0.03	114.9 ± 9.48	72.74 ± 5.74
Diclofenac	1.48 ± 0.02	121.7 ± 4.53	85.32 ± 1.58
Piroxicam + Ibuprofen	1.44 ± 0.06	131.8 ± 17.27	72.09 ± 1.68
Piroxicam + Diclofenac	1.47 ± 0.04	130.7 ± 19.43	71.94 ± 4.65
Ibuprofen + Diclofenac	1.45 ± 0.02	146.5 ± 26.41	79.61 ± 2.25
Piroxicam + Ibuprofen + Diclofenac	1.43 ± 0.02	147.8 ± 15.71	76.48 ± 3.66
	P=0.9264, F=0.3346	P=0.7150, F=0.6430	P=0.0890, F=2.215

Values are expressed in mean ± standard error of mean

DISCUSSION

In the rural communities, especially where accessibility to medical facilities or trained medical personnel are hard to come by, patients take to self-medication by buying analgesic drugs over the counter and from patent medical stores in order to kill or manage pains (39). For quick relief, they take more than one of the NSAIDs without the knowledge of the adverse effect of these drugs when taken singly or in combination in the body especially the liver where they are metabolized (5,31). Drugs administered

orally pass through the portal vein to the liver even before entering systemic circulation, hence the liver becomes the first tissue of contact with the drug. Liver plays a major role in metabolism of drugs and other xenobiotic, processing them for elimination from the body (17). It is the target organ which gets exposed to drugs in higher concentration than other organs of the body when administered orally (27). Hence, it is most vulnerable to be injured by drugs or chemicals which may damage hepatic cells, hence affect body metabolism (5).

Tissue and body weights following administration of drugs may be a useful indicator of drug toxicity to specific tissues or the body generally. The recent study showed an increase in the body weight of all the experimental groups after drug administration when compared to the weights before drug administration. The difference in body weight was significant except in ibuprofen and combined piroxicam, ibuprofen and diclofenac treated groups. The increased weight after drug administration may be attributed to the nutritional status of the animals having been fed ad libitum throughout the course of the study. Body weight increase is therefore a positive indicator of healthy nutrition. The non-significant status of body weight before and after administration of the ibuprofen and combined piroxicam, ibuprofen and diclofenac treated groups may be due to the high variance in the body weight of the individual animals in the group. Report by Wongrakpanich (46), which studied that NSAIDS increased weight in the elderly, supports our present study, but contrarily, report by Okamoto (26) observed that NSAIDs cause decrease in body weight.

Decrease in organ weight following administration of drug or toxic substances is an indication of organ specific toxicity. The liver, being readily exposed to toxic substance is more susceptible to toxicity. The result of the present study showed a general decrease in liver weight compared to the control with significant decreases observed in liver weight of groups administered piroxicam only, combined piroxicam and Ibuprofen as well as combined ibuprofen and diclofenac treated groups when compared with control. The toxic effect may be due to generation of free radicals in the course of the drug metabolism consequently initiating lipid peroxidation of the hepatocyte membrane hence reduction in weight. Specifically, NSAID induced liver toxicity may result from acidic moiety of NSAIDs or reactive adducts of NSAIDs metabolites binding to host proteins causing cellular injury in susceptible individuals (38). Traversa et al. (43) in their cohort study that recruited patients receiving various NSAIDs, confirmed that ibuprofen has a low liver toxicity rate: only two out of 26 patients that took the NSAIDs showed ibuprofen-associated liver injury. This is probably because ibuprofen is characterized by a high safety profile and very low toxicity incidence, and this is based on the fact that ibuprofen has short plasma half-life and does not form pathological metabolites (44). The mechanism of this NSAIDs hepatoxicity involves alteration of covalent protein by reactive metabolites (20,37), oxidative stress generation (41) and mitochondrial injury (21). This supports recent reports that drugs like NSAIDs actually induce hepatoxicity which results in reduction of the liver tissue (29,2,40).

There was no significant difference in serum total bilirubin, ALT and AST activities in NSAID treated groups in comparison with the control. This possibly imply that the NSAIDs had no significant deleterious effect on these parameters. Although damage to the liver is supposed to be evidenced with increased concentration of bilirubin and activities of ALT and AST but that is not the case in the present study. This observation may be a function of the degree of toxicity inflicted on the liver by the drugs. The mild decrease in liver weight therefore indicates that the liver

toxicity induced by the NSAIDs was mild hence the non-deleterious effect on the biochemical parameters of liver function. Contrarily, Rostom et al. (33) found that increased doses of NSAIDs did appear to increase the risk of elevated levels of aminotransferases with diclofenac (33). The authors defined hepatic toxicity as aminotransferase elevations, and concluded that diclofenac and rofecoxib had higher rates of aminotransferases, three times greater compared to other NSAIDs. This was supported by a case control study on NSAIDs that highlighted diclofenac as a drug associated with an increased risk of liver damage and disruption of function (4), and report by Fokunang et al. (12) also observed that NSAIDs have high risks on liver functions. However, Chalasani et al. (9) reported that patients with elevated liver enzymes are not at higher risk hepatotoxicity and stastin.

The mechanism of liver injury is not well understood, and it has been proposed that acidic moiety of NSAIDs or reactive adducts of NSAIDs metabolites may bind to host proteins and cause cellular injury in susceptible individuals (38). Histologically, Piroxicam administration showed congestion of portal vein. This is in agreement with a report that Piroxicam induced hepatic and renal histopathological changes in mice (11). Sadeq (35) also confirmed that Piroxicam administration induced hepatoxicity in rats. Histology of rats administered with Ibuprofen showed haemorrhage on bile duct and general distortion of the portal triad of the liver. Though reports of liver toxicity with ibuprofen are rare, subacute hepatic failure requiring orthotropic liver transplant has been reported in a 59-year-old female taking 600mg of ibuprofen (32). The division of viral hepatits at the National Centers for Disease Control and Prevention (CDC) has also clearly identified Ibuprofen has a cause of liver inflammation (8).

Diclofenac administration also showed distortion of the portal triad and sinusoids in the liver of the rats. Aithal and Day (3) proposed a multistep theory for diclofenac induced liver injury; the metabolites of diclofenac penetrates the liver as 4-hydroxydiclofenac and hydroxylated frames, after glucuronidation and sulfation, the metabolites are discharged in the urine and bile but on high dosage or long term use, can present hepatic injury from mild to fatal. In their report, the liver injury was dose-dependent and seen mostly at the dose of 150mg or higher (3). However, our findings are not aligned with the systematic review conducted by Rubenstein and Laine (34) which indicated no hepatoxicity from diclofenac (34).

Dilatation of bile duct, distortion of portal area, congestion of portal vein and hepatic artery, were observed in co-administration of piroxicam and ibuprofen. Congestion of portal vein and elongation of sinusoids was also observed in co-administration of piroxicam and diclofenac. Ibuprofen and Diclofenac co-administration showed dilatation of the portal area and sinusoids. There was also marked congestion of hepatic artery, also degenerative features on the hepatocytes following combined administration of the three NSAIDs were observed. Reports have shown that NSAIDs causes histological damages, inflammation, ulcers, gastroenterology, strictures, haemorrhage and eosinophilic colitis (13,45,6).

CONCLUSION

Findings from the present study revealed that NSAIDs had detrimental effect on the histomorphology of liver of Wistar rats as the liver weight reduced and histological alterations were observed without accompanying increase in bilirubin concentration and aminotransferases activities. This research work will serve as awareness to the government, health

parastatals and medical officers on the effect of co-administration of NSAIDs on the liver. This will also aid in public enlightenment on the long term use of NSAIDs which can lead to liver cirrhosis and cancer.

RECOMMENDATIONS

We recommend that combined ingestion of these drugs, and over a long period of time, should be discouraged to allow the liver cells regenerate or recover from drug induced toxicity. We also recommend further research to be done on adjuvants or ethomedicinal plants that will treat or reduce adverse effects caused by NSAIDs.

CONFLICTS OF INTEREST

The authors affirmed that there are not any conflicts of interest.

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