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TIME COURSE STUDY OF COMBINED N-ACETYL-P-AMINOPHENOL AND DICLOPHENAC INDUCED-HEPATOTOXICITY AND OXIDATIVE STRESS IN WISTAR RATS

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Abstract
The abuse of combined acetaminophen or N-acetyl-p-aminophenol (APAP) and diclofenac (DIC) due to their analgesics, anti-inflammatory and antipyretic properties is a predominant cause of hepatotoxicity and oxidative stress. This study investigated the time-course effects of APAP, DIC and their combination on biomarkers of hepatic function and oxidative stress in rats. Forty male Wistar rats were randomly divided into four groups of 10 animals each as follows; control (distilled water), APAP only, DIC only and APAP + DIC for 4 weeks. Indices of liver damage (serum ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase and bilirubin) were measured. Oxidative stress biomarkers (MDA, malondialdehyde; NO, nitric oxide; CAT, Catalase activity; SOD, superoxide dismutase activity; GSH content, reduced glutathione), GR, glutathione reductase, and GST, glutathione-S-transferase) were also determined using spectrophotometric methods. Statistical analysis was done using one-way ANOVA with $p < 0.05$ considered significant. Acetaminophen and diclofenac caused marked liver damage as noted by time-dependent significant ($p < 0.05$) increased activities of serum ALT, AST, ALP, GGT, and bilirubin levels as well as significant ($p < 0.05$) increase in hepatic MDA and NO levels as compared to the control group. Hepatic GSH content, SOD, CAT, GPx, GST and GR activities were decreased significantly ($p < 0.05$) in all acetaminophen and diclofenac-treated groups compared to normal control in a time-dependent manner. These findings suggest that prolonged administration of diclofenac, acetaminophen or their combination may induce hepatotoxicity, oxidative stress and alteration of hepatic antioxidant status in a time-dependent manner.
Keywords: Acetaminophen, diclofenac, non-steroidal anti-inflammatory drugs, hepatotoxicity, oxidative stress, antioxidant status.

INTRODUCTION

The regulation of different physiological functions, including bile secretion, metabolic processes, clotting factor production, protein synthesis, glycogen, and vitamin A storage, as well as detoxification and excretion of endogenous and exogenous substances, such as chemotherapeutic agents are well established attributes of the liver, while drug-induced hepatotoxicity is a serious health concern, as it is the main cause of death among patients with acute liver failure worldwide [1].

N-acetyl-p-aminophenol (APAP) (also known as acetaminophen or paracetamol) and diclofenac (DIC) are one of the commonly prescribed antipyretic, analgesic and anti-inflammatory over-the-counter drugs and has been categorized as the most widely used drugs. It may cause acute and chronic liver injury if the recommended doses are exceeded [2, 3]. Generally, over-the-counter analgesics are regarded as safe for the majority of patient when used at therapeutic levels. However, the therapeutic doses may also cause hepatotoxicity and oxidative damage in human and experimental animals. It is more prominent in individuals with malnutrition or association with alcohol ingestion [4, 5]. The exorbitant exposure to toxins, xenobiotic and drugs like acetaminophen and diclofenac or their combination may lead to liver damage as a result of excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation, resulting in perturbation of the hepatocyte membrane [6].

A large portion of the administered acetaminophen is metabolized through glucuronidation or sulfation in the liver and then excreted in the urine; therefore, the

remaining part of the dose of is metabolized by the cytochrome p450 system to a reactive metabolite, N-acetyl-P-benzoquinone imine (NAPQI). In normal physiological conditions, NAPQI is metabolized into a non-reactive metabolite via the glutathione (GSH) pool. When GSH is depleted, NAPQI attacks the intracellular organelles, resulting in oxidative stress, altered mitochondrial permeability, perturbed calcium homeostasis, and ATP depletion, leading to necrosis and, ultimately, to cell death [2, 7, 8]. Also, diclofenac being a non-selective NSAIDs has severe pathologic effects such as peptic ulceration, gastrointestinal bleeding, hepatotoxicity, renal papillary necrosis, and renal failure on long-term exposure to the drug [3]. The pharmacological actions of NSAIDs have long been established to be via inhibition of cyclooxygenase (COX) enzyme activity [4].

There has been a trend over recent years for combining diclofenac with acetaminophen for the management of various conditions such as pain, stress, fever, inflammation and general body weakness [9]. However, the therapeutic superiority of a combination of acetaminophen and NSAID over either drug alone remains controversial. Due to the widespread and frequent usage of NSAIDs, acetaminophen or their combination, regular toxicological evaluation becomes essential. Also, the literature review has revealed a lack of sufficient reports on the combined acetaminophen and diclofenac-induced hepatotoxicity. Hence, the current study aimed to investigate the combined administration of acetaminophen and diclofenac-induced hepatic intoxication in rats.

MATERIALS AND METHODS

Chemicals and Reagents

Ethanol (C_2H_5OH), disodium hydrogen phosphate (Na_2HPO_4), dihydrogen potassium phosphate anhydrous (KH_2PO_4), thiobarbituric acid, sodium azide (NaN_3), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiol-bis-2-nitrobenzoic acid (DTNB), and hydrogen peroxide (H_2O_2) were obtained from Sigma Aldrich Chemical Company (London, United Kingdom). Assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (γ-GT), lactate dehydrogenase (LDH), bilirubin and total protein were obtained from Randox laboratories limited (Antrim, United Kingdom). All other chemicals and reagents used were of analytical grade.

Drugs

The tested drugs, acetaminophen (paracetamol) (each tablet contains 500 mg active drug, May and Baker Pharmaceuticals, Otta, Nigeria) and diclofenac sodium (each tablet contains 50 mg diclofenac sodium, Novartis Pharmaceutical, Cairo, Egypt) were obtained from a public pharmacy store (Idera pharmacy, Abeokuta, Nigeria). Each tablet was crushed to a fine powder and dissolved in distilled water at appropriate concentrations.

Experimental Animals

Forty healthy male Wistar rats weighing 120-180 g were used for this study. The animals obtained from the animal house facility, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria, were housed in wire-floored cages under standard ambient laboratory conditions of 12 hours light-dark cycle, room temperature. Animals were supplemented daily standard pelleted diet and were given water *ad libitum*. The animals were acclimatized to laboratory conditions for two weeks preceding the commencement of the experiment. Handling of the experimental animals is consistent with international principles on the care and use of experimental animals

Experimental design

The rats were randomly divided into four groups of ten (10) animals each as follows;

- Group A served as control and administered distilled water only.
- Group B was administered acetaminophen (1000 mg/kg b.w.) only.
- Group C was administered diclofenac (100 mg/kg b.w.) only.
- Group D was administered a combination of acetaminophen (1000 mg/kg b.w.) and diclofenac (100 mg/kg b.w.).

The drugs and distilled water were administered by oral gavage twice daily for four weeks. Five (5) rats per group were sacrificed at the end of two weeks.

Sacrifice and preparation of serum

At the end of treatment, animals were fasted overnight under standard laboratory conditions but allowed free access to distilled water. All

Animals were weighed and anesthetized under light ether. Blood was collected via cardiac puncture into plain centrifuge tubes and allowed to stand for 30 minutes to confirm complete clotting. The clotted blood samples were centrifuged at 3000 rpm for 10 minutes and the clear serum supernatant were aspirated off and stored frozen at 4 °C until assayed for serum biochemical estimation.

Preparation of tissue homogenate

Livers were excised, washed thoroughly in ice-cold saline to remove the blood and dissected to remove connective tissues. They were then gently blotted between the folds of a filter paper and weighed in an analytical balance. Ten percent of homogenate was prepared in 0.05M phosphate buffer (pH 7.4) using a polytron homogenizer at 4ºC. The homogenate was centrifuged at 3000 g for 20 minutes to remove the cell debris, unbroken cells, nuclei, erythrocytes, and mitochondria. The supernatant thus obtained were stored at -70ºC until used for biochemical estimation of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) activities and the levels of reduced glutathione (GSH), lipid peroxidation, nitric oxide (NO) and total protein.

Biochemical analysis

Liver function parameters

Biomarkers of liver functions including serum ALT, AST, ALP, GGT activities and bilirubin levels were spectrophotometrically measured using Randox diagnostic kits according to the manufacturer's specifications.

Determination of lipid peroxide level:

Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) described by Polizio and Pena [10] and modified by Simeonova *et al*. [11]. The assay is based upon the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product of polyunsaturated fatty acid oxidation, to form pink-coloured TBA-MDA complex in acidic and boiling temperature condition. The TBA-MDA complex is measured by spectrophotometer at 532 nm.

Determination of nitric oxide level

Determination of nitric oxide (NO) involves the spectrophotometric measurement of its stable decomposition products NO_3^- and NO_2^- as described by Bryan and Grisham [12]. This method requires that NO_3^- is first reduced to NO_2^- and then NO_2^- determined by the Griess reaction. The griess reaction is a two-step diazotization reaction in which the NO-derived nitrosating agent, dinitrogen trioxide (N_2O_3) generated from the acid-catalyzed formation of nitrous acid from nitrite (or autoxidation of NO) reacts with sulfanilamide to form a chromophoric azo product that absorbs strongly at 540 nm.

Determination of superoxide dismutase activity

The superoxide dismutase (SOD) activity was measured by the method of Marklund and Marklund [13]. This method was based on the ability of superoxide dismutase to inhibit the auto-oxidation of pyrogallol in alkaline medium, measured at 420 nm. One SOD unit (U) was considered the quantity of enzyme that will able to promote 50 % inhibition and the results were expressed as U (mg protein)$^{-1}$.

Determination of catalase activity

Catalase (CAT) activity was measured by the method of Hadwan and Abed [14]. This method is based on the reaction of undecomposed hydrogen peroxide with ammonium molybdate to produce a yellow colour, which has maximum absorbance at 374 nm.

Determination of glutathione peroxidase activity

Glutathione Peroxidase (GPx) activity was assayed sby the method of Mohandas *et al.* [15]. The glutathione peroxidase (GPx) activity was estimated by measuring the change in absorbance at 340 nm due to NADPH consumption in the presence of H_2O_2, GSH, and glutathione reductase and expressed as nmol min-1 (mg protein)$^{-1}$.

Determination of reduced glutathione level

The level of reduced glutathione (GSH) was estimated by the method of Beutler *et al*. [16]. The principle is based on the reduction of disulfide compound; 5, 5 dithiol-bis (2-nitrobenzoic acid (DTNB), by sulfhydryl group of reduced glutathione (GSH) to produce a yellow compound (2-nitro-5-thiobenzoic acid). The reduced chromogen is directly proportional to GSH concentration present in the sample measured at 412 nm.

Determination of glutathione reductase activity

The glutathione reductase (GR) activity was measured by the method of Carlberg and Mannervik [17], where glutathione reductase together with its cofactor (NADPH) catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione, GSH. The oxidation of NADPH to $NADP^+$ is monitored as a decrease in the absorbance at 340 nm. The rate of decrease is directly proportional to the GR activity in the sample.

Determination of glutathione-s-transferase activity

GST activity was determined by the method of Habig *et al*. [18] using 1-chloro-2,4-dinitrobenzene as a substrate, with reduced GSH. The conjugation is accompanied by an increase in the absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample.

Determination of total protein

Total protein was assayed with the use of Randox diagnostic kits (TP 245). The assay was measured by Biuret method described by Weichselbaum [19]. The principle of the assay is based on the cupric ions in an alkaline medium that interacts with protein peptide bonds resulting in the formation of a colored complex.

Statistical analysis

All data were expressed as means ± standard error (SE). Statistical analysis was done using Statistical Package for Social Sciences (SPSS) computer software (version 22), IBM software, USA. One-way analysis of variance (ANOVA) test was used to elucidate significance among group means, followed by Duncan's post-hoc test and least significance difference (LSD) to compare mean values pair-wise. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Effects of combined acetaminophen and diclofenac on liver function tests

The needless consumption, or abuse, of specific medications, is strongly associated with mild-to-acute progression of liver dysfunction. Excessive dosage or chronic use of acetaminophen and diclofenac sodium provides a well-known example of this association [3, 5]. The widespread and chronic use of acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) particularly diclofenac sodium have been reported to increase the prevalence of their adverse effects [4, 5].

The estimation of these hepatic enzymes in the serum are useful biomarkers used to monitor the extent and type of hepatocellular injury. Their increase in serum is a marker of cellular outflow and lack of the functional integrity of cell membranes of the hepatocytes [3]. In this study, repeated administration of double dose of acetaminophen alone, diclofenac alone and their combination induced hepatotoxicity as noted by time-dependent significant increased specific activities of serum ALT, AST, ALP, GGT as well as direct and total bilirubin levels in rats administered double dose of acetaminophen, diclofenac, and combined acetaminophen and diclofenac compared with the control group (Figure 1 – 6). The results of this study were consistent with previous studies on acetaminophen and diclofenac induced hepatotoxicity and other biochemical perturbations in rats [3, 20, 21, and 22].

The hepatotoxicity resulted from non-steroidal anti-inflammatory drugs (NSAIDs) especially diclofenac could be due to its acidic moiety or reactive metabolites that bind to host proteins causing cellular injury. Further, acetaminophen is metabolized into a toxic reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) that binds to and depletes glutathione in the hepatocyte. This result to the impairment of mitochondrial function which in turns causes hepatocyte damage and necrosis. This damage may lead to cellular leakage and loss of functional integrity of cell membrane in the liver releasing high levels of serum biomarkers in the blood, notably ALT, AST, ALP, GGT that represent larger percentage of total enzyme and better index of hepatotoxicity [3]. The observed time-dependent significant increase in serum ALP activity may be due to increased synthesis of bile salts resulting from increased biliary pressure caused by defective excretion of bile by hepatocytes [23, 24]. The marked elevation in serum bilirubin levels may be caused by the production of more bilirubin than the liver can process as a result of injury to the liver via obstruction of excretory ducts which impairs its ability to excrete normal amounts of bilirubin.

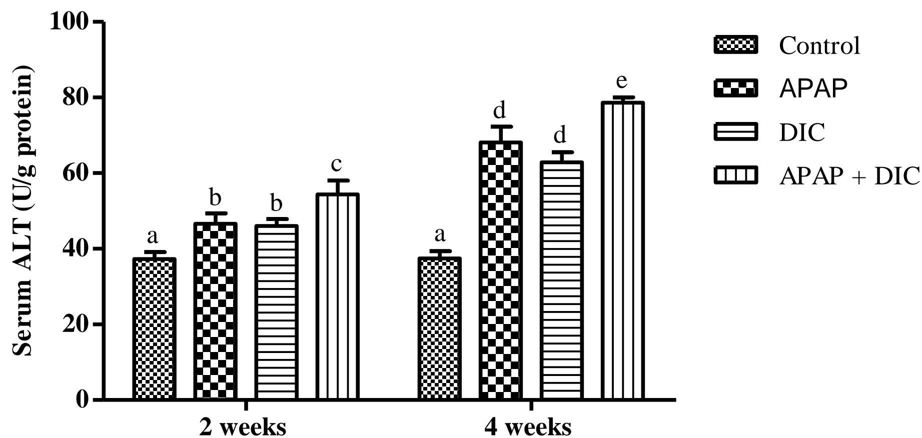

Figure 1: Effects of combination of acetaminophen and diclofenac on serum alanine aminotransferase (ALT) activity.

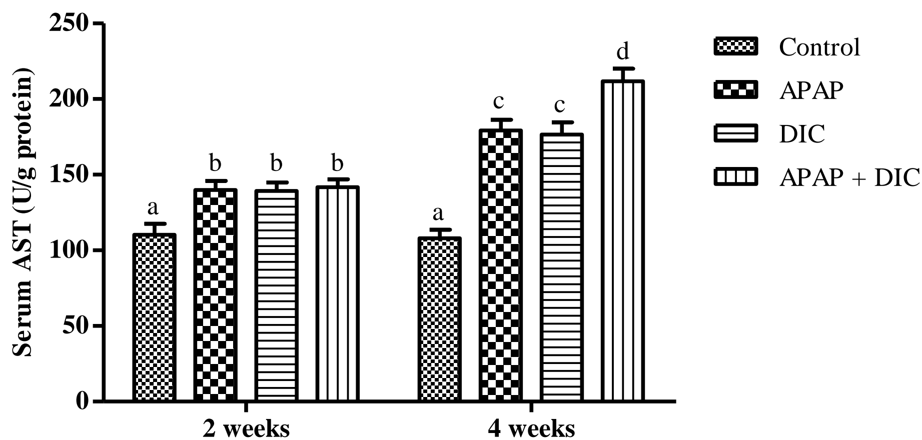

Figure 2: Effects of combination of acetaminophen and diclofenac on serum aspartate aminotransferase (AST) activity.

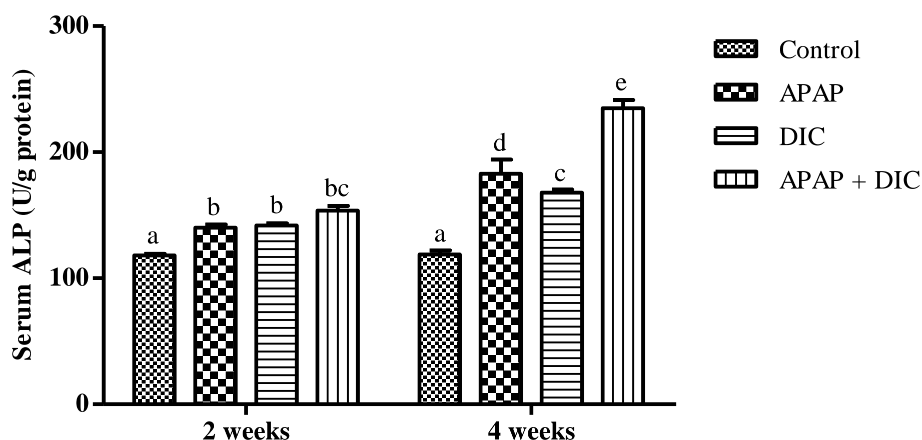

Figure 3: Effects of combination of acetaminophen and diclofenac on serum alkaline phosphatase (ALP) activity.

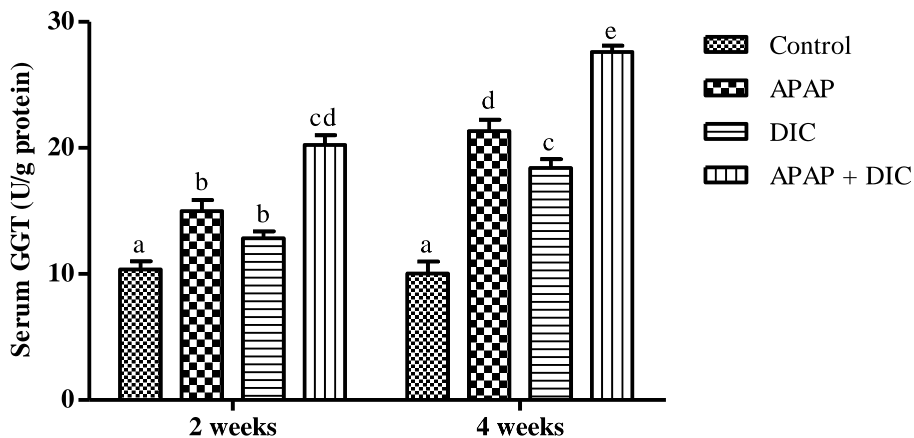

Figure 4: Effects of combination of acetaminophen and diclofenac on serum gamma-glutamyl transferase (GGT) activity.

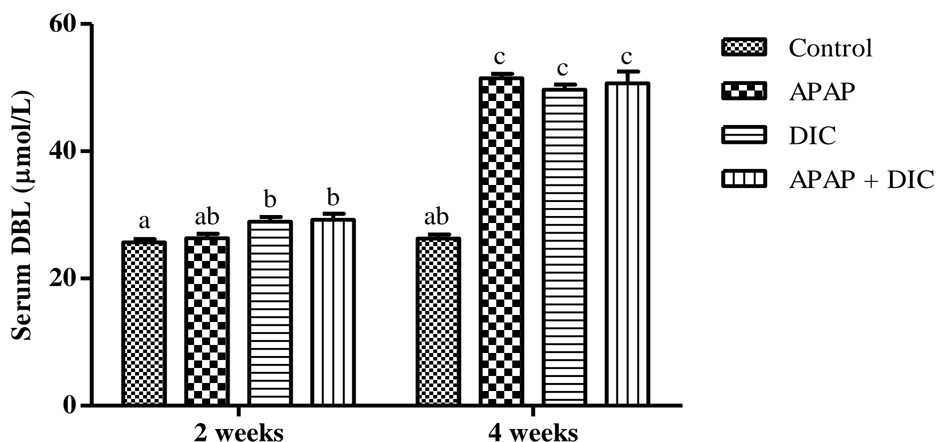

Figure 5: Effects of combination of acetaminophen and diclofenac on serum direct bilirubin concentration.

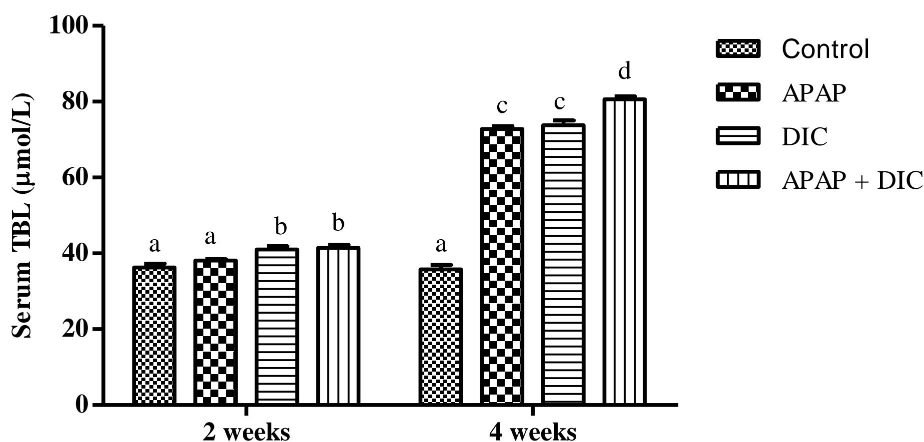

Figure 6: Effects of combination of acetaminophen and diclofenac on serum total bilirubin concentration.

Oxidative stress has been reported to play a fundamental role in acetaminophen and diclofenac-induced hepatic injury. In this study, administration of acetaminophen,

diclofenac and their combination for 2 and 4 weeks induced oxidative damage as noted by time-significantly increased levels of hepatic MDA and NO with concomitant dose and time-dependent significant decreased GSH content and alteration of enzymatic antioxidant defense system including SOD, CAT, GPx, GST, and GR. (Table 1 and 2). In response to chemical intoxication and subsequent ROS production, both enzymatic and non-enzymatic antioxidant defense systems are activated [25]. Also, in hepatocellular dysfunction, generation of excessive ROS and depletion of antioxidants have been reported [5]. Excessive administration of acetaminophen and diclofenac has been reported to cause ROS generation and this, in turn, results in lipid peroxidation, with consequent structural and functional alterations of the hepatocyte membrane, formation of reactive aldehydes, and GSH depletion [5, 22]. Following the elevated nitric oxide (NO) production, excess NO may interact with superoxide, thereby promoting the generation of peroxynitrite a known potent oxidant, thereby causing deleterious cytotoxic effects and cell death [26]. The reactive metabolites derived from APAP and DIC metabolism has been reported to attack cellular macromolecules and inactivated endogenous antioxidants [22, 27, and 28].

Table 1: Effects of combination of acetaminophen, diclofenac and their combination on hepatic MDA, NO and GSH levels in rats.

	MDA (nmol/g tissue)	NO (nmol/g tissue)	GSH (U/mg tissue)
		2 WEEKS	
Control	127.08 ± 6.46[a]	91.61 ± 3.35[a]	90.31 ± 3.10[e]
APAP only	151.92 ± 4.95[b]	168.19 ± 17.25[b]	61.69 ± 2.26[d]
DIC only	151.67 ± 6.77[b]	207.71 ± 12.82[c]	55.10 ± 0.75[cd]
APAP +DIC	188.72 ± 6.84[c]	340.72 ± 3.71[d]	48.70 ± 1.91[bc]
		4 WEEKS	
Control	128.59 ± 4.27[a]	92.29 ± 2.55[a]	90.55 ± 3.66[c]
APAP only	264.41 ± 13.47[d]	420.00 ± 15.08[e]	43.96 ± 1.24[ab]
DIC only	256.92 ± 8.47[d]	419.76 ± 11.95[e]	49.31 ± 1.85[bc]
APAP +DIC	397.56 ± 3.21[e]	600.96 ± 7.97[f]	39.09 ± 2.47[a]

Values are expressed as Mean ± S.E.M. Values with different alphabetic lettering down the groups are significantly different at $p < 0.05$.

The time-dependent significant decrease activities of hepatic SOD and GPx in rats administered acetaminophen, diclofenac and their combination may be attributed to the consumption of SOD and GPx in reactive oxygen species (ROS) detoxification. It has also been reported that SOD and GPx are inhibited by oxygen-derived free radicals [29]. NAPQI inhibits GSH synthesis which involved in the protection of cells against oxidative damage and in various detoxification mechanisms [29]. The reduction in the catalase (CAT) activity may results in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide [24]. Also, the reduction of glutathione-S-transferase (involve in GSH utilization) effectiveness may be due to its inactivation by reactive nitrogen species or the decrease in reduced glutathione (GSH) concentration. Glutathione reductase (involved in GSH redox-cycling) activity impairment may be as a result of the action of ROS or the conjugate between NAPQI and GSH [29, 30].

Table 2: Effects of combination of acetaminophen, diclofenac and their combination on hepatic enzymes specific activities in rats.

	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)	GST (U/µg protein)	GR (U/µg protein)
2 WEEKS					
Control	7.13 ± 0.12[d]	22.04 ± 0.63[a]	78.31 ± 1.43[d]	42.82 ± 1.38[e]	53.58 ± 1.63[f]
APAP only	4.48 ± 0.33[c]	17.92 ± 0.35[c]	70.24 ± 1.31[bc]	33.42 ± 1.52[c]	44.54 ± 0.85[de]
DIC only	5.70 ± 0.24[c]	17.77 ± 0.48[c]	72.44 ± 1.26[c]	37.91 ± 0.77[d]	46.49 ± 0.98[e]
APAP +DIC	4.82 ± 0.20[bc]	16.36 ± 0.81[c]	70.85 ± 2.59[c]	21.32 ± 1.02[b]	41.75 ± 1.23[d]
4 WEEKS					
Control	7.12 ± 0.17[d]	22.40 ± 0.67[a]	78.34 ± 1.17[d]	42.11 ± 1.85[e]	54.18 ± 1.13[f]
APAP only	4.14 ± 0.41[ab]	13.44 ± 0.51[b]	66.28 ± 1.34[ab]	11.59 ± 0.42[a]	33.30 ± 0.94[b]
DIC only	4.39 ± 0.20[ab]	13.97 ± 0.52[b]	68.46 ± 0.71[bc]	13.36 ± 0.44[a]	37.87 ± 1.54[c]
APAP +DIC	4.27 ± 0.14[a]	11.56 ± 0.63[a]	63.74 ± 1.11[a]	10.21 ± 0.75[a]	26.79 ± 1.69[a]

Values are expressed as Mean ± S.E.M. Values with different alphabetic lettering down the groups are significantly different at $p < 0.05$.

CONCLUSION

From the results of the present study, it could be concluded that repeated administration of double dose of acetaminophen, diclofenac or their combination-induced hepatotoxicity and oxidative stress in a time dependent manner.

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