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Expression of Inducible Nitric Oxide Synthase in N-Acetyl-p-aminophenol-induced Hepatotoxicity following Ameliorative Role of Ascorbic Acid in Wistar rat.

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ABSTRACT

Recent findings show conflicting evidences of both beneficial and deleterious effects of inducible nitric oxide synthase (iNOS). Ascorbic acid (ACA) is a source of antioxidant. This study seeks to examine the role of iNOS in acetaminophen (APAP)-induced liver damage following treatment with ACA. Twenty (20) female rats (150-200g) were used for the study, which were divided into four groups (A-D) n=5. Group A received normal saline while groups B-D received 2700 mg/kg of paracetamol for 21 days. Later, Groups C and D were orally treated with 200 mg/kg and 500 mg/kg body weight of ACA respectively for 21 days and group B was left untreated. Rats were sacrificed 24hours after the last administration. Blood and liver tissues were collected for biochemical, immunohistochemical and histological assays. One-way ANOVA was used to analyze data. Graph pad Prism 5 was used for analysis, *p*-values $\leq$ 0.05 were considered statistically significant. iNOS was strongly expressed across the groups. Collagen fibre was densely expressed in groups A and C while elastic fibre was densely distributed in A and D. ALT showed significant (p= 0.05) increase in APAP group while AST was increased only in ACA-treated groups. SGPT was increased significantly in ACA-treated groups while SGOT showed no differences across groups. TBARS was increased significantly (p=0.05) in group ACA-treated groups when compared with control. We conclude that iNOS is not only expressed in damaged tissues, as other underlying processes could lead to its expression in normal tissue. ACA was instrumental in ameliorating APAP liver damage.

Keywords: iNOS; Acetaminophen; Hepatoprotective, Ascorbic acid, hepatotoxicity; Ameliorative

INTRODUCTION

N-Acetyl-p-aminophenol (APAP), acetaminophen, or paracetamol is an extensively prescribed and over-the-counter (OTC) analgesic and antipyretic drug, and it is used either as a single agent or in combination with other drugs (s)[1,2]. It has been reported that more than 1 billion tablets are sold annually in the United States alone[3]. In recent times, the safety of acetaminophen even at therapeutic doses has generated considerable debate[4]. Over 56,000 emergency visits and nearly 500 deaths occur in the US annually, resulting from acetaminophen toxicity, owing to either intentional or accidental overdoses[3]. The metabolism of the ingested therapeutic dose of APAP is mainly accomplished by cytochrome P450 followed by glucuronidation or sulfation whereas N-acetyl-p-benzoquinoneimine (NAPQI) is conjugated with glutathione (GSH)[5]. On the other hand, a high dose saturates the detoxification pathways of APAP due to glucuronidation and sulfation insufficiency[6,7]. In addition, it was reported that APAP could induce organ damage by activating apoptotic death and infiammation which were manifested by an increase in the expression of caspase-3, caspase-9, protein 53 (p53), nuclear factor-kappa B (NF-κB), and inducible nitric oxide synthase (iNOS) as well as by a decrease in B-celllymphoma-2 (Bcl-2) expression[8,9]. Reactive oxygen species (ROS) production was found to be associated with excessive and long-term APAP administration and biotransformation[10].

Liver is a vital organ that plays a role in controlling critical biochemical and physiological activities including homeostasis, growth, energy and nutrient supply, detoxification of drugs and other xenobiotics, and also combating infections[11,12]. Therefore, liver damage may be a result of distortion of the metabolic functions[13]. Liver injury due to overdose of paracetamol is the most common cause of acute liver failure resulting in hepatotoxicity[14].

It has been noted that, the serum levels of several biomarkers such as alkaline phosphatase (ALP), transaminases, bilirubin, triglycerides, and cholesterol are increased in hepatic diseases[15]. Ascorbic acid (ACA), popularly known as vitamin-C has been reputed as one of the major sources of antioxidants in the human body[16]. It has been published that ACA protects the liver prophylactically against APAP-induced liver toxicity. Initiation of chronic liver diseases commonly involves an inflammatory phase, which progresses to fibrosis after continuous oxidative stress. Under these conditions, inducible nitric oxide synthase (iNOS) is upregulated,

leading to the production of large amounts of nitric oxide (NO)[17]. The role of iNOS in fibrosis formation in hepatotoxicity is not clearly understood due to the fact that report from animal studies of fibrosis or non-alcoholic steatohepatitis-related liver fibrosis have shown conflicting results in iNOS revealed both beneficial and deleterious effects[18,19,20]. What brought about these effects is still a puzzle to be answered. Therefore, the present study was undertaken to elucidate the implication of iNOS expression in the pathophysiology of liver damage due to APAP high dose consumption under the protective role of ACA.

MATERIALS AND METHODS

Drugs: Drugs used in the experiment include acetaminophen, ascorbic acid and normal saline. All other reagents used in this study were of analytical grade.

Animals: Twenty (20) female Wistar rats weighing between 150-200g were used for this research; they were housed in standard animal house of Babcock University Remo, Nigeria and allowed free access to food (Premier Feed Mill Co. Ltd., Ibadan, Nigeria) and water *ad libitum*. The rats were kept in controlled room temperature (24 ± 2 ºC) and humidity (65 - 80%) under a 12/12h light-dark cycle (light on 06:00h) for 7 days before the commencement of experiments. Ethical approval was obtained from Babcock University ethical review committee with a reference number (BUHREC 035/19). Rats were divided into four groups A-D (n=5). Group A received normal saline, while groups B, C, and D received 2700 mg/kg of paracetamol for two weeks. Group C and D, were later treated with 200 mg/kg and 500 mg/kg of Vitamin C orally respectively for 21 days.

Drugs preparation and use: Ten tablets (1000mg each) of paracetamol and vitamin C were ground into powder and 3g was measured using sensitive weighing balance (METTLER TOLEDO, China) and dissolved in 50 ml normal saline for each preparation. Based on the weight of each animal, the dosage was calculated and administered to the animal using oral cannula mounted on a 2ml.

Animal sacrifice and blood collection for serum Alanine transaminase (AST), aspartate transaminase (ALT), thiobabituric acid reactive species (TBARS) for lipid peroxidation, serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT): Twenty four hours after the last day of administration, the rats were sacrificed through cervical dislocation. Blood samples were obtained by cardiac puncture using 2ml syringe needle by piercing the pericardium to gain access into the right atrium after the thorax was cut open using scissors. The blood samples obtained were collected into plain sample bottles and centrifuged at 3000 rev/min. for 30 minutes to separate sera. AST, ALT, SGPT, SGOT and TBARS were determined using an Enzyme-Linked Immunosorbent Assay (ELISA) kit according to the manufacturer's instructions (ALPCO Diagnostics, Salem, NH, USA). The absorbance was measured at 450 nm using a mircroplate ELISA reader (Bio-Rad Laboratories, Inc.).

Liver Harvest and Gross Examination: Gross examination reveals that the liver of the control shows normal texture and appearance in terms of color while liver of the rats that were given APAP appeared dark-red with hardened and coarse texture.

Liver Weight Measurement: The whole liver was immersed in normal saline (to rinse it) and immediately removed and then placed on filter paper to drain the fluid. It was later place on a sensitive weighing balance to take the measurement in grams.

Histopathological studies: Excised liver tissues from each group were rinsed with normal saline and then were fixed in 10% formal saline, the fixed liver tissues were then processed following the routine procedures involved in histological tissue analysis. It was processed into 5 µm thick sections and stained for Haematoxylin and Eosin (H&E) to demonstrate the general histopathology of the liver. Verhoeff-Van Gieson stain (VVG) was used to demonstrate elastic fibres while Masson Trichome (MT) stain was used in the detection of collagen fibres in tissues.

Immunological Determination of Inos: The sections were incubated with proteinase K (0.02 mg/ml of 10 mm Tris, pH 8) for 20 mins for antigen unmasking and then with 1.5% normal goat serum to the slides for iNOS for 30 min at room temperature to block non-specific binding. IgG fractions purified from rabbit polyclonal antibody against rat liver iNOS) was applied in phosphate-buffered saline containing 0.1% bovine serum albumin overnight at room temperature. The slides were subsequently incubated with biotinylated secondary antibodies and avidin-biotin-peroxidase complex in Elite ABC kit according to the manufacturer's instructions. Binding was visualized by incubation with 0.06% diaminobenzidine (Sigma D5637) dissolved in tap water containing 0.01% H_2O_2 for 3-5min. The nuclei were counterstained with hematoxylin.

Photomicrography: Slides were viewed using LEICA DM 750 microscope connected to a digital camera (LEICA ICC50) (Olympus, New Jersey, USA) and a desktop computer.

iNOS Reactivity Quantification: ImageJ software was used for the stain intensity. Three different regions of the liver tissue on the immunohistochemical slides were focused under the microscope and photomicrographs were taken which were later imported into the ImageJ software after setting scale. The RGB-stack option was adopted under the image icon and then it was adjusted and threshold was clicked followed by measure. This generated the data (mean areas) in micrometer that was then analyzed using graph pad prism.

Statistical Analysis: Results were presented as Mean ± SEM and analyzed using descriptive and inferential

statistics. One-way ANOVA was used to analyze data, followed by Student Neuman-Keauls (SNK) test for multiple comparisons. GraphPad Prism 5 (GraphPad Software, CA, USA) was the statistical package for data analysis and *p*-values $\leq$ 0.05 were considered statistically significant.

RESULTS

Body and Liver Weight--Effect of APAP and ACA treatment on the body weight changed: Figure 1 shows the result of the body weight change. There is a significant ($p<0.05$) increase in the body weight change of APAP and the groups treated with graded doses of vit C following APAP intake.

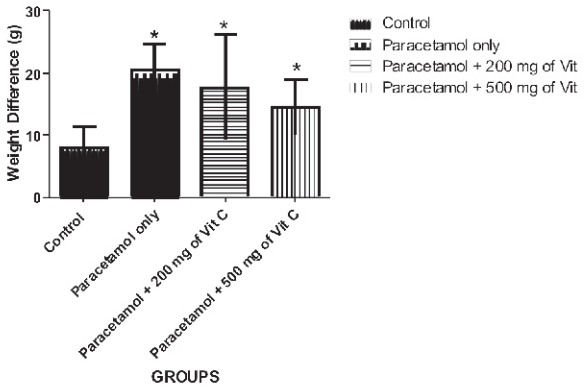


Figure 1: Effect of APAP and ACA treatment on the body weight change in APAP-induced toxicity in rats. Data are expressed as mean ± SEM (n=5) and analyzed by One-way ANOVA followed by Student Neuman-Keauls test for each parameter separately.*$p<0.05$ as compared to control group.

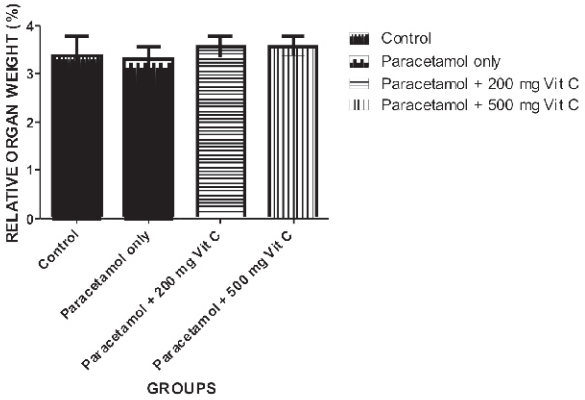
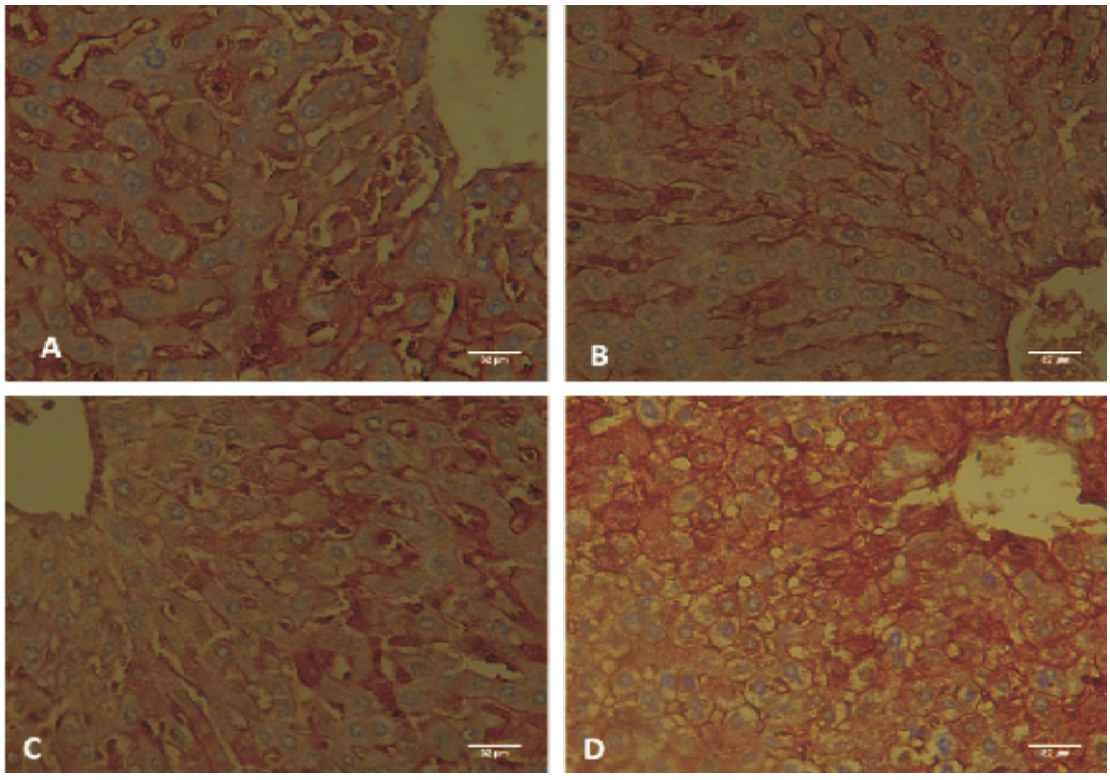


Figure 2: Effect of APAP and ACA treatment on relative liver weight in APAP-induced toxicity in rats. Data are expressed as mean ± SEM (n=5) and analyzed by One-way ANOVA followed by Student Neuman-Keauls test for each parameter separately.



Photomicrograph of the liver. Inducible nitric oxide synthase antibody for Immunohistochemistry, Scale bar is 32µm.

Plate 1: From the photomicrograph, there is the evidence of positive reactivity of iNOS across the groups as indicated by the brownish colorations on the hepatocytes, Kupffer cells and the sinusoids. Though the intensity appears very high in group D when compared with other groups. However, the interesting aspect of this result is the positive reactivity of the control group.

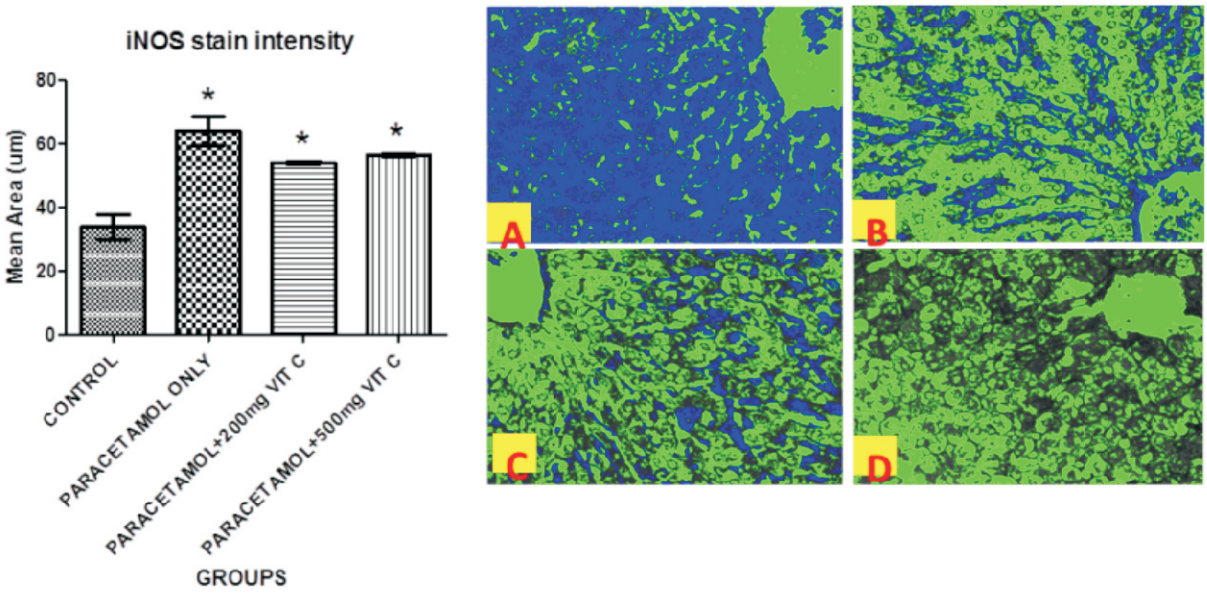
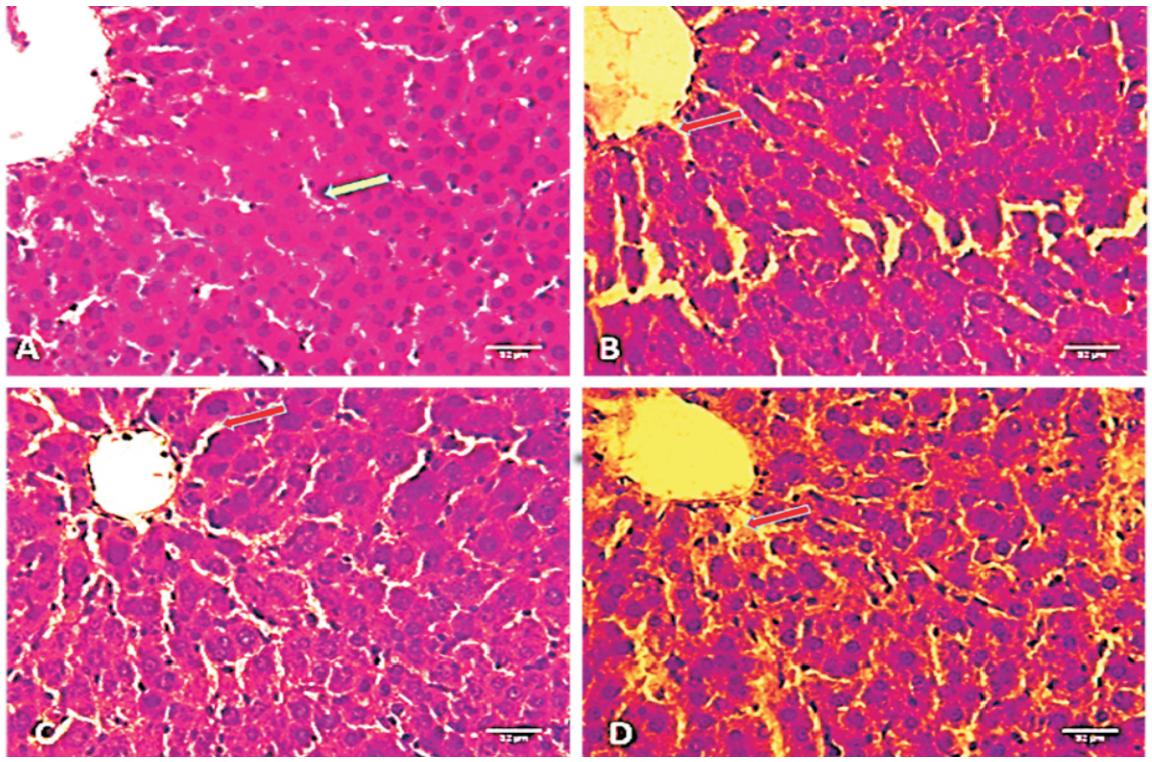
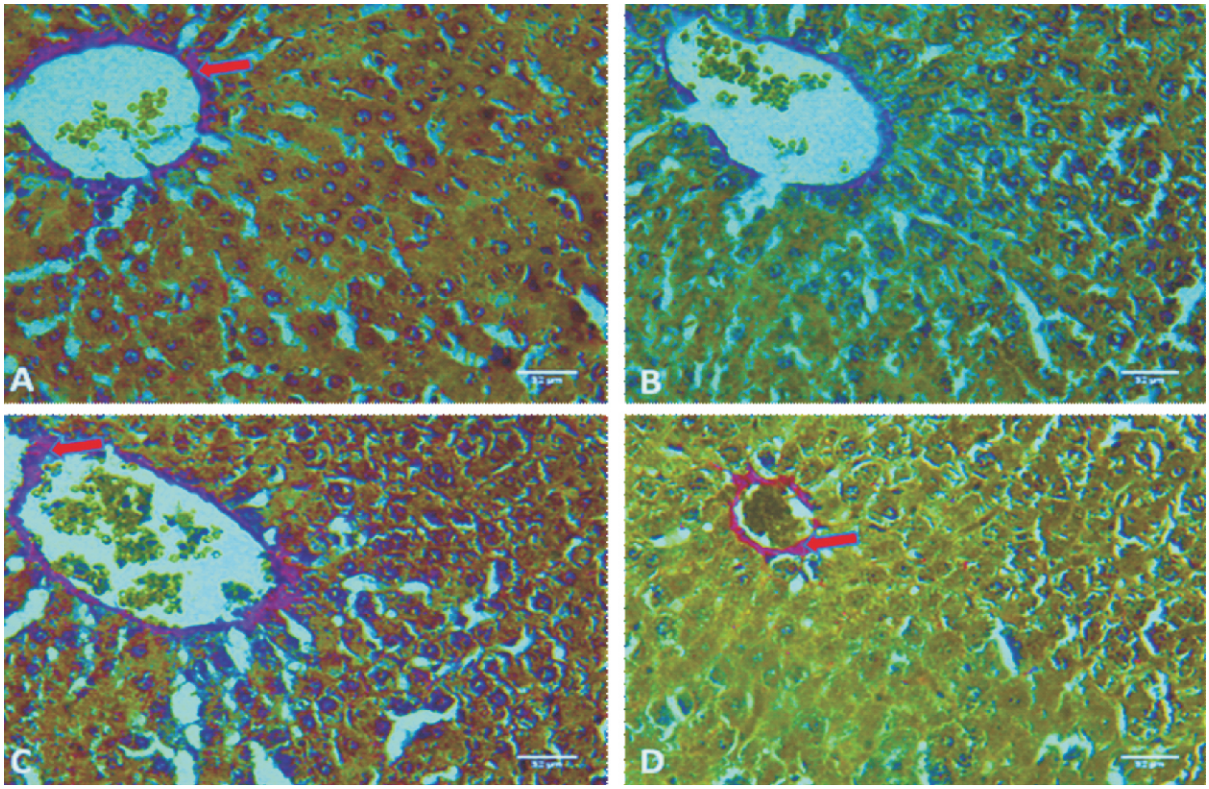


Figure 3: Quantification of iNOS reactivity using ImageJ software. Data are expressed as mean ± SEM (n=5) and analyzed by One-way ANOVA followed by Student Neuman-Keauls test for each parameter separately.*p<0.05 as compared to control group



Photomicrograph of the liver. Group A shows normal histology of the central vein and the hepatocytes (yellow arrow). Groups B, C and D however show some signs of dilated sinusoid (red arrows) when compared with the control group. The hepatocytes also show some level of necrosis (mild). H & E, Scale bar is 32µm.

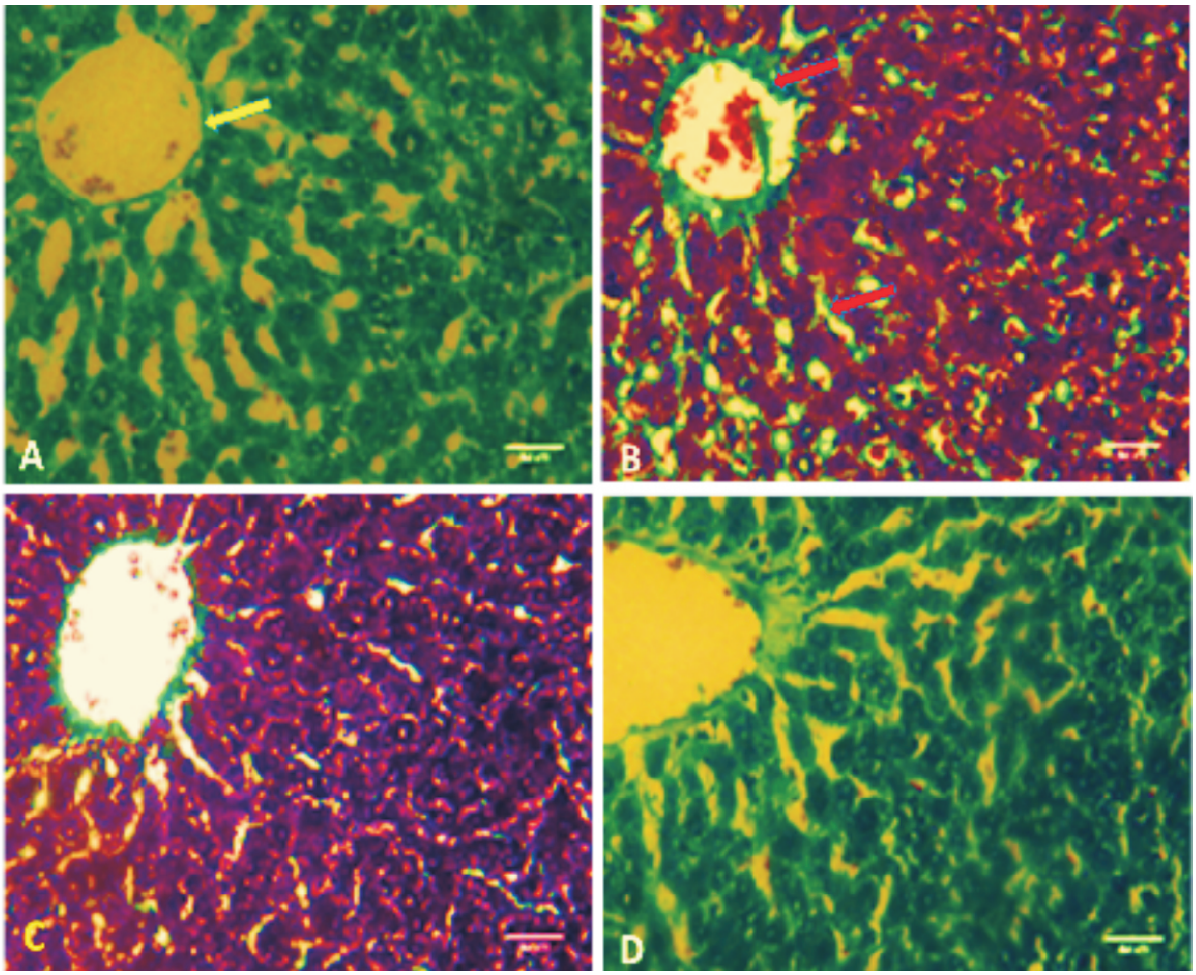
Plate 2: This plate shows the demonstration of general histopathology of the liver.



Photomicrograph of the liver. Group A shows normal distribution of collagen fibres around the central vein (red arrow). Groups B however shows poorly stained and distributed collagen fibres around the central vein and along the radiated sinusoids. Group C and D also show densely stained collagen fibres around the vein (red arrows). Masson Trichrome, Scale bar is 32µm.

Plate 3: This plate shows the effect of APAP and vit C treatment on the distribution of collagen fiber in the liver tissue.

Verhoeff's van-Gieson stain-- Plate 4: This plate shows the effect of APAP and vit C treatment on the distribution of elastic fiber in the liver tissue.



Photomicrograph of the liver. Group A shows normal distribution of elastic fibres especially around the edges of the central vein (yellow arrow). Groups B however shows more of the elastic fibres both around the central vein and along the radiated sinusoids (red arrows). Group C on the other hand shows poorly stained elastic fibres content. Group D also present the same feature as the control group. Verhoeff's van Gieson, Scale bar is 32µm.

Plate 4: This plate shows the effect of APAP and vit C treatment on the distribution of elastic fiber in the liver tissue.

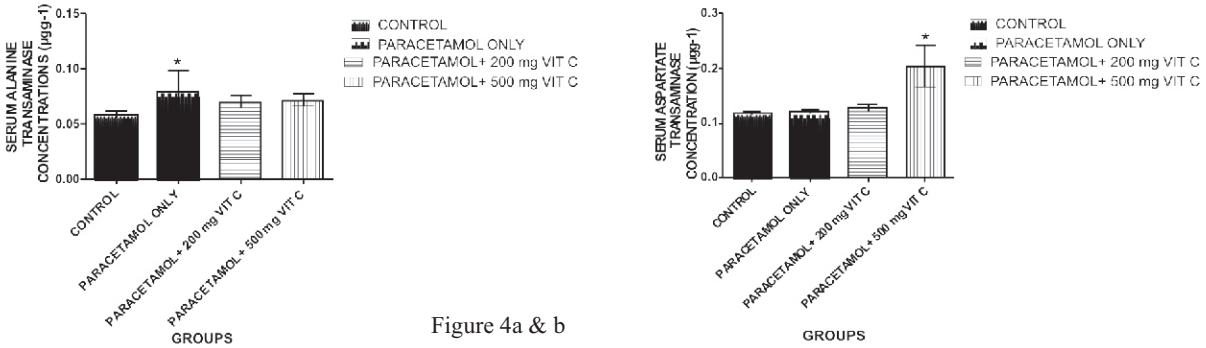


Figure 4a & b

Figure 4a&b: Effect of APAP and ACA treatment on the levels of ALT and AST in APAP-induced toxicity in rats. Data are expressed as mean ± SEM (n=5) and analyzed by One-way ANOVA followed by Student Neuman-Keauls test for each parameter separately.*p<0.05 as compared to control group.

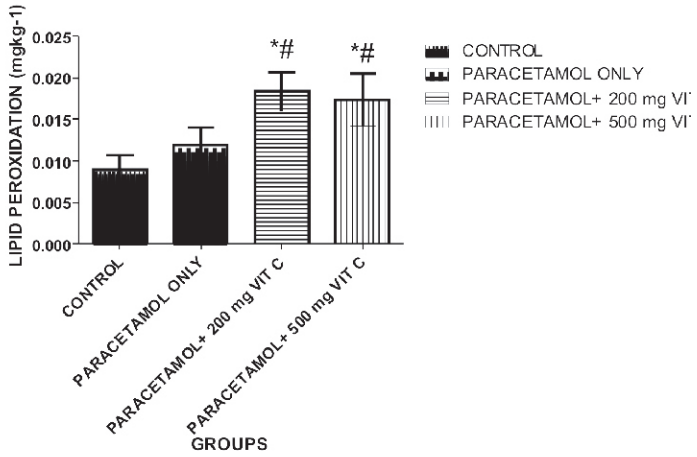


Figure 5: Effect of APAP and ACA treatment on the levels of TBARS in APAP-induced toxicity in rats. Data are expressed as mean ± SEM (n=5) and analyzed by One-way ANOVA followed by Student Neuman-Keauls test for each parameter separately.*p<0.05 as compared to control group, #p<0.05 as compared to APAP.

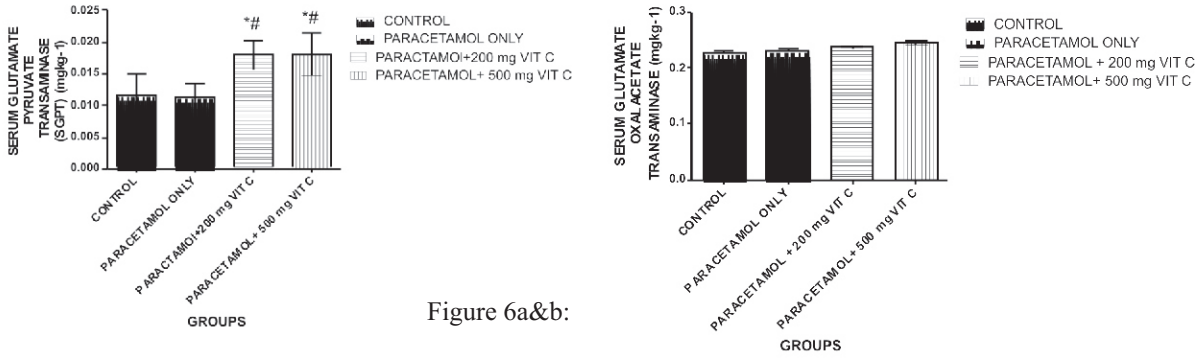


Figure 6a&b:

Figure 6a&b: Effect of APAP and ACA treatment on the levels of **SGPT and SGOT** in APAP-induced toxicity in rats. Data are expressed as mean ± SEM (n=5) and analyzed by One-way ANOVA followed by Student Neuman-Keauls test for each parameter separately.*p<0.05 as

DISCUSSION

iNOS was immunohistochemically demonstrated on the liver of APAP-induced hepatotoxicity followed by ACA treatment in this present study. iNOS is not a constitutive enzyme and its expression may be induced by stimuli such as lipopolysaccharide (LPS) or proinfiammatory cytokines (i.e. tumor necrosis factor alpha (TNF-a), interleukin-1, and interferon-g). Unlike the other two isoforms of nitric oxide synthases i.e neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) which are ubiquitous in many tissues, iNOS was first found in macrophages but has been identified in other cell types (e.g. hepatocytes)[21]. iNOS has been shown to play a role in the pathophysiology of hepatic failure[22]. However, it's role in liver fibrosis formation is still contestable as results from *in vivo* studies have shown both beneficial and deleterious outcomes[17-18,23]. Our result shows a mild positive reactivity in the control group and a significant positive reactivity in APAP and ACA treated groups (Figure 3 and Plate 1). This is difficult to understand as iNOS is expected to be expressed majorly in APAP group due to induced oxidative stress and presence of free radicals. However, it was also expressed in a mild way in the control group and this is in tandem with previous report that normal subjects had slight iNOS expression in Kuppfer cells and hepatocytes as found in fuminant hepatic failure patients[22]. This poses a puzzle indeed; suggestive that iNOS may actually have both positive and negative roles in both normal and damaged liver tissues. Anavi *et al* (2015)[24] have reported lower levels of liver fibrosis in iNOS-deficient mice and in mice with pharmacological inhibition of iNOS, given high cholesterol diet for 6 weeks which is expected. However, and interestingly, they found that despite a decrease in liver fibrosis, α-smooth muscle actin (α-SMA) levels were not reduced in comparison with control animals. Indicating that iNOS could have a role in the late phase of rat liver stellate cell line (HSC-T6) activation, during perpetuation, rather than in the activation step. Demonstration of elastic fiber using Verhoeff's stain showed that there is a dense distribution of this fiber in the liver of APAP group and the group that received low dose of ACA (group C) whereas, elastic fibers were only demonstrated around the rims of the central veins in the control and the group treated with higher dose of ACA (group D) (Plate 4). In the same vein, only group D did not show much deposition of collagen in the sinusoids and hepatocytes when compared with the control and the other treated groups (Plate 3). General histology however shows that group D is severely distorted when compared with others as demonstrated by H & E stain (Plate 2).

As a complementary but integral to any liver research, we also assayed for the basic liver function markers that helped to detect oxidative stress. Liver serum enzymes are biochemical markers of liver injury and the elevations of these markers are usually associated with impaired liver function[25]. Our result shows a significant increase in the level of ALT in APAP group when compared to the control and the other treated groups which is in agreement with previous reports[26]. Schellman, (2001)[27], had reported that paracetamol toxicity can likely generate free radicals hence; the elevated level of ALT. ACA was able to reduce the level of ALT as shown in the figure (Figure 4) indicating it ability to mop up the free radicals in the blood stream of the animals. This is not so for AST as it was up regulated in the group that was treated with the higher dose of ACA suggesting that ACA at higher dose can also induce oxidative stress. ALT is localized primarily in the liver whereas AST is found in a wide variety of other tissues, thus, ALT is considered the most reliable marker of hepatocellular injury as disease of skeletal and cardiac muscles, kidneys and the brain can equally elevate circulating levels of AST[28]. Lipid peroxidation has been reported to be up-regulated as well in liver damage associated with necrosis[26]. Result from the present study shows an increase in the level of lipid peroxidation which kept on increasing significantly in the ACA treated groups (Figure 5). This shows that ACA was unable to correct the increased level in the liver. As shown in figure 6, there is a significant increase in the level of SGPT in the ACA treated groups when compared with APAP and

the control. This follows the same pattern as ALT. However, there is no significant difference in the level of SGOT across all the groups.

CONCLUSION

The outcome of the present investigation indicates that iNOS is not only expressed in damaged tissues, as other underlying processes could lead to its expression in normal tissue pointing to a probable beneficial role. Also, ACA was instrumental in ameliorating APAP liver damage in dose-dependent manner (low dose in particular) through its antioxidant activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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