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Comparing Effects of N-Acetylcysteine, Carvedilol and Losartan against Paracetamol -Induced Hepatotoxicity in White Albino Rats

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Abstract: Hepatotoxicity induced by paracetamol is one of the most serious health problems. The present study aimed to evaluate and compare the protective effects of N-acetylcysteine (NAC), carvedilol and losartan on paracetamol-induced hepatotoxicity in rats. Thirty male adult albino rats were randomly divided into five groups, six animals each. Group I (control) was received distilled water orally. Groups II, III, IV and V were orally received; distilled water (hepatotoxic group), NAC (300 mg/kg/day), carvedilol (10 mg/kg/day) and losartan (10 mg/kg/day) respectively for 7 consecutive days. After an overnight fasting, the last four groups were received single oral dose of paracetamol (2 g/kg). At the end of the experiment, serum was separated for estimation of liver functions; serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total and direct bilirubin and albumin levels, as well as serum tumor necrosis factor- alpha (TNF-α). Moreover, liver homogenate was used for estimation of hepatic malondialdehyde (MDA) and glutathione (GSH) levels. On the other hand, histopathological examination of the liver tissues was performed. Rats pre-treated with NAC, carvedilol and losartan showed significant improvement in liver functions, significant decrease in serum TNF-α and hepatic MDA levels, as well as a significant increase in hepatic GSH level compared to hepatotoxic rats. Histopathological examination strongly supported the results of the biochemical tests. The present study demonstrated that NAC, carvedilol and losartan could be considered as a hepatoprotective against paracetamol- induced hepatic toxicity due to their anti- inflammatory effect and their abilities to reduce lipid peroxidation and enhance the antioxidant defense status.

Keywords: Hepatotoxicity**,** Paracetamol, N-acetylcysteine, Carvedilol, Losartan, Oxidative stress, Tumor necrosis factor-alpha.

1 Introduction

Liver is the major organ responsible for detoxification in the body. However, continuous exposure to certain drugs can trigger liver injury and eventually lead to various liver diseases [1]. Susceptibility of the liver to injury is much higher than any other organ because of its significant role in metabolism [2].

Paracetamol is one of the regularly used analgesics and antipyretics that can be obtained without a prescription. It is a safe drug when used at therapeutic levels. However, studies showed many disadvantages of this drug including the most serious one, centrilobular necrosis with an overdose leading to liver failure [3,4]. Much of the therapeutic dose of paracetamol is directly conjugated with glucuronic acid or sulfate and then excreted. The remaining part is metabolized by the cytochrome P450 enzymes to produce the reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which reacts with glutathione (GSH) and then eliminated by the kidney. Therefore, the earliest effect of paracetamol over dose is an intensive depletion of hepatocellular GSH. Once GSH is depleted, any remaining NAPQI will react with anther targets, particularly cellular and mitochondrial proteins leading to liver damage and necrosis [5,6]. Moreover, excess production of NAPQI lead to stimulation of the pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-1beta, which

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in turn reinforces tissue necrosis [7]. N-Acetylcysteine (NAC) is a standard antidote for the prevention of paracetamol hepatotoxicity [8]. It is a thiol containing antioxidant which acts as a direct scavenger of free radicals [9]. As a source of sulfhydryl group NAC consequentially promotes GSH biosynthesis and re-establishes the intracellular GSH concentration which is reduced during oxidative stress and inflammation [10]. Moreover, NAC inhibit the induction of pro-inflammatory cytokines and can also block TNF-α-induced apoptotic cell death [11].

Carvedilol, a non-selective β-blocker with α blocking ability, is a vasodilating agent used in treatment of hypertension and congestive heart failure. It has a greater benefit as an antioxidant, anti-inflammatory and anti-fibrotic agent in cardiac and hepatic studies [12, 13]. The anti-fibrotic effect of carvedilol is related to improvement of oxidative stress in carbon tetrachloride-induced hepatotoxicity [13].

Losartan is the first discovered angiotensin II type 1 receptor blocker. It has a protective effect in carbon tetrachloride-induced hepatic fibrosis through reducing serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and hepatic hydroxyproline. It also inhibits histopathological fibrotic changes and decreased TNF-α and TGF-β1 levels in culture supernatants of Kupffer cells [14, 15]. Moreover, losartan decreases the expression of the angiotensin II-activated NADPH oxidase in the inflammatory areas of the liver and consequently suppressed the oxidative stress [16].

Since NAC is the standard clinical antidote for paracetamol- induced hepatic toxicity. Evaluation of a potential antidote, such as carvedilol and losartan, would necessitate a comparison to NAC. The aims of the present study are to determine the protective effect of NAC, carvedilol and losartan on paracetamol induced hepatotoxicity in rats and to compare the efficacy among them.

2 Materials and Methods

2.1 Materials

Paracetamol, carvedilol and NAC were purchased from AK Scientific, Inc. (USA). Losartan was purchased from Sigma-Aldrich (USA). TNF-α kits was purchased from Wuhan EIAab Science Co. Ltd (China). Kits for determination of oxidative stress markers, Malondialdehyde (MDA) and GSH were purchased from Bio-diagnostic Company, Egypt. Kits for measuring liver functions were obtained from Egyptian Company for Biotechnology, Cairo, Egypt.

2.2 Animals

Male white albino rats (n=30, weight=150-200g) were purchased from the animal house, Faculty of Medicine, Sohag University, Egypt. They were housed in animal facility, Faculty of Medicine, Sohag University, maintained in a controlled environment under standard conditions of temperature (25±2°C) and a time controlled system provided 12 hours light/dark cycle. All rats were given ad libitum access to rodent chow diet.

This study was executed according to the Institutional Animal Care and Use guidelines of the Faculty of Medicine, Sohag University, Egypt. Animal handling and experimental protocol was approved by the Research Ethical Committee of Faculty of Medicine, Sohag University, Egypt (Approval No. 2/2019).

2.3 Experimental Protocol

Rats were left for one week-acclimatization period and then divided into five groups, six animals each. Both group I (control group) and group II (hepatotoxic group) received distilled water orally (p.o.) by gastric tube daily for seven days. Group III received NAC at the dose of 300 mg/kg (p.o.) [17]. Group IV received carvedilol at the dose of 10 mg/kg (p.o.) [18] Group V received losartan at the dose of 10 mg/kg (p.o.) [14]. All test agents were prepared freshly in distilled water and given to the rats daily for seven days. After an overnight fasting, animals in groups II, III, IV and V received paracetamol (2 g/ kg; p.o.) [19] for the induction of hepatotoxicity. Twenty-four hours later, animals were sacrificed. Blood samples were collected from jugular vein and centrifuged at 3000 rpm for 20 min for serum separation.

The liver from all groups was removed and washed with ice-cold saline solution. Part of liver was used for measurement of oxidative stress parameters (MDA and GSH), and the remaining part was used for the histopathological examination.

Livers from all groups were homogenized in 5-10 ml cold buffer (100mM potassium phosphate, pH 7.4, containing 2mM EDTA) per gram tissue by using motor driven homogenizer. The homogenates were centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was used for the assay of MDA and GSH.

2.4 Biochemical Estimation

2.4.1 Determination of Liver Functions

Serum ALT, AST, bilirubin (total and direct) and albumin levels were assessed spectrophotometrically (Jenway 6051 colorimeter spectrophotometer).

2.4.2 Determination of Serum TNF-α Level

Serum TNF-α was measured using sandwich enzyme linked immunosorbent assay (ELISA) kits. The concentration of TNF-α in the samples was determined by comparing the O.D. of the samples to the standard curve using ELISA

microplate reader (AWARENESS Stat Fax- 2200, USA) at 450 nm. TNF-α level was expressed as pg/mL.

2.4.3 Determination of Hepatic MDA and GSH Levels

Malondialdehyde level is an indicator of lipid peroxidation. MDA in the liver tissue homogenate was measured by a colorimetric method, as described by Ohkawa et al [20]. This assay based on the reaction of thiobarbituric acid with malondialdehyde in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product. The absorbance of the resultant pink product was measured at 534 nm. MDA level was expressed in nmol/g tissues.

Hepatic GSH was measured in liver homogenate by a colorimetric method, as described by Beutler et al [21]. The method depends on the reduction of 5,5' dithiobis (2-nitrobenzoic acid) with glutathione to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm. GSH level was expressed in mg/gm tissues.

2.5 Histopathological Examination

For all groups, samples of liver tissue were excised, fixed in 10% formal saline, and dehydrated in ascending grades of ethanol, cleaned in xylene and embedded in paraffin. Liver tissue was sectioned (5 µm thick) and stained with hematoxylin and eosin (H&E).

2.6 Statistical Analysis of Data

Results were expressed as means±standard error (SE) of the mean. The significance of difference between different groups was analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test to judge the difference between groups, using SPSS program (version 20). The difference was regarded as significant when $p<0.05$.

3 Results

3.1 Effect of NAC, Carvedilol and Losartan on Liver Functions

Table (1) showed that administration of paracetamol in a single dose of 2 g/kg; p.o. caused hepatotoxicity in the rats indicated by a significant increase ($p<0.05$) in serum ALT, AST, and total and direct bilirubin levels compared to the control group. Daily oral administration of NAC, carvedilol and losartan significantly decreased the levels of these hepatotoxic biomarkers compared to hepatotoxic group ($p<0.05$). Moreover, carvedilol treatment restored serum transaminases levels back to their normal levels.

Furthermore, paracetamol administration significantly decreased the serum albumin level compared to the control group ($p<0.05$). Daily oral administration of NAC, carvedilol and losartan significantly increased the serum albumin level compared to hepatotoxic group ($p<0.05$). Moreover, carvedilol again seemed to be the most efficient, as serum albumin level was restored back to normal levels.

There was no significant difference between NAC, carvedilol and losartan regarding their effects on hepatotoxicity markers, a few differences were observed but they were negligible.

Table 1: Effect of oral administration of NAC, carvedilol and losartan on liver functions

Parameters	Control group	Hepatotoxic group	NAC treatment group	Carvedilol treatment group	losartan treatment group
Serum ALT (U/L)	38.17± 0.79	112.33± 2.91*	41.50± 0.76 [a]	38.33±0.67 [a]	43.17±1.01 [a]
Serum AST (U/L)	35.17± 0.48	277.00± 3.37*	43.33± 0.88 [a]	35.50± 0.43 [a]	45.67± 0.49 [a]
T.Bil (mg/dl)	0.73± 0.06	2.75± 0.08*	0.79± 0.04 [a]	0.76± 0.06 [a]	0.78± 0.03 [a]
Direct. Bil (mg/dl)	0.10± 0.02	0.48± 0.02*	0.18± 0.01 [a]	0.13± 0.01 [a]	0.15± 0.01 [a]
Albumin (g/dL)	3.35± 0.08	1.83± 0.09*	3.15± 0.07 [a]	3.35± 0.04 [a]	2.97± 0.18 [a]

Values are expressed as Mean±SE, (n = 6), statistical analysis was carried out by ANOVA followed by Tukey post hoc test., NAC= n-acetylcysteine, ALT= alanine aminotransferase, AST= aspartate aminotransferase, T.Bil=total bilirubin.
*Significantly different from the control group at p<0.05,
[a] Significantly different from hepatotoxic group at p<0.05.

3.2 Effect of NAC, Carvedilol and Losartan on Serum TNF-α Level

Single oral dose of paracetamol led to a significant increase in the serum TNF-α level ($p<0.05$) compared to the control group and daily oral administration of NAC, carvedilol and losartan significantly decreased serum TNF-α level ($p<0.05$) compared to hepatotoxic group (Fig.1). As with the hepatotoxicity markers no significant difference present among the three pretreated groups.

3.3 Effect of NAC, Carvedilol and Losartan on Hepatic MDA and GSH Levels

Single oral dose of paracetamol led to a significant increase in the hepatic MDA level ($p<0.05$) compared to the control group and daily oral administration of NAC, carvedilol and losartan significantly decreased the hepatic MDA level ($p<0.05$) compared to hepatotoxic group (Fig.2). In contrast, single oral dose of paracetamol led to a significant decrease in the hepatic GSH level ($p<0.05$) compared to control group and daily oral administration of NAC, carvedilol or losartan significantly increase hepatic GSH level ($p<0.05$) compared to hepatotoxic group (Fig.3). In addition, NAC treatment restored GSH level back to its normal levels.

No significant difference emerged among three groups (Groups III, IV and V), as the differences observed were again negligible.

3.4 Effect of NAC, Carvedilol and Losartan on Hepatic Histopathological Changes

Liver of control group showed normal hepatic architecture (Fig. 4A). In hepatotoxic group, liver showed severe histopathological alteration appearing as irregularly dilated and congested central vein with massive inflammatory cells and activated Kupffer cells. Hepatocytes showed cellular degeneration with derangement in the hepatic plates. Moreover, there are dilated congested sinusoids as shown in Figure 4(B, C).

Liver of rats received NAC for 7 consecutive days prior to paracetamol dose, showed normal looking of the hepatic tissue but with minimal congestion in central vein (Fig. 4D). While liver of rats received carvedilol for 7 consecutive days prior to paracetamol dose showed normal structure of the hepatic tissue but with mild degeneration in hepatic cells (Fig. 4E). As for liver of rats received losartan for 7 consecutive days prior to paracetamol dose, the hepatic tissue structure appears normal but with minimal congestion in some sinusoids (Fig. 4F).

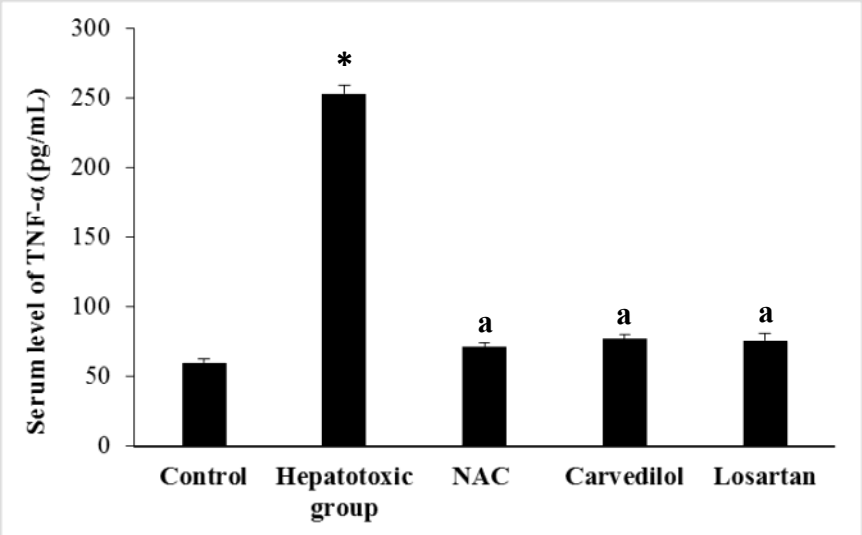

Fig. 1: Effect of oral administration of NAC, carvedilol and losartan on serum TNF-α level.

Values are expressed as Mean±SE, (n = 6), statistical analysis was carried out by ANOVA followed by Tukey post hoc test. NAC= n-acetylcysteine, TNF-α= tumor necrosis factor-alpha.
*Significantly different from the control group at $p<0.05$,
a Significantly different from hepatotoxic group at $p<0.05$.

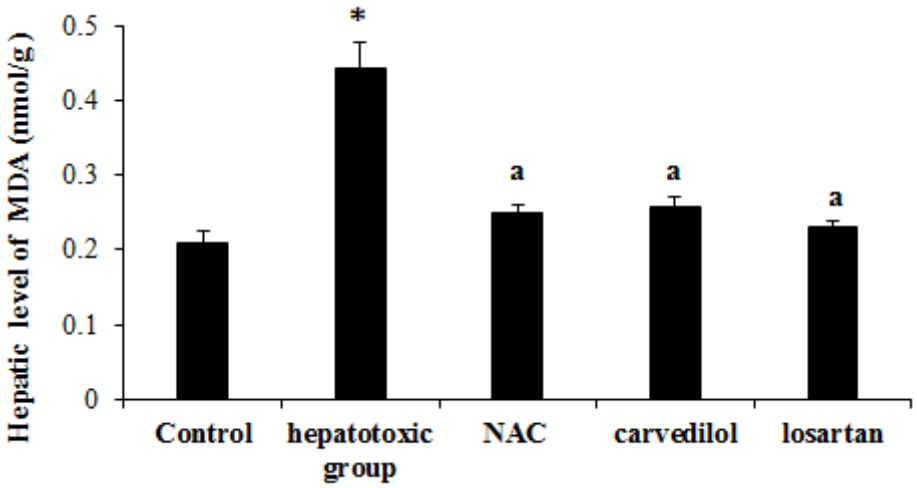

Fig. 2: Effect of oral administration of NAC, carvedilol and losartan on hepatic MDA level.

Values are expressed as Mean±SE, (n = 6), statistical analysis was carried out by ANOVA followed by Tukey post hoc test. NAC= n-acetylcysteine, MDA=malondialdehyde.
*Significantly different from the control group at $p<0.05$,
[a] Significantly different from hepatotoxic group at $p<0.05$.

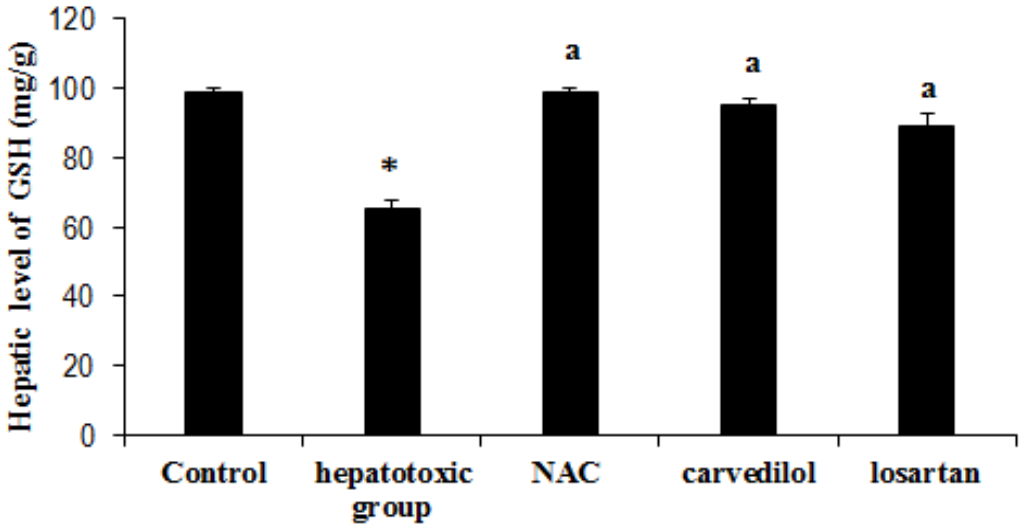

Fig.3: Effect of oral administration of NAC, carvedilol and losartan on hepatic GSH level.

Values are expressed as Mean±SE, (n = 6), statistical analysis was carried out by ANOVA followed by Tukey post hoc test. NAC= n-acetylcysteine, GSH=reduced glutathione.
*Significantly different from the control group at $p<0.05$,
[a] Significantly different from hepatotoxic group at $p<0.05$.

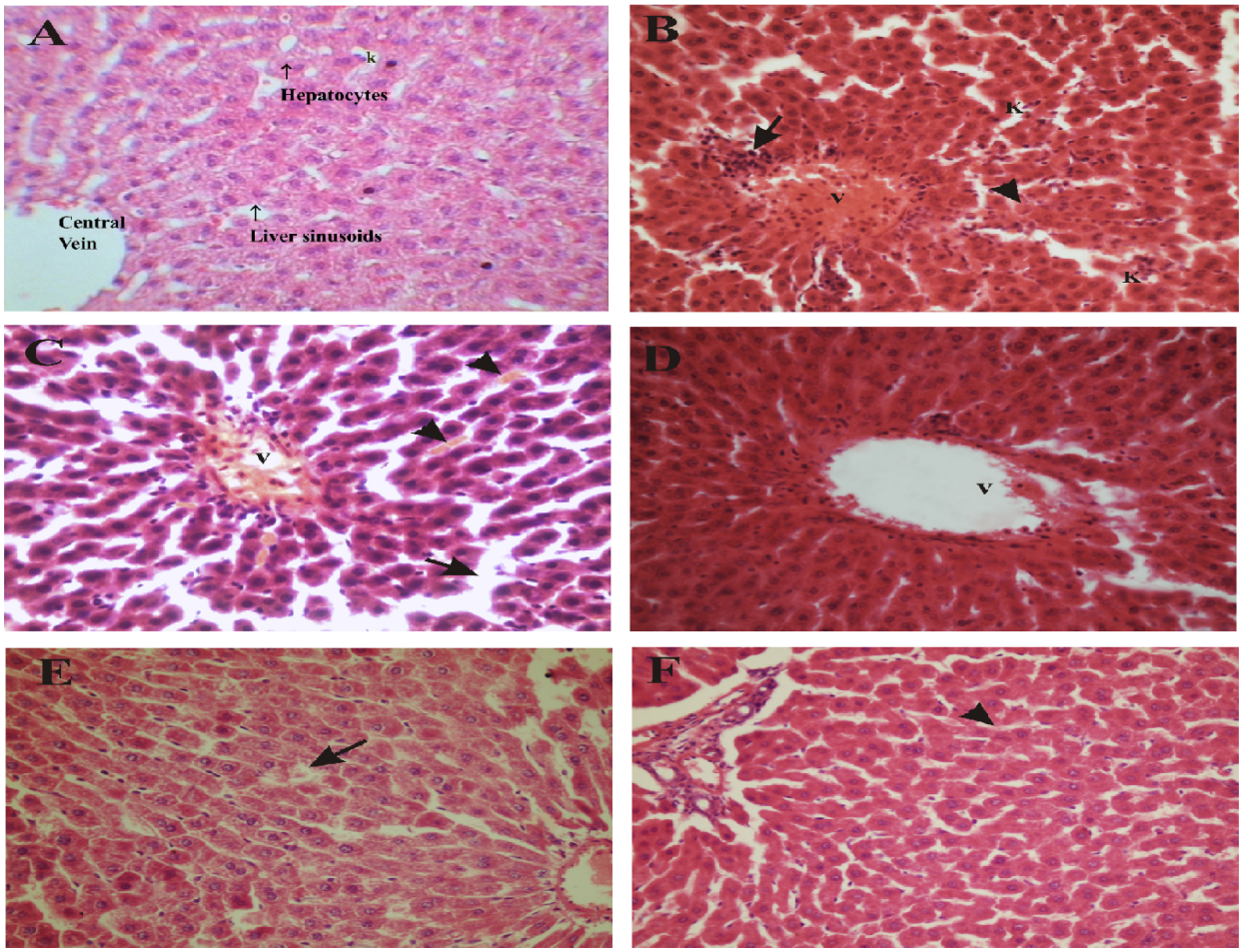

Fig. 4: Photomicrographs of the liver tissue showing effects of oral administration of n-acetylcysteine, carvedilol and losartan on paracetamol -induced hepatotoxicity.

- **A.** Liver tissue of the control group showing normal hepatic architecture, regular hepatic plates, hepatocytes have vesicular nuclei and acidophilic cytoplasm (H&E x400).
- **B.** Liver tissue of the hepatotoxic group showing severe histopathological alteration; appearing as irregularly dilated and congested central vein (**V**) with massive inflammatory cells (**arrow**) and activated Kupffer cells (**K**). Hepatocytes showed cellular degeneration (**arrow head**) (H&Ex400).
- **C.** Liver tissue of the hepatotoxic group showing severe histopathological alteration; appearing as derangement in the hepatic plates (**arrow**), dilated congested sinusoids (**arrow head**) (H&Ex400).
- **D.** Liver tissue of the n-acetylcysteine group showing normal looking of the hepatic tissue but with minimal congestion in central vein (**V**) (H&Ex400).
- **E.** Liver tissue of the carvedilol group showing normal looking of the hepatic tissue but with mild degeneration in hepatic cells (**arrow**) (H&Ex400).
- **F.** Liver tissue of the losartan group showing normal looking of the hepatic tissue but with minimal congestion in some sinusoids (**arrow head**) (H&Ex400).

4 Discussion

In the present study, single oral dose administration of paracetamol caused acute liver damage to the rats as evidenced by significant increase in serum ALT, AST and both total and direct bilirubin levels coupled with a significant decrease in the serum albumin level. Similar results were previously reported [22,23,24,25]. ALT and AST are cytoplasmic liver enzymes which are released into the blood after hepatic cell damage, and their marked

leakage into the circulation is indicative of severe damage to the membranes of the hepatic tissue during paracetamol intoxication [26]. Furthermore, the higher level of AST compared to ALT is a confirmation of the involvement of not only the cytosol but also the mitochondria where AST occurs in both compartments [27].

Bilirubin is one of the most paramount clinical indicators to the severity of hepatic damage and its significant increase is a measure of binding, conjugation and excretory capacity of hepatocyte [28].

Albumin is the most considerable serum protein produced in the liver and transferred to the blood. Any damage to the liver decreases albumin production and hence, its serum level, which indicates that the synthetic function of the liver to make albumin and transfer it to the blood has been disturbed [29].

N-acetylcysteine, carvedilol and losartan pretreatment reduced paracetamol -induced hepatotoxicity, because the ALT, AST, both total and direct bilirubin levels and serum albumin level were restored to nearly the control levels. However, there was insignificant difference between them, but the positive effect of carvedilol was much higher than that for the other medications. Therefore, the ability of NAC, carvedilol and losartan to prevent the disturbance in the activities of the previous parameters is the primary evidence of their hepatoprotective activity. Even though we have no reported experimental study about losartan as a hepatoprotective agent against paracetamol induced hepatotoxicity. Losartan was reported to have hepatoprotective effect in CCl4-induced hepatotoxicity [14].

Tumor necrosis factor-α, another factor contributing to liver damage, is a pleiotropic proinflammatory cytokine rapidly produced by macrophages in response to tissue damage [30]. In the present study, inflammation was initiated by paracetamol-induced hepatotoxicity resulted in a significant increase in the serum levels of TNF-α. This result is in harmony with finding reported by Mahmoud et al [31]. This finding may be due to activation of kupffer cells by paracetamol overdose and oxidative stress leading to increased release of such pro-inflammatory cytokines [32].

In the present study, it was found that NAC, carvedilol and losartan significantly decreased serum TNF-α level. NAC has an anti-inflammatory effect through reducing nuclear factor-kappa B (NF-κB) activation which in turn decreases the overproduction of TNF-α and the expression of inflammatory mediators [33]. Moreover, carvedilol significantly decreased serum level of TNF-α which may be due to the suppressing effect of carvedilol on its mRNA expression and protein production [34]. Angiotensin II produces massive proinflammatory effects in the liver [35]. In particular, angiotensin II activates intracellular signaling pathways leading to increased expression of inflammatory cytokines [36]. Furthermore, the pro-inflammatory properties of angiotensin II are mediated by AT1R and these effects are markedly blunted by AT1 receptor blockers [37].

Lipid peroxidation is closely related to paracetamol induced tissue damage and MDA is a good indicator of the degree of lipid peroxidation. In the present study, there was a significant increase in the hepatic MDA level of the hepatotoxic group compared to the control group. This observation agrees with other previous studies [38,39].

Furthermore, in case of paracetamol toxicity, the increased production of NAPQI depletes GSH and inhibits its synthesis [5] may explain the significant reduction in hepatic GSH level in the present hepatotoxic group compared to the control group. These findings are in agreement with other studies [25,31,40]. However, the present findings are in disagreement with a study done by Betto et al [41], who investigated effects of treatment with enalapril on hepatotoxicity induced by paracetamol in mice, showing a significant increase of hepatic GSH concentrations after paracetamol administration. Betto et al [41] explained the increase in GSH levels as an attempt to restore the lost oxidative balance, following the high production of NAPQI under acute liver injury.

In the present study, the increased hepatic MDA level in hepatotoxic group was significantly inhibited in all three drug groups. Moreover, it was found that the depletion in hepatic GSH level due to paracetamol in hepatotoxic group was significantly prevented by pretreatment with NAC, carvedilol and losartan. In addition, NAC able to restore GSH level back to its normal levels. No statistically significant differences existed among three drug groups.

The ability of the studied drugs to prevent paracetamol-induced oxidative stress can be attributed to their antioxidant constituents and confirmed the fact that disturbances of oxidant/antioxidant level in hepatic tissue underlies acute paracetamol hepatotoxicity [13,42,43].

N-acetylcysteine diminishes hepatic paracetamol toxicity by increasing glutathione levels and reducing the extent of hepatic glutathione depletion [44]. Glutathione is critical for conjugation with the reactive metabolite of paracetamol and treatment with NAC allows generation of sufficient levels of glutathione to react with the toxic metabolite (i.e. NAPQI) and prevent hepatic necrosis [45].

The antioxidant effect of carvedilol is related to the carbazole moiety in its structure and it is approximately 10-fold more potent as an antioxidant than vitamin E [46]. Moreover, the antioxidant effect of losartan was related to the ability of its metabolite EXP3179 to inhibit phagocytic NADPH oxidase–dependent O_2^- production [47].

The present biochemical findings were strongly supported by the histopathological changes in the liver of the hepatotoxic group, where central vein appeared irregularly dilated and congested with massive inflammatory cells and activated Kupffer cells. Hepatocytes showed cellular

degeneration with derangement in the hepatic plates with dilated congested sinusoids. These results are remarkably similar to those observed by Betto et al [41] which showed necrosis in the hepatocytes accompanied by centrilobular degeneration of liver tissues in paracetamol treated group. The present study showed that administration of NAC, carvedilol and losartan greatly protect the hepatic tissue from the effect of paracetamol by showing nearly normal histological structure of hepatic tissues. Such histopathological findings support the biochemical findings and could be due to the anti- inflammatory and the antioxidant activity of the studied drugs that minimize the histopathological alterations and restore the normal physiological state of liver.

5 Conclusions

Based on our results, the study confirm that NAC is the standard treatment in the paracetamol- induced hepatic toxicity, but the studied drugs (carvedilol and losartan) have the same effect and may have the same role in the prevention of paracetamol -induced hepatic toxicity and their role at any rate as important as n-acetylcysteine. Carvedilol and losartan could protect against paracetamol-induced hepatic toxicity by their anti- inflammatory effect and their abilities to reduce lipid peroxidation and restore hepatic GSH level and normalization of the histopathological abnormalities.

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