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Paracetamol Overdose Induces Acute Liver Injury accompanied by oxidative stress and inflammation

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Abstract
The concept of paracetamol as a safe drug has become very misleading as this has led to a high rate of paracetamol toxicity. Hepatotoxicity and liver failure have been reported even with doses just more than the maximum therapeutic dose, which was obviously noticed in the (COVID-19) pandemic. Oxidative stress plays an important role in paracetamol hepatotoxicity. The current study investigates the mechanism of action through which paracetamol induces hepatotoxicity and implements an alarming sign for the unsupervised use of paracetamol. Twenty albino rats were equally divided into a normal control group and paracetamol treated group where rats received paracetamol at a dose of 2g/kg b.wt once orally for 24 hours. Oral administration of paracetamol resulted in a significant elevation of liver enzymes in serum such as glutamate pyruvate transaminase and glutamate oxaloacetate transaminase when compared with the results of the control group. In terms of oxidative stress biomarkers, the group that received an overdose of paracetamol showed a significant increase in the tissue level of 4-Hydroxynonenal accompanied by a significant decrease in the activity of the anti-oxidant markers Paraoxonase and Catalase. Histopathological examination revealed focal necrosis in the hepatocytes, Centri-lobular necrobiotic changes, and dilated congested portal vein. Immunohistochemical investigation for the Nuclear factor-kappa B showed strong positive expression in the nuclei of the hepatocytes of rats that received an overdose of paracetamol. Our study suggests that an overdose of paracetamol could attenuate the endogenous antioxidant defense mechanisms and augment the hepatic tissue inflammation; both factors may contribute to the observed increase in apoptosis-related signaling and cell death.
Keywords: Hepatotoxicity; Paracetamol; Oxidative stress; NF-κB

Introduction

Paracetamol, also known as acetaminophen, N-acetyl-p-aminophenol (APAP), P- acetamido phenol or it is N-acetyl P-aminophenol is one of the most commonly used medications. It is available over the counter both as a single-entity formulation and in combination with other medications, as well as by prescription when combined in various quantities with opioids. While paracetamol is a safe and effective drug at recommended doses, it has the potential for causing hepatotoxicity and acute liver failure (ALF) with overdose [1].

Paracetamol hepatotoxicity is still a significant public health concern and a common indication for urgent liver transplantation, it is the most common cause of ALF in the United States of America and the United Kingdom [2]. According to the primary Poison Control Center at Ain Shams University Hospitals (PPCASUH) in Egypt, "paracetamol is one of the top ten most frequently involved exposure substances received in PCC-ASUH during 2019". There's a great growing concern about paracetamol toxicity as it has been considered, according to World Health Organization (WHO) pain ladder, to be the first-line antipyretic and analgesic for COVID-19 patients. [3]. When paracetamol is consumed at therapeutic doses, the majority (80%–90%) is conjugated with glucuronic acid or sulfate and excreted through the kidneys. A minor component is acted upon by cytochrome P450 enzymes such as Cyp2E1 and

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Cyp1A2 to form a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) [4]. In paracetamol overdose, NAPQI is produced in excess of glutathione (GSH) detoxification capacity, and only part of it can be detoxified by conjugation with GSH. The remaining part of NAPQI subsequently binds to liver proteins and induces oxidative stress, mitochondrial dysfunction, and necrotic cell death. Oxidative stress is recently reported to play a major role in paracetamol hepatotoxicity [5]**.** paracetamol-mediated hepatotoxicity is closely related to oxidative stress, inflammatory response, and apoptosis. Excessive paracetamol exposure can cause mitochondrial dysfunction and severe energy debt which induces reactive oxygen species (ROS) and further damage to hepatocytes [6]**.**

4-Hydroxynonenal (4-HNE) derives from oxidation of membrane *n*-6-polyunsaturated fatty acids (PUFAs), essentially arachidonic acid and linoleic acid, two of the most important fatty acids in membranes. HNE has been commonly acknowledged as an inducer and mediator of oxidative stress. The toxicity of aldehydes results from alterations in a number of cell functions which predominantly depend on the formation of covalent adducts with cellular proteins [7].

PON (paraoxonase) is a calcium-dependent serum esterase and a member of a multigene family with at least three types. PON1 is mainly expressed in the liver, PON1 plays an important role as an endogenous free-radical scavenging molecule [8].

Catalase (CAT) is present in all body cells, and it can be found inside peroxisomes, organelles which are mostly active in hepatocytes. This enzyme avoids excessive accumulation of hydrogen peroxide (H_2O_2), a toxic oxidizing agent similar to NAPQI, as it breaks it down to water and oxygen [9].

Nuclear factor kappa B (NF-κB), is a key mediator of proinflammatory signaling pathways triggered by cytokines, both directly by deacetylation and indirectly through the induction of antioxidant systems that prevent ROS-induced NF-κB nuclear translocation [10]. The current study aimed to investigate the mechanism through which paracetamol induces hepatotoxicity and it alarms about evident risk of unsupervised use of paracetamol.

Materials and methods:

Experimental Animals

This experimental study was conducted at the National Research Centre (NRC), Giza, Egypt. The animal study protocol was reviewed and approved by the ethical committee, NRC. Ethical approval code [19-017]. Animal study protocols followed the guidelines of the NRC animal research ethics committee. Rats were kept in merit conditions, including 12 hours in the dark and 12 hours in light, and a temperature of 22 ± 2 °C. Rats had free access to water and a standard pellet diet. The animals were acclimated under these conditions for two weeks before the start of the study.

Experimental Design

After the acclimatization period, Twenty albino rats were randomly assigned into two groups; Group (I) Normal control; healthy rats received saline, and Group (II) Paracetamol treated group; healthy rats received Paracetamol (Acetaminophen) A5000, which meets USP (United States Pharmacopeia) testing specifications, at a dose of 2g/kg b.wt once orally for 24 hours [11]. Paracetamol was purchased from Sigma Aldrich, 98.0-102.0% powder.

At the end of the experiment, rats were sacrificed (after 24 hours of paracetamol administration), and blood samples were freshly obtained from the retro-orbital venous plexus of the eye using capillary tubes. Blood samples were centrifuged at 3000 rpm for 10 min, and the serum was separated and stored at -20°C for biochemical analysis. Liver tissue was removed, quickly washed with cold saline and was plotted on a filter paper to be divided into two parts; first part was homogenized to be used for biochemical analysis, and the other part was fixed in 10% formalin saline for pathological and immunohistochemical examination.

Biochemical analysis:

1. **Determination of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT)**:
   Levels of liver enzymes (GPT and GOT) are detected using the colorimetric method previously described by Huang et al. [12]. Serum GPT and GOT levels were measured using glutamate dehydrogenase using glutamate amount formed in 20 µl serum incubated for 45 min at 37°C. The dehydrogenation of glutamate reduces the diazonium salt, which is estimated at absorbance 520 nm.
2. **Determination of 4-Hydroxynonenal (4-HNE) "oxidative stress marker":** 4-HNE is one of the most prevalent and active lipid peroxides, generated from the peroxidation of n-6 polyunsaturated fatty acids such as arachidonic and linoleic acids. The serum level of 4-HNE was detected by ELISA kit (BioVision, China, Catalog #: E4645) according to the manufacturers' protocols.
3. **Determination of Paraoxonase (PON) activity "anti-oxidant marker":** The activity of the PON1 enzyme was evaluated using phenylacetate as a substrate to determine arylesterase activity. Briefly, the arylesterase activity was measured using 1 mmol phenylacetate (Aladdin, Beijing, China) as the substrate in 20 mmol/l Tris-HCl buffer (pH 8.0) with 1 mmol/l CaCl2; the activity was expressed as U/l.
4. **Determination of liver catalase activity:** The functional catalase activity of the tissue lysates

was estimated by a colorimetric method using Bio- Diagnostic kit (Giza, Egypt, CAT. No# CA 25 17).

Histopathological and immunohistochemical examination

Liver tissue samples were removed, washed with saline, and excised into small tissue sections. Liver sections were dried using filter paper and preserved in 10% formaldehyde, and then embedded in paraffin blocks. Preserved liver tissue in paraffin was cut into 5µm thick sections and stained with hematoxylin-eosin for histological examination by light microscope.

For immunohistochemical examination, liver tissue sections cut from the formalin-fixed paraffin-embedded blocks were mounted onto positively charged adhesive slides. The tissue slides were subject to immunohistochemical staining, including deparaffinization, rehydration, blocking, and staining with an NF-κB/p65 polyclonal antibody. Stained tissue sections were visualized using a streptavidin-horseradish peroxidase technique. Tissue sections were examined, and images were captured by light microscopy. For detection of NF-κB expression we used NF-κB/p65 polyclonal antibody (Thermo Fischer Scientific, Catalog # 51–0500).

Statistical Analysis

Results of the present study are presented as mean ± SD. Statistical analysis was assessed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences with p values < 0.05 were considered statistically significant.

Results

1) Biochemical analysis

Liver function: Liver function tests were done to rats in both groups and they indicate acute liver insult as the levels of plasma markers, including GOT and GPT, were significantly increased in the paracetamol group compared with the control group, as shown in Fig. (1).

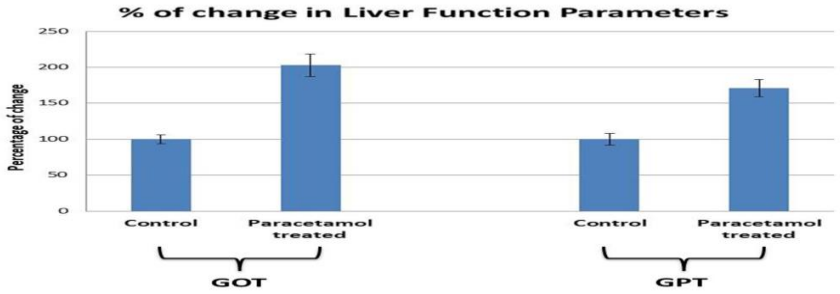

Figure (1): The percentage of change in liver function parameters between control and paracetamol group.

1) 4-HNE(4-Hydroxynonenal):

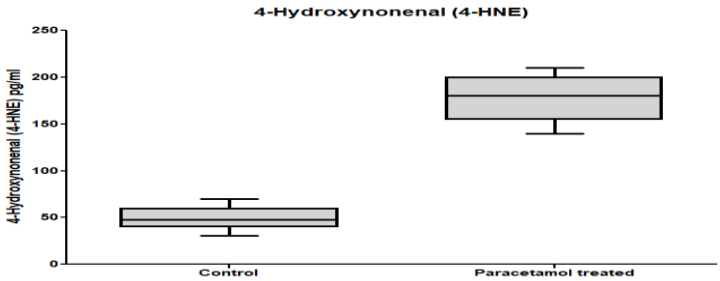

Figure (2): Level of 4-Hydroxynonenal (pg/ml) in control and paracetamol treated group. Data expressed as mean ± S.D. A significance difference (P-value ≤ 0.05) is detected between the two group.

2) Paraoxonase (PON) activity:

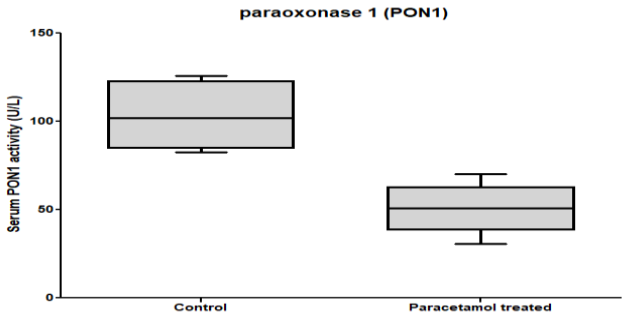

Figure (3): Paraoxonase activity (U/L) in control and paracetamol treated group. Data expressed as mean ± S.D. A significance difference (P-value ≤ 0.05) is detected between the two group.

3) Catalase

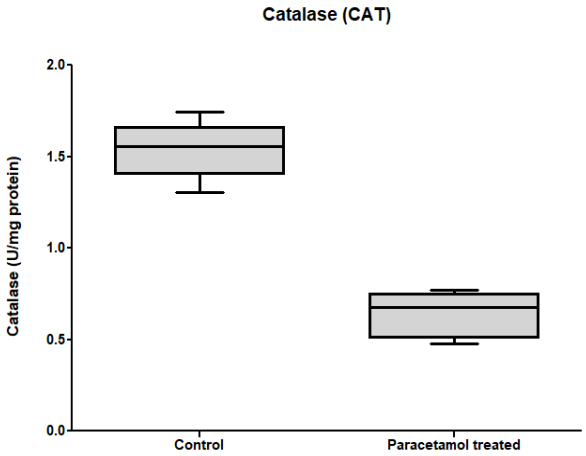

Figure (4): Catalase activity (U/mg protein) in control and paracetamol treated group. Data expressed as mean ± S.D. A significance difference (P-value ≤ 0.05) is detected between the two group.

Table (1): Shows the statistical difference between the means in both control group and paracetamol group as regard the oxidative stress marker (4HNE) and anti-oxidant markers (PON and CAT)

Parameters	Control group Mean ± SD	Paracetamol group Mean ± SD	P value
4-Hydroxynonenal (4HNE)	**50.06 ± 13.05**	**176.29 ± 26.61**	**0.000***
Paraoxonase (PON)	**103.5 ± 19.2**	**50.74 ± 14.37**	**0.001***
Catalase (CAT)	**1.53 ± 0.16**	**0.65 ± 0.12**	**0.000***

As shown in table (1), there are significant statistical differences between the control group and the paracetamol group regarding the oxidative stress markers and the anti-oxidant markers, with a P-value ≤ 0.001 (using T-test).

II. Histopathological examination (H&E stain)

Control Group

There was no histopathological alteration and the normal histological structure of the central vein and surrounding hepatocytes in the parenchyma were recorded in (Fig.5).

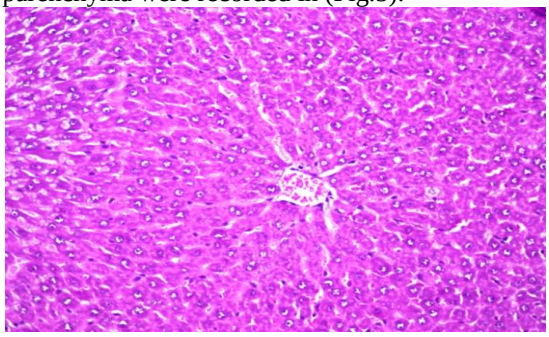

Figure (5): Shows normal hepatic histology

Paracetamol Group

Centri-lobular necrobiotic changes were detected in the hepatocytes surrounding and adjacent the dilated central vein (Fig.6). The hepatic parenchyma showed focal necrosis in the hepatocytes (Fig.7). There was vacuolar degeneration in diffuse manner all over the hepatocytes in the parenchyma (Fig.8). The portal area showed dilatation and congestion in the portal vein with thickening in the surrounding area of the bile ducts (Fig.9).

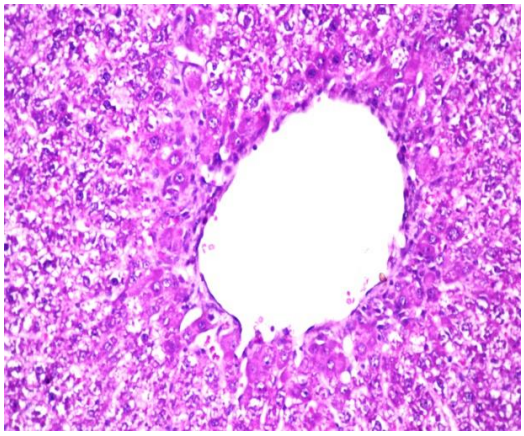

Figure (6): Shows the centrilobar necrobiotic changes

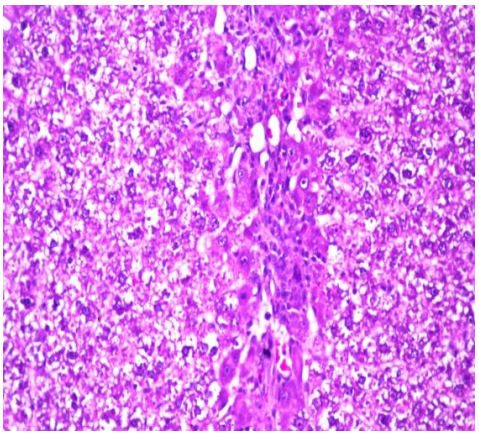

Figure (7) shows the hepatic parenchymal focal necrosis

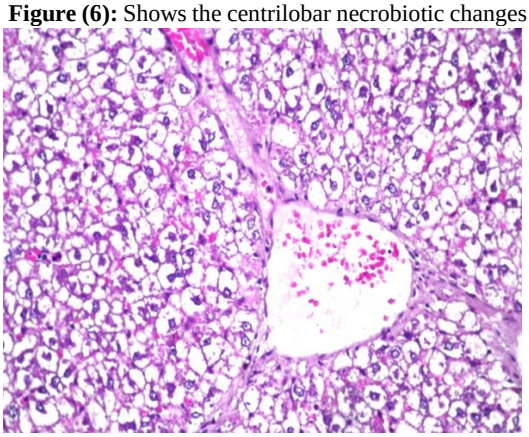

Figure (8): shows vacuolar degeneration

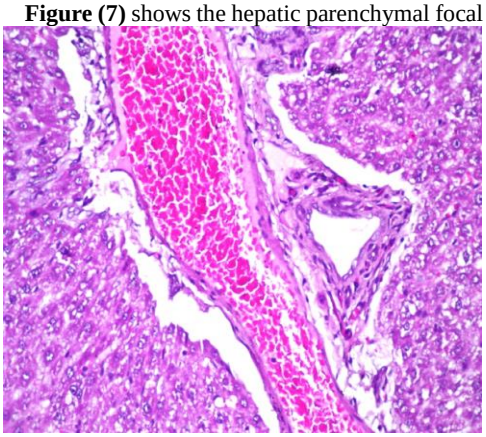

Figure (9): shows portal dilatation and thickening

III. Immunohistochemical examination for NF-κB/p65

Liver of rats served as control group showed negative expression in hepatocytes (Fig.10)., while liver of rats in paracetamol group showed strong positive expression in the nuclei of hepatocytes (Fig.11).

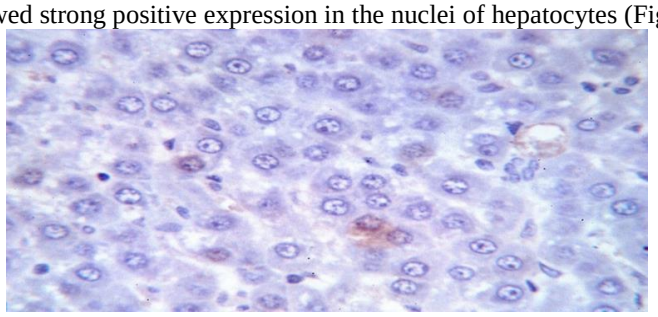

Figure (10): Normal control group showing negative expression for hepatocytes

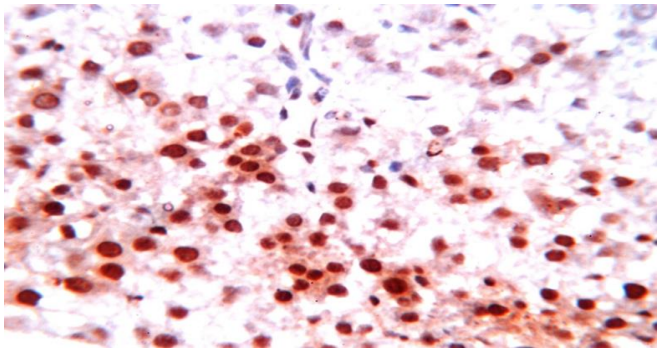

Figure (11): Paracetamol group showing positive expression for NF-κB/p65 in hepatocytes nuclei

Discussion:

Paracetamol is widely used as an analgesic and anti-fever drug globally. Although, hepatotoxicity induced by an overdose of paracetamol is a common cause of acute liver failure. The oxidative stress is the major reason of liver injuries. Excessive oxidative stress and inflammation induced by paracetamol are the main causes of acute liver failure [13].

In the current study, paracetamol overdose induced deterioration of the liver function, which was manifested as a significant statistical elevation of serum GOT and GPT activity in the paracetamol group of rats in comparison with the control group, this agreed with Bouhlali et al [14]. study that reported paracetamol-induced hepatotoxicity revealed a significant increase in serum levels of GPT and GOT. Bessems and Vermeulen explained the effect toxic paracetamol dose that leads to consumption of the stored GSH and sulfate through converts excess level of paracetamol to the CYP-450 oxidase system, which will lead to formation of more reactive intermediate (NAPQI), that will make bonds to protein macromolecules intracellularly leading to hepatic cells injury [15]**.** This mechanism leads to initiation of programmed cell death (apoptosis), which leads to hepatic necrosis and dysfunction in the form of elevation of GPT and GOT [16].

Another study was held by *Allam et al* to investigate the paracetamol toxic effects upon the liver and oxidative stress, they found out that there was a significant statistical increase in liver function as serum GPT and GOT in paracetamol group compared to the control groups [17]. Also, they showed that toxicity of paracetamol resulted in significant decline in CAT activity as compared to control group.

Moreover, induction of oxidative stress and increased ROS formation, after reduced glutathione depletion and diminished endogenous antioxidation mechanisms represent the mainstay of paracetamol-induced liver injury [18]. In this study, 4-HNE as an oxidative stress marker was significantly elevated, the anti-oxidant marker CAT, PON1 was significantly reduced in the paracetamol group compared to the control group.

4-HNE a major end-product of free-radical activated peroxidation of polyunsaturated fatty acids. It is more stable than free radicals and can diffuse within the cell or leave it and react with targets far from the initial site. These reactive aldehyde species are considerably reactive, producing multiple intra- and inter-molecular covalent adducts with biomolecules such as proteins, DNA and phospholipids. It can propagate and amplify oxidative injury and promote mitochondrial dysfunction and cell death [7].

Studies have confirmed that oxidative stress is the central mediator of paracetamol-induced hepatotoxicity **[**19**].** paracetamol-induced liver injury activates biochemical signaling pathways that originate mainly from CYP2E1-mediated formation of the reactive metabolite NAPQI [20**].** It is well known that CYP2E1 has the greatest effect on acute hepatotoxicity caused by paracetamol, which is a potent inducer of 4-HNE lipid peroxide. Therefore, we evaluated the oxidative stress injury caused by an overdose of 4-HNE expression [21]. When the equilibrium between the created reactive oxygen species (superoxide anions, hydrogen peroxide and hydroxyl radicals) and antioxidants is disturbed, the free radicals react with macromolecules leading to disruption of cell functions [22]**.**

PON1 has esterase and lactonase activities and protects against xenobiotics. Reduction mechanism of serum PON1 activity in paracetamol toxicity and can be driven by several factors. First, antioxidant action of PON1 is associated with enzyme inactivation; through this process, free sulfhydryl group of PON1 reacts with specific oxidized lipids, and finally PON1 is inactivated probably due to invasion of free radicals (ROS). Other mechanism associated with reduced PON1 activity can be due to suppressed synthesis of enzyme caused by genetic defects or may arise from down regulation of transcription in liver [23].

Decreased serum PON1 enzyme activity may have a role in the etiopathogenesis of acetaminophen intoxication, and the increased susceptibility to oxidative stress was observed in acetaminophen intoxication [8]. This comes in harmony with our study that showed decrease in PON1 activity within paracetamol group.

CAT is a highly efficient enzyme; it is conceivable that the moderate increase in its activity following paracetamol exposure may offer a much-enhanced protection against ROS generated in ischemia/reperfusion injury and prooxidant challenge [24]. CAT is one of the most essential enzymes which enhance oxygen metabolism [25]. Catalase is suppressed by extra superoxide radical and lipid peroxides **[**26**]** and [27].

The accumulation of ROS is the main cause of oxidative stress. The levels of ROS accumulation, antioxidant factors and oxidative stress factors were measured to assess the state of oxidative stress induced by paracetamol and emphasize that the antioxidant enzymes were significantly lower in the paracetamol group [28].

These results went in hand with what ***Ge Z. and his co-worker*** concluded, as they tested the levels of oxidative stress in liver tissues. Levels of catalase, and GSH were both reduced after treatment with paracetamol [29]. Their results also concur with our study results as regard the histopathological findings and acute liver insults in the paracetamol administrated group of rates.

In light of previous studies, mitochondrial oxidative stress is regarded as the dominant cellular event in paracetamol-induced acute hepatotoxicity [30][31].

Also, the obtained findings are reinforced by ***Yousef et al.*** who stated that administration of paracetamol produced significant lowering of antioxidant enzyme; CAT in rat plasma [32]. That also goes in harmony with ***Pradhan and his co-workers,*** who stated that paracetamol overdose led to a significant decline in the CAT level in plasma [33].

The histopathological findings in our current study showed, centri-lobular necrobiotic changes in the hepatocytic surrounding and adjacent to the dilated central vein, also the hepatic parenchyma showed focal necrosis in the hepatocytes. There was also vacuolar degeneration in diffuse manner all over the hepatocytes in the parenchyma, and the portal area showed dilatation and congestion in the portal vein with thickening in the surrounding area of the bile ducts. This pathological alterations in liver tissue sections was similar to the finding of **Du et al. and Tsuji et al.** research work that indicates effect of paracetamol on liver toxicity [34][35].

Previous research had indicated that paracetamol-induced hepatocyte apoptosis also plays an important role in the pathogenesis of acute liver injury [36][37].

There are two predominant theories for paracetamol to cause cell death:

1. First one is the theory of oxidative stress where paracetamol metabolites induce oxidative stress in the target cell which leads to its death. Lipid peroxidation which is generated by ROS is thought to be a major cause of disruption to cell membranes [38].
2. The other one is the theory of covalent binding where highly reactive metabolites of paracetamol bound to cellular macromolecules causing death of the cell [39].

The main finding recorded in paracetamol toxicity is liver centrilobular necrosis. The explanation for that finding could be that the central zone liver cells are rich in CYP2E1, so they are most susceptible to injury by paracetamol toxicity [40]**.**

These findings were in accordance with ***Abdel Zaher and his colleges*** results. Where there was marked vacuolar degeneration and moderate centrilobular necrosis in liver histopathological examination following ingestion of paracetamol overdose [41]. The present study findings were agreed by ***Mahmoud et al.,*** they noticed centrilobular necrosis, vacuolar degeneration in a moderate manner and infiltration of inflammatory cell in liver microscopic examination when paracetamol was administered to the rats with a dose of 3 g/kg orally (acute overdose) [42]. Also, ***Allam et al,*** concluded that paracetamol induced marked dilatation and central vein congestion and congestion of the portal venules, and marked hepatocytic vacuolar degeneration [17].

In the present study, APAP-induced hepatotoxicity was accompanied by an enhanced inflammatory response as illustrated by the increased expression of the NF-κB. Oxidative stress can upregulate pro-inflammatory gene expression and then inflammatory cells can trigger ROS overproduction, which would form a vicious circle and trigger the development of liver damage [43]**.** NF-κB is a major transcription factor, participating in immunity and inflammation processes, regulating apoptotic genes expression, and then causing apoptosis [44]. The NF-κB pathway is a critical signaling axis mediating the expression of inflammation-related mediators such as via NF-κB-binding motifs in their promoters [45]. The activation of the NF-κB protein has been previously reported to be associated with paracetamol misuse [46].

Previous literature reported that extracellular stimuli induced the rapid phosphorylation of I-κB (Inhibitory kappa B) and lead to the dissociation of NF-κB from I-κB [47]. Subsequently, activated NF-κB caused transcription of some inflammatory genes, including Tumor necrosis factor (TNF-α) and interleukin-1β [48, 49].

Our results go in hand with **Long and his colleges,** who conducted a study that states that APAP-treated mice displayed significantly increased hepatic NF-κB p65 expression when compared to the control [18]. Also, **Jiang et al,** concluded that the phosphorylation of NF-κB was increased by paracetamol treatment [28]. In agreement to our results **wang and his co-workers,** conducted their study on Male ICR mice (institute of cancer research mice) and found out that paracetamol overdose resulted in evidently higher levels of phosphorylated NF-κB [50].

Conclusion: Although paracetamol is considered a relatively safe drug, non-intentional misuse may result in mitochondrial dysfunction, oxidative stress, cellular necrosis, and apoptosis and eventually acute hepatic failure. Therefore, new strategies are recommended to healthcare providers to aware patients with all the needed information about paracetamol to lessen the possibilities of overdosage aimed to reduce drug induced hepatotoxicity.

Conflicts of interest:

There are no conflict of interests among authors.

Funding sources:

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