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ORIGINAL ARTICLE

Evaluation of the Efficacy of Glutathione Administration in Acetaminophen-Induced Hepatotoxicity in Experimental Rats

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KEYWORDS

GSH;
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Comet assay

ABSTRACT: One of the most common causes of acute liver failure is acetaminophen overdose. The antidote N-acetylcysteine acts by scavenging the reactive metabolite, but its therapeutic limitation necessitates the development of additional therapeutic approaches that can benefit late-presenting patients. Glutathione (GSH) is the most abundant intracellular nonprotein thiol that has an important role in the regulation of many cellular physiologic functions such as redox-homeostatic buffering. This study aims to evaluate the efficacy of GSH supplementation in the recovery of deteriorated liver functions in induced acute acetaminophen toxicity rats; in addition to determining its value in the preservation of DNA integrity in such toxicity**.** This experimental study was done on 36 albino rats which were divided into three groups (n=12 rats / group) as follows, group1: Control group, group 2: Acetaminophen (APAP) treated group, group 3: APAP and glutathione treated group. Each group was subdivided into 2 subgroups (n=6) and they were sacrificed at 12 hours and 24 hours sequentially. The extent of hepatic inflammation, oxidative stress, and DNA damage was evaluated using histopathological study, and comet analysis, and biochemical markers (ALT, GSH, and MDA). GSH supplementation (APAP and glutathione treated group) significantly improved liver functions resulting in; a statistically significant decrease in ALT levels, reducing Malondialdehyde (MDA) levels, and preserving DNA integrity. GSH is a highly effective alternative in the treatment of APAP hepatotoxicity.

INTRODUCTION

As an effective oral analgesic and has few side effects. Paracetamol is a famous worldwide OTC analgesic. Nevertheless, this medication has potentially fatal toxicity, and it is a well-known factor that contributes to acute liver cell failure in both the USA and UK [1]. Metabolism of therapeutic paracetamol dose is mainly by conjugation to be converted to inactive metabolites. The main problem is that when it is given or ingested in toxic doses, the liver's cytochrome P450 enzyme family oxidizes paracetamol to produce the poisonous reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI forms a covalent bond with the

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sulfhydryl groups that can be provided by glutathione. In case of depletion of the glutathione stores, NAPQI bonds covalently with proteins in the cells, and cell injury occurs that is mediated by free radicals. Following cell death, an inflammatory response occurs and that determines the prognosis of the case [2, 3]

Mitochondria play a crucial function in pathogenesis as the alkylation of proteins of mitochondria in a specific way starts the formation of the reactive oxygen species causing oxidative stress that leads to mitochondrial membrane permeability transition pores that disturb the mitochondrial membrane potential causing depletion to ATP and oncotic necrosis and cell death, in addition to alkylation of electron transport chain protein [4-6].

In addition, this permeability results in the mitochondrial release of endonuclease G. Nuclear DNA fragmentation is brought on by the endonuclease's translocation to nuclei. The effector caspase-3 cannot be activated by the apoptosome when ATP is depleted. Consequently, liver failure and centrilobular hepatocyte necrosis occur. [7].

Because of the important role of SH groups in the mechanism of paracetamol- induced hepatotoxicity, therapy is mainly directed to measures that can restore it. Acetylcysteine (also known as N-acetylcysteine) was proven to prevent hepatic injury. It can be used as an antidote for acetaminophen toxicity, it acts as a scavenger that forms an adduct with NAPQI to prevent its binding with the hepatic proteins [8]. Oral administration of N-acetylcysteine is not tolerated by the patient, that's why limits its use; the intravenous preparation can be used as it has fewer adverse effects and better to be tolerated. Important to be mentioned that only a few patients if professionally managed can still develop acute fulminant hepatic failure [9].

Glutathione has significant functions in biological processes like cellular growth, gene expression regulation, and apoptosis, antioxidant defense, DNA, and protein synthesis, signal transduction, and nutrient metabolism. Lack of glutathione causes oxidative stress, which can speed up aging and the pathogenesis of many diseases like liver disease, diabetes, and cancer [10].

Lipid peroxidation is the bad sequela due to increasing levels of oxidative species and the occurrence of what's called oxidative stress, it is a chain phenomenon that results in cellular damage. To monitor the occurrence of lipid peroxidation and monitor the prognosis of the case, Malondialdehyde (MDA) levels are used in different biological systems as an important biological indicator [11].

MDA level monitoring is used in studies for numerous diseases (e.g. diabetes, hypertension, atherosclerosis, heart failure, and different types of cancer), patients suffering from glaucoma, complex regional pain syndrome, and cancer, reported higher levels of MDA making it as a reliable biomarker in detecting the oxidative stress-related pathologies[12].

The comet assay (single-cell gel electrophoresis), which combines the single-cell approach of cytogenetic assays with the simplicity of biochemical techniques used for detecting DNA single-strand breaks and/or alkali-labile sites, is one of the most simple and useful techniques for studying DNA damage, and also repair [13].

The comet assay's simplicity, speed, and sensitivity in identifying DNA damage are further benefits, in addition to being used to analyze data at the cellular level, the sample needed is extremely small, and low cost of this assay is with reliable results. For all these reasons, the test is widely used in genotoxicity testing, DNA damage and repair investigations, monitoring the human population, and environmental biomonitoring [14].

Aim of the current study is to evaluate the efficacy of GSH supplementation in the recovery of deteriorated liver functions in induced acute acetaminophen toxicity rats; in addition to determining its value in the preservation of DNA integrity in such toxicity.

MATERIALS AND METHODS

Experimental animals

Thirty- six white Albino rats that were 6 weeks old and weighed between 120 and 150 grams were included in this study., the sample size was calculated using G power software, based on Bajt et al., 2003[15]. The power is 80% and α probability error is 0.05.

The handling and care of the animals complied with the guidelines for laboratory animals published by the US National Institute of Health (NIH publications No. 8023, amended 1978) and authorized by the faculty of medicine at Cairo University's animal research ethics committee.

Rats were cared for in a pathogen-free environment with a 12:12 hrs, light/dark cycle in an air-conditioned animal housing where they had unrestricted access to food and water. Every day, the behavioral and morphological changes in rats were observed; but no significant behavioral alteration or morphological changes in rats were observed.

Rats were divided into three groups:

1. Control group (n=12): rats were injected with only warm saline as a vehicle to serve as a control.
2. Acetaminophen (APAP) treated group (n=12): APAP was dissolved (15mg ml^{-1}) in warm saline and injected at (300mg kg^{-1}) [16].
3. APAP and glutathione treated group (n=12): Glutathione was injected after being dissolved in phosphate buffer saline (PBS) through an intravenous route once (200mg kg^{-1}) at 1.5h after APAP injection [15].

Each group was split into two smaller groups (n=6) and they were sacrificed at 12 hours and 24 hours sequentially.

Biochemical assays

Blood was drawn from the retro-orbital vein to evaluate plasma ALT level (enzymatic colorimetric method, Spectrum, Germany). Furthermore, glutathione peroxidase (Egale Bioscience Inc. Catalog Number: GLU39-K01) and lipid peroxidation (MDA) (Egale Bioscience Inc. Catalog Number: LIP39-K01) levels were assessed in all the studied groups.

Analysis of nuclear DNA and mitochondrial DNA:

Mitochondrial hepatic DNA was isolated with Biovision, Inc. (Life Science Company San Francisco) according to the manufacturer's recommendations. The genomic nuclear DNA was further isolated with Qiagen (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was assessed from absorption at 260 nm. To quantify mtDNA and nDNA, a standard microscope to assess apoptosis was used. After an initial layer of 0.5% normal agarose was applied to the slides, a washing buffer was used to extract and wash the lymphocytes. The slides were immediately covered with coverslips after a 50 l aliquot of the cell sample was added and 100 l of 0.5% low melting point agarose was added. All slides were placed in a lysing solution (2.5 M NaCl, 100 M EDTA, 10 M Tris, NaOH to pH 10, 1% N-Lauryl Sarcosine, to which 1% Triton X-100 and 10% DMSO were freshly added) for 1 hour at 4 C after the cover glass was removed.

The slides were put in an electrophoresis tank with newly made alkaline buffer (three hundred mM sodium hydroxide, 1 mM EDTA, pH > 13), and the electrophoresis was run for 20 min at 300 mA and 25 V at room temperature. The slides were removed from the tank after the electrophoresis stage and washed three times over, for five minutes with neutralizing buffer (pH 7.5, 0.4 M Tris).

Each was then washed with ethanol in order to perform fixation. Ethidium bromide was used to stain the DNAs (60 l of 20 l ml^{-1} solution). For each sample, two slides were made, and 50 randomly selected cells were counted using a Leica fluorescence

microscope and the Comet Assay automatic image analysis system. All results were evaluated in terms of nine image-analysis parameters [17].

Histopathology

In 40 g L^{-1} paraformaldehyde in PBS at 4°C, liver samples were collected and fixed overnight. Hematoxylin and eosin (H&E) were used to stain successive liver sections.

Statistical analysis

Using the statistical package SPSS version 22. Data were coded and entered into the program. Data were summarized using mean and standard deviation.

Analysis of variance (ANOVA) was used for comparisons where there were more than two groups to be compared, along with multiple comparisons post hoc test. Using a paired sample t-test, the two durations within the same group were compared. A value less than 0.05 is regarded as significant [18]. Significant change from the control group indicated by (*). Significant deviation from the APAP group is indicated by a (#).

RESULTS

GSH significantly improves hepatic function **in both 12hrs and 24hrs durations**

ALT level in the APAP group and the APAP+GSH group increased statistically significantly (p-value<0.001) when compared to the control group, and its level significantly decreased in the APAP+GSH group when compared to the APAP group (p-value = 0.01 12hrs and <0.001 in 24hrs) (Figure 1A). ALT level was significantly increased over time in the untreated APAP group and decreased in the GSH-treated APAP group (p value <0.001) (Figure 1B).

GSH significantly amelieorates hepatic oxidative stress induced by the APAP **in both 12hrs and 24hrs durations**

GSH level was decreased statistically significantly in the APAP group compared to the control group (p-value < 0.001), but there was no significant statistical difference between the APAP+GSH group and the control group (p-value = 0.5) in 12 hours duration only, But significant increase in GSH level in the APAP+GSH group when compared to the control group (p-value < 0.001). Statistically significant increase in GSH level in the APAP+GSH group compared with the APAP group (p-value < 0.001). (Figure 1c). The level of GSH was significantly elevated over time in GSH treated APAP group (p-value <0.001) (Figure 1D).

Regarding MDA level, MDA level in the APAP and the APAP+GSH groups was increased statistically significantly when compared to the control group. (P-value = 0.00, 0.01) respectively in 12 hrs, duration, while no significant difference between the APAP+GSH group and the control group (p- value = 0.5) in 24hrs duration only. A statistically significant decrease in MDA level in the APAP+GSH group compared to the APAP group (p-value <0.001). (Figure 1E). The level of MDA was significantly elevated over time in the untreated APAP group and decreased in the GSH treated APAP group (p-value <0.001) (Figure 1F).

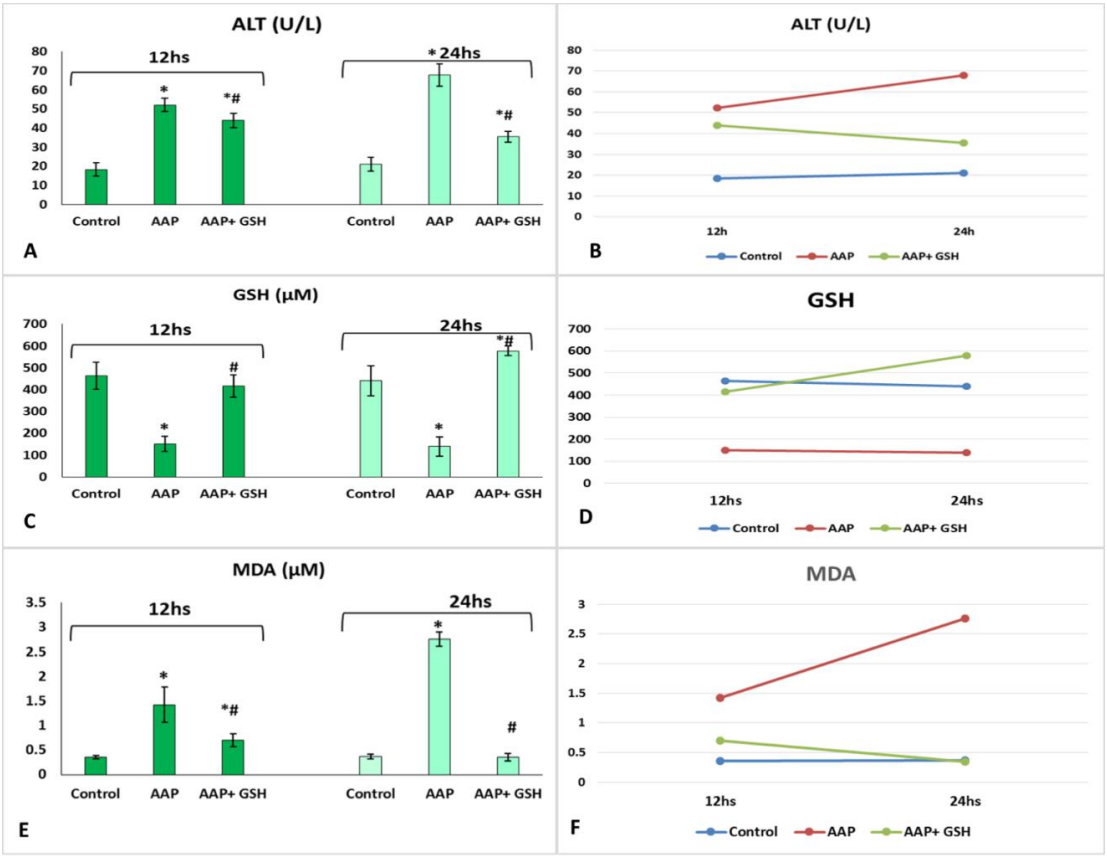

Figure 1. Biochemical analysis of ALT, GSH, and MDA. (*) Denotes significant difference versus the control group. (#) Denotes significant difference versus APAP group.

GSH maintains the integrity of both nuclear and

mitochondrial DNA

Tailed DNA

Regarding nuclear DNA, in both 12 and 24 hrs**,.** There was a statistically significant increase in tailed DNA percent in the APAP group compared to the control group (p -value< 0.001) while no significant differences between the APAP+GSH and the control group (p-value = 0.99). a statistically significant decrease in tailed DNA percent in the APAP+GSH group compared to the APAP group (p-value< 0.001), a statistically significant increase in tailed DNA percent in the APAP group in 24 hrs**.,** duration compared to the APAP group in 12 hrs**.,** duration (p-value< 0.001)(Figure 2A)

Regarding mitochondrial DNA, in both 12 and 24 hrs., there was a statistically significant increase in tailed DNA percent in the APAP group compared to the control group (p-value <0.001) while no significant differences between the APAP+GSH and the control group (p-value = 0.9). a statistically significant decrease in tailed DNA percent in the APAP+GSH group compared to the APAP group (p-value <0.001). a statistically significant increase in tailed DNA percent in the APAP group in 24 hrs**.,** duration compared to the APAP group in 12 hrs**.,** duration (p-value< 0.001) (Figure 2A)

Tailed DNA length

Regarding nuclear DNA, in both 12 and 24 hrs. There was a statistically significant increase in tail length in the APAP group compared to the control group (p-value= 0.001) while no significant differences between the APAP+GSH and the control group (p-value = 0.99). A statistically significant decrease in tail length in the APAP+GSH group compared to the APAP group (p-value= 0.004). A statistically significant increase in tail length in the APAP group in 24 hrs**.** duration compared to the APAP group in 12 hrs., duration (p-value<0.00) (Figure 2B)

Regarding mitochondrial DNA, in both 12 and 24 hrs., there was a statistically significant increase in tail length in the APAP group compared to the control group (p-value< 0.001) while no significant differences between the APAP+GSH and the control group (p-value = 0.95). A statistically significant decrease in tail length in the APAP+GSH group compared to the APAP group (p-value= 0.007). A statistically significant increase in tail length in the APAP group in 24 hrs**.** duration compared to the APAP group in 12 hrs., duration (p-value<0.001) (Figure 2B)

Untailed DNA

Regarding nuclear DNA, in both 12 and 24 hrs., there was a statistically significant decrease in untailed DNA percent in the APAP group compared to the control group (p-value= <0.00) while no significant differences between the APAP+GSH and the control group (p-value = 0.99). A statistically significant increase in untailed DNA percent in the APAP+GSH group compared to the APAP group (p-value <0.001). A statistically significant decrease in untailed DNA percent in the APAP group in 24 hrs, duration compared to the APAP group in 12 hrs, duration (p-value <0.001) (Figure 2C)

Regarding mitochondrial DNA, in both 12 and 24hrs, there was a statistically significant decrease in untailed DNA percent in the APAP group compared to the control group (p-value <0.00) while no significant differences between the APAP+GSH and the control group (p-value = 0.97). A statistically significant increase in untailed DNA percent in the APAP+GSH group compared to the APAP group (p-value< 0.001). A statistically significant decrease in untailed DNA percent in the APAP group in 24 hrs, duration compared to the APAP group in 12 hrs, duration (p-value<0.001) (Figure 2C).

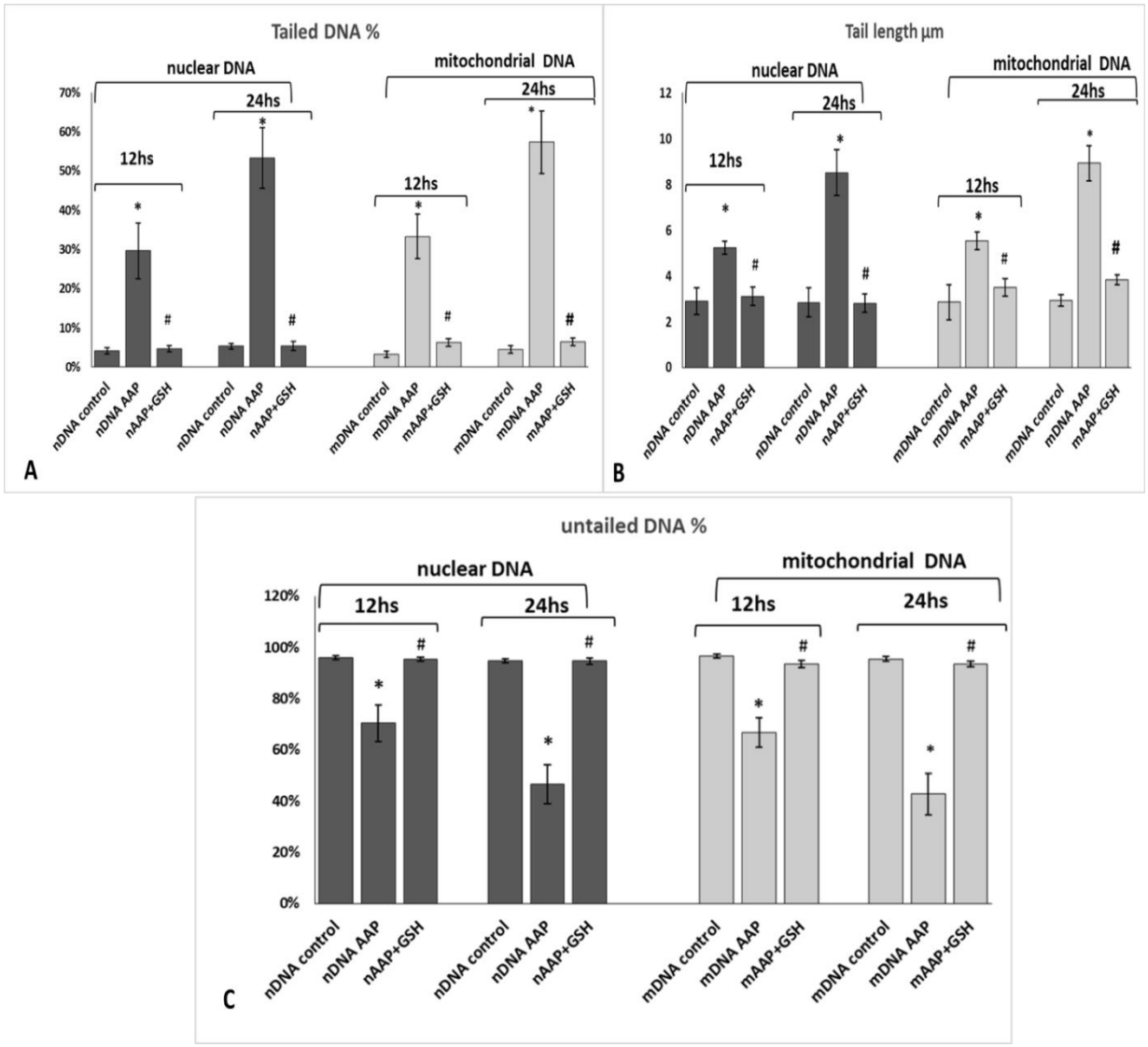

Figure 2. Comet analysis for nuclear and mitochondrial DNA. (*) Denotes significant difference versus the control group. (#) Denotes significant difference versus APAP group.

Comet assay

The comet assay revealed increased detaching tail length and DNA concentration during the hepatotoxicity by APAP in both durations (12hrs and 24hrs) when compared with the control group and the GSH treated group (Figure 3).

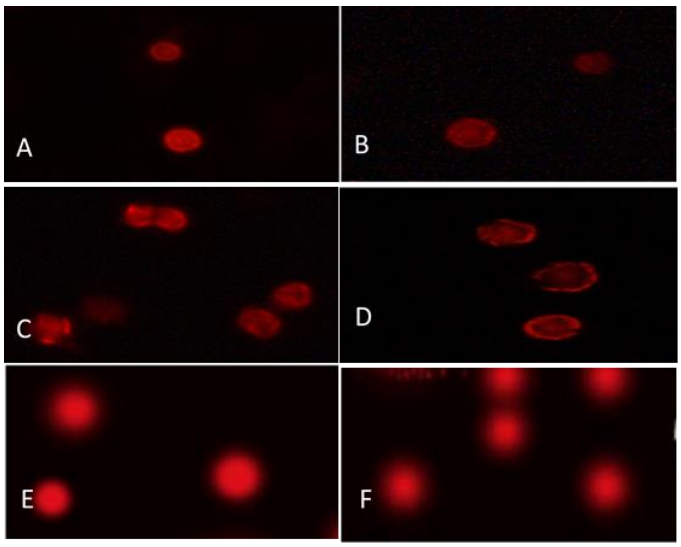

Figure 3. Comet analysis: A-Control 12hs. B- Control 24hs. C- APAP 12hs. D- APAP 24hs. E- APAP + glutathione 12 hrs. F- APAP+ glutathione 24 hrs.

GSH treatment alleviates hepatic inflammation Histo-pathologically

The normal hepatic lobule shows normal hepatic architecture, normal hepatocytes with no signs of inflammatory cells, and no apoptotic cells (Figure 4A). APAP induces hepatic inflammation which increases over time shown as marked cell swelling (black arrow), and apoptotic cells (white arrows). The apoptotic cells did not rupture which indicates the inflammatory response (Figure 4B, C). GSH reduces hepatic inflammation with no focus on inflammation and marked reduction of apoptotic cells (white arrow) in 12hrs, (Figure 4D) with marked improvement in 24hrs, duration as no cell swelling and no apoptotic cells (Figure 4E).

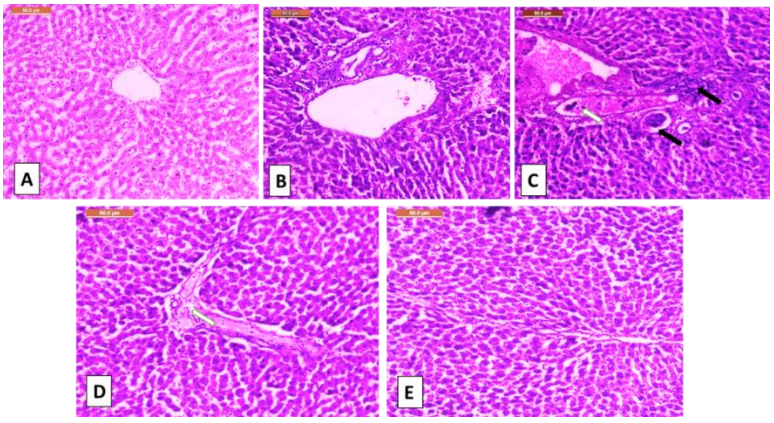

Figure 4. Histopathological examination of studied groups stained with hematoxylin and eosin (H&E). A: Normal hepatocytes with no signs of inflammatory cells and no apoptotic cells (200x).B Hepatocytes in APAP (12hrs) treated group with a small focus of inflammation (arrow). (200x). C: Hepatocytes in APAP (24hrs) treated group with a small focus of inflammation (arrowhead) (200x).D: A: Hepatocytes in APP+glutathione (12hrs) treated group with no focus of inflammation and marked reduction of apoptotic cells (white arrow). (200x)E: Hepatocytes in APAP +glutathione (24hrs) treated group with no cell swelling and no apoptotic cells (200x).

DISCUSSION

A leading factor in acute liver failure is acetaminophen overdose. APAP overdose patients are treated for up to 48 hours with the antidote N-acetylcysteine (NAC). NAC acts by scavenging of the reactive metabolite NAPQI, and also by regeneration of GSH, however, the undesirable side effects of NAC limit its uses [19]. Thus, the development of novel therapeutic approaches that produce well-tolerated and broad protective effects against late-presenting patients is needed.

The primary goal of this study was to assess GSH's potential to reduce oxidative liver damage

That gets induced in rats due to the administration of high-dose paracetamol. The results showed that the intravenously administrated paracetamol led to obvious oxidative hepatic stress. In normal physiological conditions, Antioxidants preserve the advantage in the equilibrium between oxidants and antioxidants. Any disturbance in this equilibrium might cause severe tissue damage (oxidative stress) [1].

According to the current study, ALT levels statistically significantly increased in the APAP group and the APAP+GSH group when compared to the control group, although they statistically significantly decreased in the APAP group when compared to the APAP+GSH group. The same results have been reached by a previous study that reported the elevation of liver enzymes and oxidative stress in APAP hepatic toxicity [20]. Another study reported the same results on the hepatotoxic effect of APAP [21].

MDA level, is a reliable indicator for increased free oxygen radicals and subsequent lipid peroxidation [2, 22]. Following previous studies [23 - 26] which discovered elevated MDA levels following a high dose of APAP therapy. While the MDA level in the rats groups which were given GSH was decreased when compared to the level in the untreated group.

In paracetamol overdoses, very high amounts of NAPQI get formed which GSH is unable to effectively detoxify. Acute liver damage was caused by the formation of NAPQI in proportions greater than GSH's capacity to metabolize it. The findings of this study are consistent with other research that found that APAP therapy in cell cultures and animal models reduced GSH levels. [8]. these published data support our research, which showed that APAP treatment significantly reduced GSH levels and cell viability in hepatic cells. This suggests that the reduced cell viability was caused by an increase in oxidative stress, which was brought on by the reduced GSH levels. Following APAP therapy, a considerable decrease in GSH levels may be caused by conjugation with NAPQI during the detoxification pathway. [27].

The findings of this study are consistent with other research that found that APAP therapy applied to cell cultures and animal models reduced GSH levels [28-31].

It has been reported that GSH helps the liver function return in a short amount of time by enhancing anti-oxidative stress resistance and repairing the membrane structure of liver cells. GSH can minimize the aggregation of activated effector cells, stop effector cell activation, stop the production of cytokines, and lessen the harm to hepatic cells brought on by cytokines [32]. These can explain our histopathological finding regarding the improvement of hepatic inflammation in the GSH- treated group**.** These findings agreed with a previous study reported that treatment with GSH is more efficient than treatment with NAC in lowering the elevated hepatic oxidative stress after APAP overdose. [33]. Another study reported that treatment with GSH reduces the serum levels of transaminases, total Bilirubin, tumor necrotic factor-α, transforming growth factor β_1, interlukin-6, and interleukin-8 [32].

Mitochondrial DNA and other associated proteins are important targets for nitration and oxidative stresses. It was demonstrated to have a substantial loss of mtDNA

following the occurrence of protein nitration [34].

These previous studies support our results that showed significant DNA damage to both mitochondrial and nuclear DNA in the APAP group, while it shows no difference between the APAP+GSH group in comparison to the control group. This agreed with a study that was performed previously stated that the cytoplasm, nucleus, and mitochondria of mammalian cells contain subcellular pools of glutathione, a key element of the antioxidant defense mechanism [35]. A further study reported that the protective function of GSH presumably does not appear to include the repair of classic DNA damage but may relate to alteration of DNA damage dependent signaling. GSH performs a vital protective role in maintaining both mitochondrial and nuclear DNA functional integrity. [36].

Further research which plasma from APAP-overdose patients was examined for the presence of indicators of mitochondrial damage, including glutamate dehydrogenase, nuclear DNA, and mitochondrial DNA fragments were higher than in the control group. Other studies in mice found a correlation between these plasma indicators and tissue damage. Apoptosis is not brought on by an APAP overdose. Thus, hepatotoxicity in humans and necrotic cell death are caused by APAP and are primarily caused by mitochondrial damage and nuclear DNA fragmentation. That is validated because there was no evidence of caspase-3 activity or cleaved caspase-3 in mice or overdose patients' plasma [37].

CONCLUSIONS

Glutathione protects from liver damage produced by APAP. APAP induces the production of oxygen-free radicles during its metabolism with subsequent lipid peroxidation, causing GSH depletion and DNA damage. Thus it seems to be beneficial to supplement GSH to replenish the GSH store and correct the oxidant/antioxidant balance.

CONFLICT OF INTERESTS

No conflict of interest

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