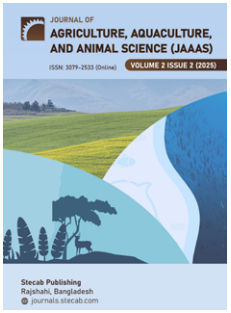

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Research Article

Toxic Effect of Frequent Low and High Doses of Acetaminophen on Liver Function in Mice

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About Article

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ABSTRACT

Acetaminophen is pharmacologically an active chemical entity, which is used safely and most widely as an over-the-counter analgesic drug, has been on the increase for the past few years-a trend that is predicted to continue. The study aims to examine experimental assessment the toxic effect of frequent low and high doses of acetaminophen on liver function through serological measurement of hepatic enzymes and antioxidants in mice. Forty adult male albino mice were equally assigned to NC (distilled water), and three acetaminophen groups; HAD (1000 mg/kg/day), RAD (500 mg/kg/day), and LAD (250 mg/kg/day). All mice were injected daily for 28 days; and finally, they chloroform- euthanized and directly blood sampled to obtaining the sera that utilized for measurement of hepatic enzymes (ALP, ALT, AST, and GGT), antioxidants [catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD)] and lipid peroxidation [malondialdehyde (MDA)] throughout the quantitative enzyme-linked immunosorbent assay (ELISA) kits. In comparison to values of NC, though the serum levels of ALP, ALT, AST, and GGT were significantly increased in HAD, there were significant decreases in values of ALT and GGT in mice of LAD but not RAD; whereas, values of ALP and AST were differed insignificantly in mice of RAD and LAD. Among antioxidants, the findings of CAT, GSH-Px, and SOD were decreased in HAD but not RAD; however, significant elevation in values of SOD but not CAT and GSH-Px were shown in mice of LAD. For MDA, higher values were seen in mice of HAD but not in RAD and Lad. This study demonstrates that the prolonged administration of acetaminophen induces dose-dependent hepatotoxicity in mice, characterized biochemically by significant elevations in serum ALT, AST, ALP, and GGT, consistent with impaired serum antioxidants and lipid peroxidation markers. These findings underscore the hepatotoxic risk of acetaminophen overdose and prolonged use, emphasizing the necessity of strict adherence to therapeutic dosages, vigilant clinical monitoring, and early intervention strategies to mitigate liver injury.

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1. INTRODUCTION

One of the broadest-selling analgesic antipyretic medications is acetaminophen or paracetamol (N-acetyl-p-aminophenol, APAP), first synthesized in (1893) by reacting p-nitrophenol with glacial acetic acid and tin. During the 1880s, phenacetin and acetaminophen were discovered to have antipyretic and subsequently analgesic effects (Brune *et al.*, 2015; Bunchorntavakul & Reddy, 2018; Tejo, 2021). Phenacetin was more popular than acetaminophen at first, but with the development of the serious side effects mentioned before, especially hemolytic anemia and the formation of methemoglobin, its use in the clinic declined, and people started paying attention to acetaminophen (Alanazi, 2017; Ogemdi, 2019; Grgic, 2022). Consequently, acetaminophen was made freely available since the 1950s and has since become the most common over the counter non-narcotic analgesic agent used in treating mild to moderate pain and fever (Anoopkumar-Dukie, 1999; Vannacci *et al.*, 2017). Subsequently, acetaminophen takes over the market non-narcotic analgesics drugs following its safety profile in therapeutic doses and especially following the onset of aspirin use decline since the 1960s because of its gastrointestinal toxicity and causation of Reye Syndrome in children (Fitzgerald, 2007; Schrör, 2007; Singh & Vyas, 2022). Acetaminophen is nowadays the first-line intervention in many forms of acute painful diseases, which encompass headache, musculoskeletal pain, period pain, osteoarthritic pain, back pain, dental pain also in the management of postoperative pain (Moore *et al.*, 2015; Amaechi *et al.*, 2021; Freo *et al.*, 2021).

Despite its favorable safety profile at therapeutic doses, acetaminophen remains a leading cause of drug-induced liver injury worldwide, primarily due to overdose. Most studies have focused on acute high-dose toxicity, which is known to cause hepatocellular necrosis through the accumulation of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). However, there is limited understanding of how frequent exposure to low or fluctuating doses of acetaminophen may influence liver function over time, especially under conditions that mimic real-life scenarios of repeated self-medication.

Therefore, this study aims to investigate the toxic effects of frequent low and high doses of acetaminophen on liver function in mice, to better understand the sub-chronic hepatotoxic potential of repeated exposure. By comparing biochemical and histological changes across dosing regimens, this research provides novel insights into how both therapeutic and supratherapeutic patterns of acetaminophen use may compromise liver integrity over time. The findings are expected to contribute to the ongoing discourse on safe dosing limits and the risks of repeated, unsupervised use of this widely available drug.

2. LITERATURE REVIEW

Various in vivo studies in both experimental animals and human subjects corroborated the hypothesis that pharmacological activities of acetaminophen are mediated by the selective inhibition of a centrally expressed cyclooxygenase (COX) enzyme (Esh *et al.*, 2021; Zakiyah *et al.*, 2022; Jasim & Mustafa, 2024). Moreover, it was found that the analgesic effect of acetaminophen was not associated with peripheral inhibition of prostaglandin production (Przybyła *et al.*, 2021). Acetaminophen has a pk a of 9.5, which remains largely un-ionized in the physiological range of pH, and is a moderately lipid-soluble, weak organic acid, allowing it to be absorbed rapidly by passive diffusion in the small intestine, enter the cellular membranes and easily cross the blood-brain barrier (Murakami, 2017; Malungpaishrope, 2018; Yang, 2023).

In medical field, the standard therapeutic dose of acetaminophen in adults is 2 tablets of 500 mg each taken orally every 4 hours up to a maximum of 8 tablets for any 24-hour period; while in children, acetaminophen is marketed in dosages depending on age and range from 60 mg (2–3 months) to 480–750 mg (12–16 year olds), (Abourbih *et al.*, 2016; Aitken *et al.*, 2019). Giving more than the recommended dose, acetaminophen can lead to toxicity, liver damage, nausea, vomiting, breathing problems, abdominal pain, and even death if untreated (Ciejka *et al.*, 2016; Caragea *et al.*, 2022). Hence, this study aims to experimentally assess the toxic effect of frequent low and high doses of acetaminophen on liver function through serological measurement of hepatic enzymes and antioxidants in mice.

While the acute toxic effects of single high doses of acetaminophen have been extensively documented, evidence regarding the chronic or sub-chronic effects of repeated low-dose exposure remains limited and inconsistent, particularly in animal models. Some long-term studies in rodents have focused on single daily dosing regimens or short exposure durations (often <14 days), which may not adequately simulate frequent clinical use or self-medication patterns in humans (e.g., low-dose but frequent intake for chronic pain or fever management). For example, certain sub-acute studies reported mild oxidative stress and minor histopathological changes at doses near the therapeutic range, but the findings vary widely depending on duration, strain, and dosing frequency (Chiew *et al.*, 2018; Wang *et al.*, 2020).

Therefore, there remains a clear research gap in understanding how frequent administration of low versus high doses of acetaminophen over an extended period (e.g., 28 days) influences hepatic enzyme levels and antioxidant status in animal models. The lack of comparative, dose-dependent, and time-controlled studies under such conditions limits our ability to predict the cumulative effects of repeated exposure.

Hence, the present study aims to bridge this gap by experimentally evaluating the toxic effects of frequent low and high doses of acetaminophen administered over 28 consecutive days in mice, focusing on alterations in liver function through serological measurements of hepatic enzymes and antioxidant biomarkers. This approach provides a more realistic model of habitual or sub-chronic acetaminophen use, helping to clarify its potential cumulative hepatotoxicity at both therapeutic and supratherapeutic dosing levels.

3. METHODOLOGY

3.1. Ethical approval

This study was licensed by the Scientific Committee in the College of Veterinary Medicine (University of Wasit).

3.2. Study design and samples

A total of 40 BALB/c adult male mice aged ≤ 4 months and of 25-40 gm weight were bought and transported to the Animal

House in the College of Veterinary Medicine (University of Wasit) and acclimatized after one week (fed pellet, received tap water and subjected to 12/12 dark / light). In turn, the study mice were separated into four groups in equal and random proportions by use of plastic cages as follows:

i. Negative control (NC): Mice were injected daily with a single dose of distilled water throughout the study period (28 days), and exposed to the standard conditions including light, food and water availability.

ii. Low-acetaminophen dose (LAD): Mice were injected daily with a single low dose of acetaminophen (250 mg/kg/day) for 28 days, and exposed to the standard conditions including light, food and water availability.

iii. Regular-acetaminophen dose (RAD): Mice were injected daily with a single regular dose of acetaminophen (500 mg/kg/day) for 28 days, and exposed to the standard conditions including light, food and water availability.

iv. High-acetaminophen dose (HAD): Mice were injected daily with a single high dose of acetaminophen (1000 mg/kg/day) for 28 days, and exposed to the standard conditions including light, food and water availability.

After ending the period of therapy, mice of all study groups were anesthetized with ether to drain blood directly from the heart into free-anticoagulant glass gel tubes. After centrifugation at 5000rpm for 5 minutes, the obtained serum from each sample was transferred into labeled 1.5 ml Eppendorf tube and kept frozen (-20°C) until be tested (Al-Bayati *et al.*, 2023; Hussen *et al.*, 2024).

3.3. Quantitative ELISA

According to the manufacturer working guides, SunLong Biotech, China ELISA kits were used to quantify alkaline phosphatase '[ALP (Cat No: SL0031Mo], alanine aminotransferase [ALT (Cat No: SL00229Mo)], aspartate aminotransferase [AST (Cat No: SL0086Mo)], gamma-glutamyl transferase [GGT (Cat No: SL1256Mo)], catalase [CAT (Cat No: SL0747Mo)], glutathione peroxidase [GSH-Px (Cat No: SL0241Mo)], superoxide dismutase [SOD (Cat No: SL1341Mo)], and malondialdehyde [MDA (Cat No: SL0370Mo)]. The processed and values of optical density (OD) of each kit were measured by the Automated Microplate Photometer (BioTek, USA). Then, the ODs of each marker in serum samples in addition to the concentrations and ODs of the Standard Solution of each kit were plotted on the Log Scales of Standard Curve to calculate the concentration of each marker in serum samples.

3.4. Statistical analysis

One-Way ANOVA in the GraphPad Prism Software was served for identification significant differences between values of each marker among study groups at a value of $p<0.05$, and values were represented as Mean ± Standard Errors (M±SE), (Wahab *et al.*, 2024).

4. RESULTS AND DISCUSSION

4.1. Hepatic enzymes

In comparison with values of NC (198.2 ± 19.12 pg/ml), the findings of ALP were shown a significant elevation ($p<0.0001$; 95%CI: 129.8 to 829.4) in values of HAD (801.8 ± 80.43 pg/ml) but not ($p>0.05$) in those of RAD (208.2 ± 17.31 pg/ml) and LAD (191.1 ± 15.81 pg/ml), (Figure 1).

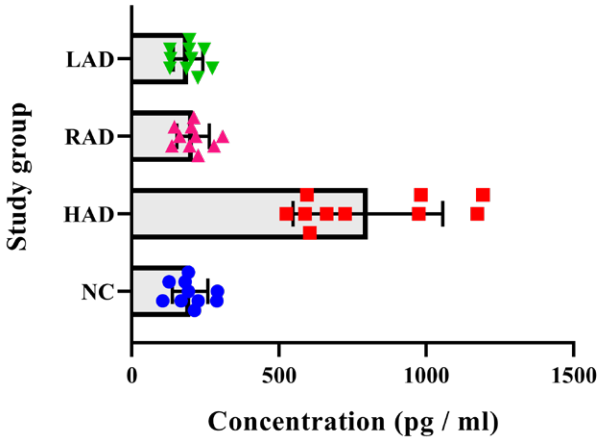

Figure 1. Levels of serum ALP in mice of various study groups

Concerning the values of ALT, there was a significant increases ($p<0.0001$; 95%CI: 112.9 to 494.5) in values of HAD (476.9 ± 26.87 pg/ml) but not ($p>0.05$) in values of RAD (101.1 ± 10.98 pg/ml); however, significant decreases ($p<0.05$) in values of LAD (85.7 ± 6.22 pg/ml) was observed when compared to values of NC (99.4 ± 6.34 pg/ml), (Figure 2).

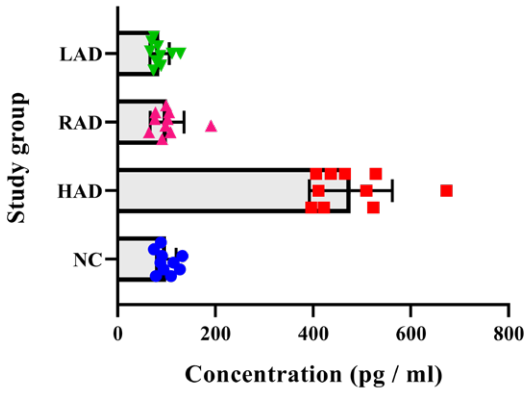

Figure 2. Levels of serum ALT in mice of various study groups

For AST, mice of HAD were recorded a significant ($p<0.0001$; 95%CI: 0.5200 to 2.644) higher values (2.552 ± 0.11 ng/ml) when compared to those of NC (0.561 ± 0.07 ng/ml); however, insignificant variation ($p>0.05$) was seen in values of RAD (0.526 ± 0.09 ng/ml) and LAD (0.608 ± 0.07 ng/ml), (Figure 3).

Significantly, the findings of GGT were elevated ($p<0.0001$; 95%CI: 565.1 to 2857) in mice of HAD (2755.2 ± 107.29 pg/ml) and reduced in mice of LAD (512.9 ± 51.76 pg/ml); whereas, insignificant differences ($p>0.05$) were appeared in mice of RAD (666.6 ± 62.8 pg/ml) when compared to those of NC (648.2 ± 60.85 pg/ml), (Figure 4).

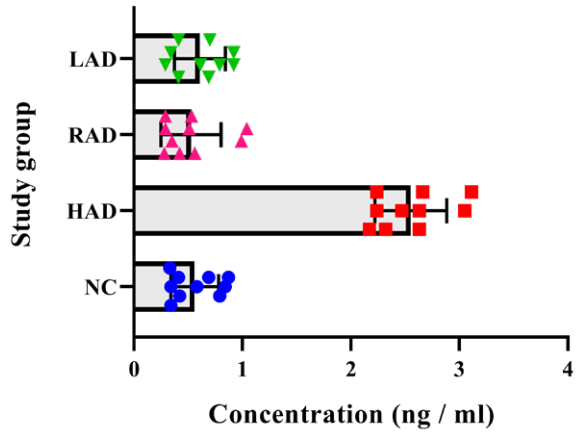

Figure 3. Levels of serum AST in mice of various study groups

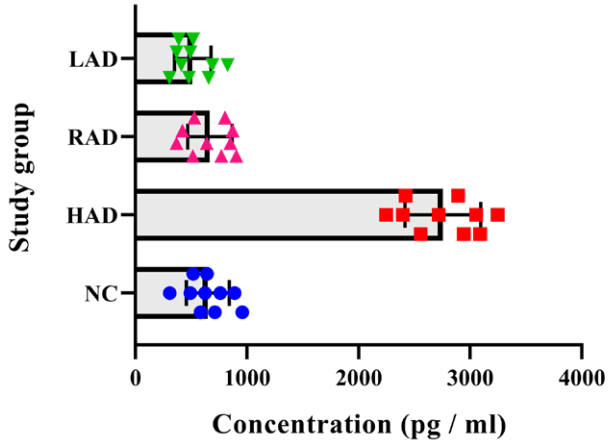

Figure 4. Levels of serum GGT in mice of various study groups

4.2. Antioxidants

The findings of CAT were revealed a significant lower values (p<0.0001; 95%CI: 146.8 to 739.3) in mice of HAD (164.9 ± 24.46 pg/ml) than those of NC (558.3 ± 30.49 pg/ml); however, insignificant variation (p>0.05) was seen in values of RAD (530.8 ± 29.9 pg/ml)' and LAD (518.2 ± 29.23 pg/ml), (Figure 5).

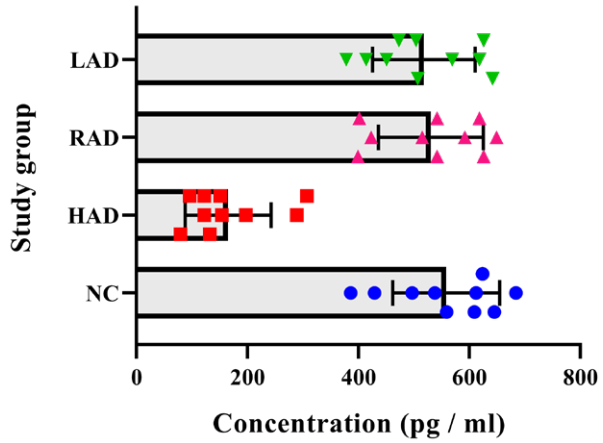

Figure 5. Levels of serum CAT in mice of various study groups

Relation GSH-Px, although the values of HAD (0.8 ± 0.06 ng/ml) were reduced significantly (p<0.0001; 95%CI: 0.6661 to 4.790) more than observed in mice of NC (3.575 ± 0.13 ng/ml), insignificant variation (p>0.05) was identified in mice of RAD (3.172 ± 0.15 ng/ml) and LAD (3.365 ± 0.25 ng/ml), (Figure 6).

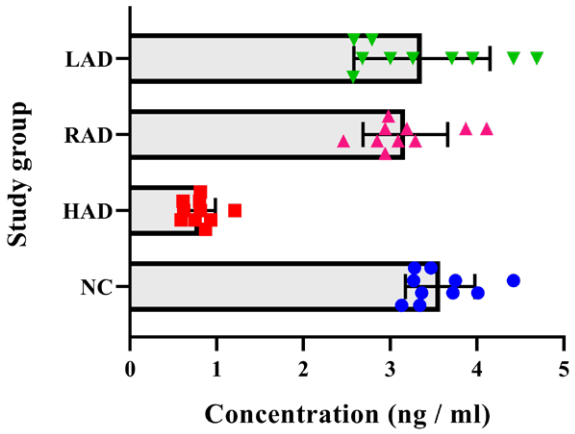

Figure 6. Levels of serum GSH-Px in mice of various study groups

The findings of SOD were shown a significant reduction (p<0.0001; 95%CI: 0.8458 to 9.994) in values of HAD (1.123 ± 0.09 ng/ml) and elevation in those of LAD (7.135 ± 0.28 ng/ml), but not (p>0.05) in values of RAD (6.872 ± 0.29 ng/ml) when compared to results of NC (6.549 ± 0.35 ng/ml), (Figure 7).

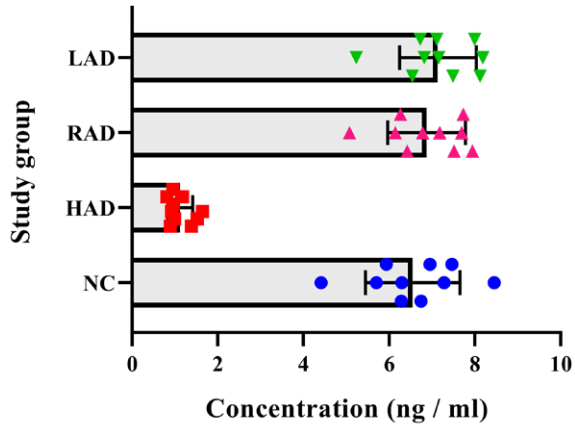

Figure 7. Levels of serum SOD in mice of various study groups

4.3. Lipid peroxidation

Significant increases (p<0.0001; 95%CI: 34.21 to 218.1) in values of MDA were detected in mice of HAD (210.8 ± 7.75 ng/ml) but not (p>0.05) in those of RAD (53.6 ± 7.63 ng/ml) and LAD (48.4 ± 6.09 ng/ml) when compared to values of NC (55 ± 7.49 ng/ml), (Figure 8).

4.4. Discussion

In this study, the findings of hepatic enzymes were shown a significant elevation in mice received a high dose of acetaminophen (HAD) compared to those received regular

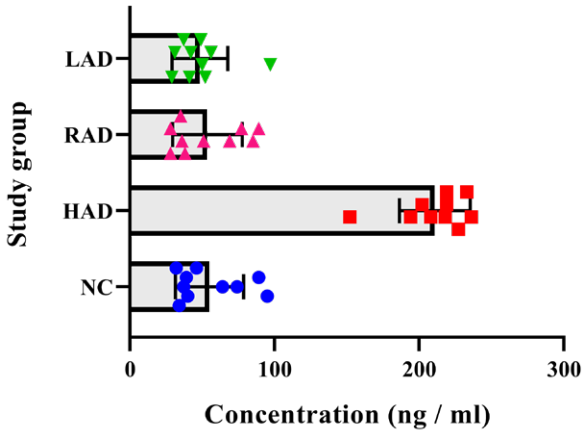

Figure 8. Levels of serum MDA in mice of various study groups

(RAD) and lower (LAD) doses suggesting the marked toxic effect of acetaminophen on liver. However, significant reduction in levels of ALT and GGT were identified in mice received lowered doses of acetaminophen suggesting that lowered doses of acetaminophen might play a protective role in hepatocytes. Worldwide, different studies showed previously that 'overuse of acetaminophen in mice and rats can cause severe and extensive necrosis cells in liver, and increased serum ALT/AST levels in rats which is in line with the results of the present study (Ray *et al.*, 1996; Sakaue *et al.*, 1996; Shon & Nam, 2004; Song *et al.*, 2004; Du *et al.*, 2017). Other researchers demonstrated that acetaminophen toxicity causes hepatocytes necrosis within the centers of liver lobules, sometimes extending throughout them (McClain *et al.*, 1999; Hinson *et al.*, 2009). Some differences are seen in sensitivity to acetaminophen within different species, so that in most rat strains acetaminophen is primarily hepatotoxic, but in others, acetaminophen shows nephrotoxic effects (Davis *et al.*, 1974; Gregus *et al.*, 1988; Jemnitz *et al.*, 2008; Neirinckx *et al.*, 2010). Mitic-Zlatkovic and Stefanovic (1999) showed that after 24 hours of a single dose administration of 900 mg/kg acetaminophen to rats intraperitoneally, there was a significant GGT activity with slight tissue damage. da Silva Melo *et al.* (2006) observed that the urinary levels of GGT, ALP and LDH enzymes were significantly higher at time 24 hours when compared to the levels at time 0 hour and returned to basal levels at time 48 and 72 hours. The GFR was significantly reduced 24, 48 and 72 hours after the drug administration. Dadkhah *et al.* (2007) conducted a study on adult and newborn rats and found liver lesions in adult rats; whereas, Ben-Shachar *et al.* (2012) used a mathematical model to evaluate the effects of different doses of acetaminophen on the GSH and liver metabolism of acetaminophen. They showed that the mathematical model could be used to study the metabolism of acetaminophen, if the expression levels of hepatic enzymes are known. In this model, the plasma ALT enzyme levels showed significant differences at different doses and times which were also consistent with the results achieved in the present study. In a study done by Heard *et al.* (2014) among 252 healthy outpatient volunteers treated with 4 g acetaminophen daily, or placebo for 16 days, 23% showed ALT elevations on acetaminophen while 2% of volunteers on placebo showed peak values (highest 191 U/L) at days 7-10. The ALT elevations in volunteers on acetaminophen were above normal in 9% and above twice normal in 3% versus none in volunteers on placebo. In another study on humans, 94 adults with asthma were treated with acetaminophen (2 g daily) or placebo for 12 weeks. ALT elevations above 3 times ULN arose in 1 subject in both groups, and mean ALT levels were minimally increased from 23.6 to 25.4 U/L, but did not change for those receiving placebo (Ioannides *et al.*, 2018). Other studies also showed that ALT/AST levels increased with low doses of acetaminophen due to cardiopulmonary and renal insufficiencies (Bonkovsky *et al.*, 1994; Lee *et al.*, 2015). James *et al.* (2003) indicated that acetaminophen adducts occur in necrotic cells following toxic doses of acetaminophen and did not cause hepatic lipid peroxidation in wild-type mice but did cause lipid peroxidation in iNOS knockout mice. In another study, Jarsiah *et al.* (2017) found a significant correlation between overuse of acetaminophen and hepatotoxicity, as well as the extent of GSH.

The findings of this study revealed that the levels of serum antioxidants were decreased significantly in mice received a higher doses of acetaminophen (HAD), while the level of lipid peroxidation MDA was elevated significantly. In comparison, Nuttall *et al.* (2003) found that the mean total antioxidant capacity was significantly reduced over the 3 hours post-dosing on both days 0 and 14; while, the results from days 4, 7 and 10 were showed a trend towards reduced antioxidant activity over time. Subsequently, on day 14, values were consistently lower compared with the corresponding times on day 0. Wang *et al.* (2017) mentioned that oxidative stress is involved in various toxicities associated with acetaminophen, and various antioxidants can be evaluated to investigate their protective roles against acetaminophen-induced liver and kidney toxicities. Ramachandran and Jaeschke (2018) has addressed the acetaminophen toxicity in relation to oxidative stress and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as a result of acetaminophen treatments, and ultimately their correlation with the toxicity and metabolism of acetaminophen. Ramachandran and Jaeschke (2021) reported that metabolism of acetaminophen involves various CYP450 enzymes, through which oxidative stress might occur, and such metabolic factors are reviewed within. The therapeutics of a variety of compounds against acetaminophen-induced organ damage based on their anti-oxidative effects is also discussed, in order to further understand the role of oxidative stress in acetaminophen-induced toxicity'. This review will throw new light on the critical roles of oxidative stress in acetaminophen-induced toxicity, as well as on the contradictions and blind spots that still exist in the understanding of acetaminophen toxicity, the cellular effects in terms of organ injury and cell signaling pathways, and finally strategies to help remedy such against oxidative damage.

5. CONCLUSION

This study demonstrates that the prolonged administration of acetaminophen induces dose-dependent hepatotoxicity in mice, characterized biochemically by significant elevations in serum ALT, AST, ALP, and GGT, along with impaired serum antioxidant activity and increased lipid peroxidation. These

biochemical alterations collectively indicate substantial hepatic dysfunction resulting from acetaminophen overdose and prolonged exposure. However, it is important to note that the present findings are based solely on biochemical markers of liver injury. Therefore, future studies should incorporate detailed histological analysis to confirm the extent and nature of tissue damage, providing more definitive insights into the cellular and structural alterations associated with acetaminophen-induced hepatotoxicity. These results underscore the hepatotoxic risk of acetaminophen misuse and highlight the necessity of strict adherence to therapeutic dosages, vigilant clinical monitoring, and prompt intervention strategies to prevent or mitigate liver injury.

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