Research Article

The Role of Alpha Lipoic Acid and Tempol Against Liver Injury after Acetaminophen Subacute Exposure in Mice

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Abstract | Acetaminophen (APAP) is a widely used analgesic and antipyretic drug, but it can cause liver damage when taken in high doses or for prolonged periods. Alpha Lipoic Acid (ALA) and Tempol are two antioxidants that have been shown to protect against various forms of oxidative stress and inflammation. This study explored the hepatoprotective effects of Alpha Lipoic Acid (ALA) and Tempol against subacutely repeated acetaminophen (APAP) exposure in a murine model. Fifty male Balb-c mice were divided into five groups. Groups received 700 mg/kg.BW of APAP and oral treatments: G1 (distilled water, negative control), G2 (no treatment, positive control), G3 (Par+Tempol, 20 mg/kg.BW), G4 (Par+ALA, 20 mg/kg.BW), G5 (Par+Tempol+ALA, 10 mg/kg.BW each). Half animals were sacrificed after 30 days, the rest after one week of treatment withdrawal. Investigation included body weight, biochemical markers (ALT, GGT, PT, INR), and liver histopathology. After 30 days, G5 showed a significant ($P \leq 0.05$) increase in body weight (29.2±1.32) compared to the positive control (20.8±1.2). After withdrawal, G5 displayed a significant ($P \leq 0.05$) increase in body weight(31.4±1.17) compared to all other experimental groups G1(29.2±0.37),G2(25.8±2.4), G3(25.0±1.38) and G4(27.2±1.02). Positive control (117 ± 42.9) had significantly ($P \leq 0.05$) increased ALT after 30 days, but all experimental groups G1(6.10 ± 1.81), G3(6.96 ± 3.33), G4(10.4 ± 5.56) and G5(7.62 ± 2.34) showed decreased ALT after withdrawal in comparison with positive control(16.1 ± 6.8). GGT levels were significantly increased in G4(2.05 ± 1.14) after 30 days, while G5(1.92 ± 0.97) showed a significant ($P \leq 0.05$) decrease after withdrawal. PT increased significantly ($P \leq 0.05$) in G4(2.05 ± 1.14) after 30 days but decreased in G3(5.01 ± 1.02), G4(3.85 ± 1.46), and G5(3.85 ± 1.46) after withdrawal. INR significantly increased in G4(16.6 ± 0.39) after 30 days but decreased in G3(13.1 ± 0.72), G4(3.96 ± 0.04), and G5(14.6 ± 0.66) after withdrawal. Liver sections displayed necrotic hepatocytes in G3 after 30 days, and G4 showed normal features. G5 exhibited normal hepatocytes after 30 days, with mild cellular swelling after withdrawal. These findings suggest the potential of ALA and Tempol in mitigating APAP-induced subacute toxicity, indicating a promising therapeutic avenue. However, further research is essential to elucidate the precise mechanisms, and caution is warranted in interpreting these preliminary results. The clinical implications could revolutionize APAP-induced toxicity management, emphasizing the need for continued research in this area.

Keywords | Subacute toxicity, Acetaminophen, Mice, Tempol, Alpha Lipoic acid, Liver Injury, Analgesic, Biochemical marker, Hepatio protective

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INTRODUCTION

Acetaminophen, also known as N-acetyl-para-aminophenol (APAP (was first used to treat both acute and chronic pain as an antipyretic and analgesic (Bandoli *et al*., 2020). It is also prescribed to patients for whom nonsteroidal anti-inflammatory drugs (NSAIDs) are contraindicated, such as those with gastric ulcers and bronchial asthma and has been used as a home analgesic for over three decades (Hegazy *et al*., 2021). In the early 1980s, in the United Kingdom, (APAP) paracetamol® supplanted aspirin as the most popular over-the-counter analgesic (Bertolini *et al*., 2006). The company's medicines are offered in many dosage forms, such as syrup, injection, suppositories, regular pills, effervescent tablets, and others (Allen and Ansel, 2013). Adults commonly take 325–650 mg of APAP every 4–6 hours or 1 g of immediate-release oral formulations every 4–6 hours as required, not to exceed 4 g per day (McEvoy, 2007). In the case of viral diseases in cattle, such as three-day sickness, cowpox, and so on, paracetamol is occasionally used as an oral pain reliever in dogs. In severe pain dogs and small mammals, APAP is used as an analgesic or antipyretic. It may be especially useful for treating chronic pain conditions in dogs with renal dysfunction, APAP should be administered orally or rectally at a dose of 10-15 mg/kg every 8 hours (Hernández-Avalos *et al*., 2020). If used long-term, which is defined as being over five days, it might be prudent to reduce the frequency of administration to every 12 hours, particularly at the lower end of the dosing range. The recommended dose for mice, rats, gerbils, hamsters, guinea pigs, and chinchillas is 1-2 mg/mL in their drinking water (Plumb, 2018). The recommended dosage for horses is 0.04-0.1 mg/kg administered intramuscularly. The administration can be repeated every 6-12 hours, however, a longer interval of 36-48 hours is preferable (Duggan and Scott, 2009; Golf *et al*., 2011). For perioperative analgesia or treatment of laminitis, the dosage is 0.01-0.05 mg/kg or 0.04 mg/kg respectively, administered by intramuscular, subcutaneous, or intravenous route (Papich, 2020). Dogs suffering from chronic pain should receive analgesics containing Paracetamol at a dosage of 10 to 15 mg/kg on a regular basis (Otero, 2013). The exposure of subacute paracetamol has led to significant hepatotoxicity in young and old mice, as observed through the use of biochemistry (ALT) and histology (Kane *et al*., 2016). Administration of either a single or double dose of N-acetlcysteine (NAC) failed to provide protection against this toxicity from subacute paracetamol in either young or old mice (Wu *et al*., 2004). This discovery holds notable clinical implications for the treatment of paracetamol toxicity in patients of all ages, and suggests the pressing need for new treatments to combat subacute paracetamol toxicity (Vliegenthart *et al*., 2015). In another study investigation, it was observed that exposing mice to a dosage of 200 mg/kg of APAP increases their susceptibility to the hepatotoxic effects caused by APAP. This heightened vulnerability is thought to stem from the elevated formation of APAP-protein adducts in the liver, consequently intensifying mitochondrial dysfunction in hepatocytes (Duan *et al*., 2016). As delineated in the study conducted by Venkatesan *et al*. (2014) the sub-acute oral toxicity of acetaminophen in Sprague Dawley rats was evaluated using doses of 250, 500, and 1000 mg/kg body weight over a period of 28 days. The results of this investigation indicated that there were no significant changes in hematological parameters, urinalysis parameters, absolute and relative organ weights, and gross pathological changes observed in the treated groups compared to the control group. However, notable alterations were detected in specific biochemical parameters related to liver and kidney function, including AST, ALT, ALP, blood urea, creatinine, glucose, and proteins, particularly within the high dose group (1000 mg/kg). These changes suggested that acetaminophen induced a certain degree of hepatotoxicity and nephrotoxicity in the rats, though not to an extent leading to mortality or clinical manifestations. According to the authors observations, the "no observed adverse effect level" (NOAEL) for acetaminophen in Sprague Dawley rats was determined to be 500 mg/kg body weight (Venkatesan *et al*., 2014).

Therefore, future research should focus on developing novel approaches to address this issue (Kane *et al*., 2016). Non-acute APAP ingestions, also known as subacute or chronic APAP ingestions. Few researchs exist to guide treatment or define who must be screened, making it challenging to handle nonacute ingestions. The majority of chronic APAP ingestions that result in hepatotoxicity are nonacute. include people who are consuming supratherapeutic doses and are at risk of developing hepatitis (Daly *et al*., 2008; Jwied, 2009).Age, total dose, duration of use, presence of intercurrent febrile illness, starvation, co-administration of cytochrome P450-inducing drugs, underlying hepatic disease, and a person's unique genetic makeup are all factors that have been proposed as potential risk factors for injury associated with chronic use ,hepatotoxicity risk associated with persistent consumption (Mohammad, 2013). On the basis of their medical history, clinical examination, and laboratory results, patients can be classified into three groups: those without hepatic injury and with no residual acetaminophen to be metabolized, those without injury but with some acetaminophen to be metabolized, and those with hepatotoxicity (Sztajnkrycer and Bond, 2001; Nafal *et al*., 2019). Alpha-lipoic acid exhibits strong antioxidant properties and demonstrates protective effects on the liver against injury (Hadi *et al*., 2020; Abd Hasan and Algareeb, 2022). Additionally employed to enhance the quality of semen after cryopreservation (Hamad, 2016). Most of the previous studies have focused on the acute toxicity of APAP and the role of antioxidants in preventing or reversing the

liver damage caused by APAP overdose. However, there is a lack of research on the subacute toxicity of APAP, which is more relevant to the clinical scenario of chronic or repeated use of APAP for pain management. Moreover, the combination of ALA and Tempol as a potential hepato protective agent against APAP-induced subacute toxicity has not been explored before, to the best of our knowledge. Therefore, this research presents a novel and significant contribution to the field of pharmacology and toxicology, as it provides new insights into the mechanisms and therapeutic options for APAP-induced subacute liver injury.The objective of this research is to examine the potential hepatoprotective properties of Alpha Lipoic Acid (ALA) and tempol in murine model subjected to repeated exposure to acetaminophen(APAP). Furthermore, this investigation could elucidate the potential hazards associated with recurrent subacute APAP overdose, thereby contributing to the body of knowledge regarding its safe administration.

MATERIALS AND METHODS

Laboratory animals

Fifty male Bulb-C mice reached the age of 8 weeks and ranged in body weight from 20-30 grams . The mice used in this study were raised and housed under standard laboratory conditions. They were maintained on a 12-hour light/dark cycle, with ad libitum access to food and water. The temperature in the animal house was kept constant at 22 ± 2°C, with a relative humidity of 50 ± 10% (Huynh *et al*., 2005). All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals (Suckow *et al*., 2023). The mice were acclimatized to the laboratory conditions for a week prior to the commencement of the experiment. Every effort was made to minimize the number of laboratory animals used and their suffering. The health and behaviour of the mice were monitored daily by trained personnel. At the end of the experiment, the mice were humanely euthanized following the approved protocols (Allen-Worthington *et al*., 2015).

Experimental design

This experiment has been carried out with the participation of fifty male Bulb-C mice. They were divided into five equal groups. All experimental groups except (G1) were administered 700 mg/Kg.BW of APAP(SDI/Samara, Iraq) and treated orally via gastric gavage needle as follows for 30 days: (G1) administered distilled water only and considered negative control group (C-ve) while (G2) did not receive any treatment and considered positive control group (C+ve), G3(Par+Tempol) treated by 20 mg/Kg.BW of Tempol (Chem Cruz, USA) (Pınar *et al*., 2018), G4 (Par+ALA) treated by 20 mg/Kg.BW of Alpha lipoic acid (ALA) (Chemical point, Germany) (Wang *et al*., 2018), while G5 (Par+tempol+ALA) treated by 10 mg/Kg.BW of each Tempol (Kwon *et al*., 2003) and ALA (Di Tucci *et al*., 2018). After that the laboratory animals have been rendered unconscious with diethyl ether (Thomas Baker, India).

Blood collection

Blood samples (1.5 mL) were collected via heart puncture (Rathkolb *et al*., 2013) from anesthetized mice by inhalation diethyl ether, using a 28-gauge, 4mm insulin syringe, taken after 30 days of treatment and one week post-treatment cessation. Samples were divided into anticoagulant-free gel tubes (gel tubes, Vaculab, China). and prothrombin time (PT) tubes (Al-Malik, Iraq). After a 10-minute incubation at room temperature, tubes were centrifuged at 5000 rpm for 15 minutes, separating serum and plasma, which were then stored at −18°C. These samples were utilized to assess biochemical markers (ALT, GGT, PT, INR) for liver function and clotting (Friedman *et al*., 2014; Friedman, 2022).

Clinical chemistry

Half of each group was sacrificed after the 30-days treatment, and the remaining half after an additional week without treatment, for liver extraction and clinical chemistry analysis. A commercial kit for spectrophotometric methods was used to assess the Serum Alanine Aminotransferase (ALT) (Jourilabs, France), (Lala *et al*., 2021). Gamma Glutammyle Transferase (GGT) (Human Gesellschaft, Germany) (McCommis *et al*., 2015). Prothrombin time (PT) Biosam, UAE (Gosselin, 2023) and international normalized ratio(INR) levels (Yuan *et al*., 2023). ALT determination uses oxaloacetate hydrazone via 2,4-dinitrophenylhydrazine. ALT kit measures α-oxoglutarate + L-alanine reaction. GGT quantification/ GGT kit with a standardized kinetic colorimetric method. GGT catalyzes glutamyl group transfer from γ-glutamyl-3-carboxy-4-nitroanilide to form glutamylglycylglycine and 5-amino-2-nitrobenzoate. The Prothrombin Time (PT) Kit assesses blood coagulation using tissue thromboplastin and Ca++. PT measures activation time, aiding in hemorrhagic disorder estimation. Thromboplastin reagent addition to citrated plasma initiates clotting, and prolonged clot formation indicates extrinsic pathway factors deficiency.

Histopathology

The histopathological patterns of the liver of all experimental mice, were examined.The process begins with the euthanasia of the mouse by diethyl ether (Thomas Baker India). The euthanasia of the mice was conducted using diethyl ether within a close chamber, also known as a dissector (Nasution *et al*., 2023). This method involves the administration of diethyl ether vapor in a controlled

environment, ensuring a rapid and humane method of euthanasia, until the mice loss of consciousness, followed by cessation of the heart and lung function (Meyer, 2015). Subsequently, a midline incision is made to expose the abdominal cavity, and enabling the careful extraction of the liver (Yokota *et al*., 2016). These organs are then immediately fixed in a 10% neutral buffered formalin) Alfa lab chemika China) for 48 h, following fixation, samples were sectioned to 0.5 cm thickness and placed in plastic cassettes (Werner *et al*., 2000). Dehydration and clearing of the tissues were automated using a histo- Line Laboratories ATP 1000 tissue processor (Italy). Subsequently, the dehydrated tissues were embedded in paraffin wax via a histo- line laboratory HESTION TEC 2900 embedding system, with temperature regulation managed by a TEC 2900 Thermal console) histo-line Laboratories, Italy (Jing *et al*., 2019). Tissue blocks were then sectioned at 4-5 µm thickness using a Hhistol line Laboratories, MRS3500 rotary microtome (Italy) (NODA and OGAWA, 1984). The sections were floated in a water bath and temperature -controlled hot plate, both regulated by the TEC 2900 Thermal console, before mounting on glass slides. staining was performed using Hematoxylin and Eosin (H and E, Dakocytomation, Denmark. The stained tissue sections were examined under a light microscope (Olympus Japan) at 40x magnifications for detailed histopathological assessment by a trained pathologist (Suvarna *et al*., 2018).

Statistical analysis

The data was analyzed using SPSS version 26 by SPSS Inc. A two-way ANOVA was conducted to determine the group differences. The LSD test was employed to identify significant differences, which were declared at a p-value of less than 0.05. The results were presented as the mean ± standard error (SE) (IBM Corp,. Armonk, NY, USA).

RESULTS AND DISCUSSION

Manifestations of subacute toxicity from APAP exposure

During the course of our research conducted on mice, we meticulously observed various clinical symptoms that were prominently evident, particularly in the Positive control Group as well as the G3 (Par+ Tempol) groups. These symptoms encompassed shallow breathing, a proclivity for lying and slow movement, sporadic convulsions, profound lethargy, that occurred within a week subsequent to the commencement of treatment. After that all toxicity signs have been relieved.

The effect of orally administration APAP, tempol, and lipoic acid for 30 days on body weight/g of male mice

The body weights of the positive control and (Par+Tempol) groups exhibited a significant ($P < 0.05$) decrease compared to other experimental groups at the initiation of treatment. After 30 days, the experimental groups, namely the positive control, (Par+Lipoic), and (Par+Lipoic+Tempol), demonstrated a significant ($P < 0.05$) decrease in body weight compared to the negative group and G5 (Par+Lipoic+Tempol). Intriguingly, animals in the treated group (Par+Lipoic+Tempol) showed a non-significant ($P < 0.05$) difference throughout this period compared to the negative control group. Moreover, only the animals in the (Par+Lipoic+Tempol) group displayed a non-significant ($P < 0.05$) change in body weight compared to the negative control group after one week of treatment withdrawal. Conversely, both mentioned groups (Par+Lipoic and Par+Lipoic+Tempol) demonstrated a significant ($P < 0.05$) increase in body weight compared to other experimental groups, the positive control, and Par+Tempol, over the same period. The body weights of all experimental groups showed non-significant ($P < 0.05$) changes when comparing their values after one week of treatment withdrawal with those at the initiation of treatment, except for the treated (Par+Tempol) group, which displayed a significant ($P < 0.05$) decrease across the periods after 30 days of treatment and one week of treatment withdrawal compared to their body weights at the initiation of treatment (Figure 1).

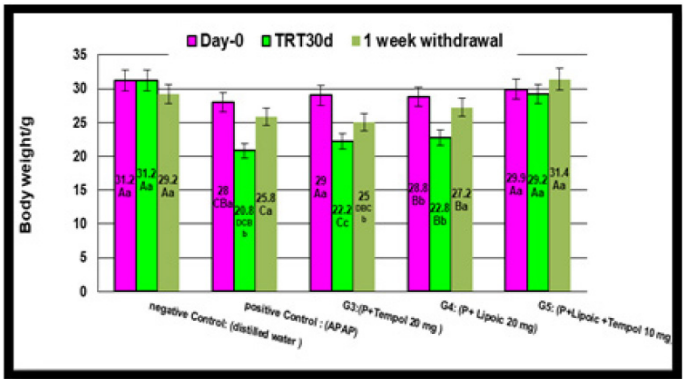

Figure 1: The effect of orally administration APAP, Tempol, and Lipoic Acid for 30 days on body weight/g of male mice. Data= Mean±SEM, A-D significant ($P < 0.05$) differences between groups, a-c significant ($P < 0.05$) differences within groups, LSD of interaction=2.529

The effect of orally administered APAP, tempol, and lipoic acid for 30 days on ALT(U/L) of experimental groups

The serum ALT activity in the treated groups, negative control (Par+Tempol), (Par+Lipoic), and (Par+Lipoic+Tempol), exhibited a significant ($P < 0.05$) decrease compared to the positive control group after 30 days of treatment. However, ALT serum activity demonstrated non-significant ($P < 0.05$) changes among all experimental groups after one week of treatment withdrawal. The positive control group showed a significant ($P < 0.05$) decrease in ALT serum activity after

one week of treatment withdrawal compared to its activity after 30 days of treatment. In contrast, all other experimental groups displayed non-significant (P < 0.05) changes across the two periods of the experiment (Figure 2).

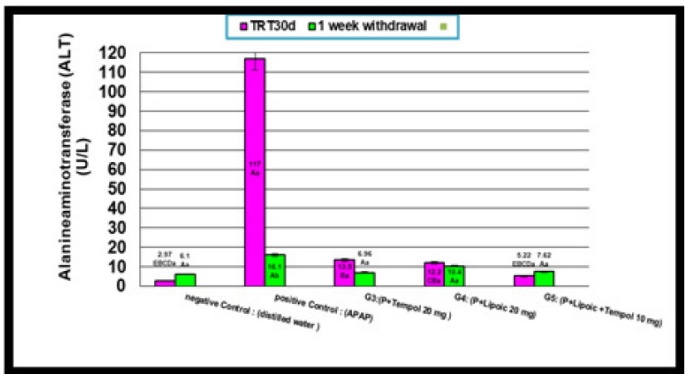

Figure 2: The effect of orally administration APAP, Tempol and Lipoic Acid for 30 days on the serum activity of ALT (U/L) in experimental groups. Mean ± SEM, A-D significant (P <0.05) differences between groups, a-b significant (P <0.05) differences within groups, LSD of interaction=40.82.

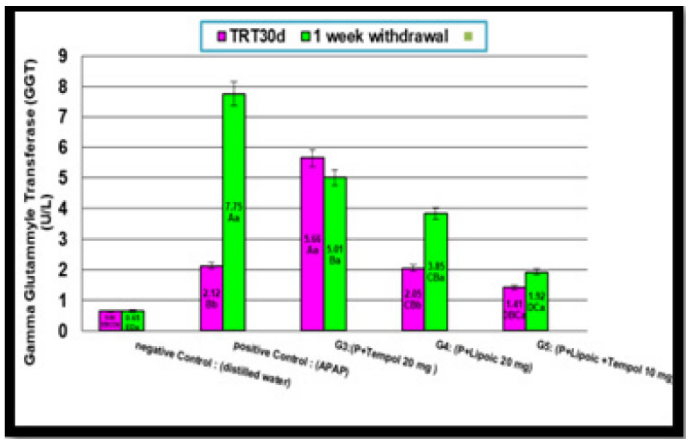

Figure 3: The effect of orally administration APAP, Tempol, and Lipoic Acid for 30 days on the GGT (U/L) in the experimental group. Mean ± SEM, A-D significant (P <0.05) differences between groups, a-b significant (P <0.05) differences within groups, LSD of interaction= 2.714.

The effect of orally administered APAP, tempol, and lipoic acid for 30 days on GGT (U/L) in the experimental groups

The serum GGT activity after 30 days of treatment in the group treated with (Par+Lipoic+Tempol) exhibited non-significant differences (P < 0.05) compared to the positive control group but showed a significant increase (P < 0.05) compared to the negative control group. Conversely, the (Par+Tempol) group demonstrated a significant increase (P < 0.05) compared to other experimental groups. After one week of treatment withdrawal, the serum GGT activity displayed a significant decrease (P < 0.05) in the (Par+Tempol), (Par+Lipoic), and (Par+Lipoic+Tempol) groups compared to the positive control group. The treated groups (Par+Tempol) and (Par+Lipoic) showed a significant increase (P < 0.05) compared to the negative control group, while only the (Par+Lipoic+Tempol) group exhibited non-significant differences (P < 0.05) compared to the negative control group. Furthermore, the serum GGT activity demonstrated a significant increase (P < 0.05) after one week of treatment withdrawal in the positive control and (Par+Tempol) groups compared to their activity after 30 days of treatment. In contrast, the negative control, (Par+Lipoic), and (Par+Lipoic+Tempol) groups showed non-significant differences (P < 0.05) between the two periods of the experiment (Figure 3).

The effect of orally administered APAP, tempol, and lipoic acid for 30 days on PT/ SEC of the experimental groups

The prothrombin time (PT) in all experimental groups exhibited a significant (P < 0.05) decrease, except for the (Par+Tempol) group, which displayed a non-significant (P < 0.05) difference compared to the positive control after 30 days of treatment. Notably, both the positive control and (Par+Tempol) groups showed a significant (P < 0.05) increase in PT compared to the negative control group. Conversely, the other treated groups, namely, (Par+Lipoic) and (Par+Lipoic+Tempol), demonstrated a significant (P < 0.05) decrease compared to the negative control group after the same treatment period. The PT of all experimental groups exhibited non-significant (P < 0.05) changes after one week of treatment withdrawal. When considering both periods of the experiment (30 days of treatment and one week of treatment withdrawal), only the positive control and (Par+Tempol) groups showed a significant (P < 0.05) decrease in PT, while the other experimental groups displayed non-significant (P < 0.05) changes (Figure 4).

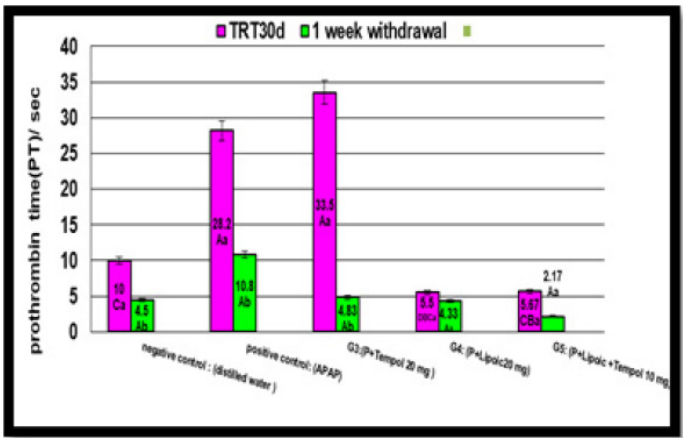

Figure 4: The effect of orally administration APAP, Tempol, and Lipoic Acid for 30 days on the PT/ sec in the Experimental group. Mean±SEM, A-D significant (P <0.05) differences between groups, a-b significant (P <0.05) differences within groups, LSD of interaction= 8.289.

The effect of orally administered APAP, tempol, and lipoic acid for 30 days on INR of the experimental groups

After 30 days of treatment, the International Normalized Ratio (INR) results revealed a significant ($P < 0.05$) decrease in the treated groups, negative control, (Par+Lipoic), and (Par+Lipoic+Tempol), compared to the positive control group. Conversely, the treated group (Par+Tempol) displayed a non-significant ($P < 0.05$) change when compared to the positive control. Following one week of treatment withdrawal, the experimental groups, namely, negative control, (Par+Lipoic), (Par+Tempol), and (Par+Lipoic+Tempol), exhibited a significant ($P < 0.05$) decrease compared to the positive control group.Within-group analysis demonstrated that the INR values of the positive control, (Par+Lipoic), and (Par+Lipoic+Tempol) groups experienced a significant ($P < 0.05$) decrease after one week of treatment withdrawal compared to their values after 30 days of treatment. In contrast, the treated group (Par+Lipoic) showed a non-significant ($P < 0.05$) change across the two periods of the experiment (Figure 5).

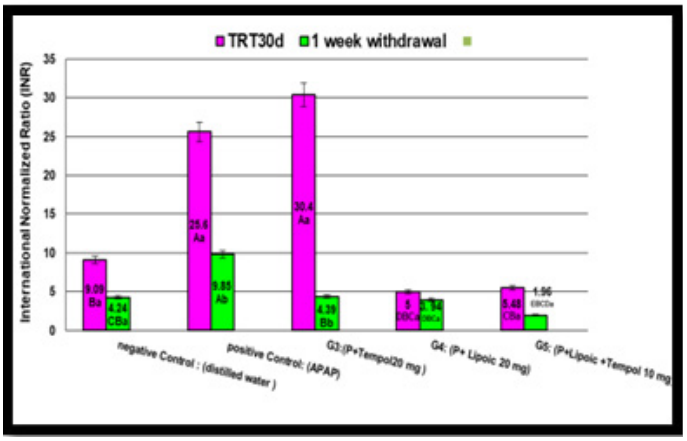

Figure 5: The effect of orally administration APAP, Tempol, and Lipoic Acid for 30 days on the INR in the experimental groups. Mean ± SEM, A-D significant ($P < 0.05$) differences between groups, a-b significant ($P < 0.05$) differences within groups, LSD of interaction= 7.175.

Histopathology

In the positive control group exposed orally to 700 mg/kg BW APAP, after 30 days of treatment liver histopathology shows dilated central vein with moderate mononuclear cell infiltration (lymphocytes and macrophages) with marked Centre lobular necrosis (Figure 6). After one week of withdrawal, the presence of a dilated central vein accompanied by lymphocytic cuffing and degenerated and necrotic. In the (Par+Tempol) group, liver histopathological examination after 30 days of treatment shows severe mononuclear cell infiltration)lymphocytes and macrophages(with marked necrotic hepatocytes (coagulative N) (Figure 7). For the (Par+ALA) group, the liver histopathological examination after 30 days shows central vein (C.V) with moderate mononuclear cell infiltration and mild centrelobular necrosis (coagulative N) (Figure 8). In the (Par+Tempol+Lipoic Acid) group, shows mild mononuclear cell infiltration (lymphocytes and macrophages) with moderate vacuolar degeneration (Figure 9).

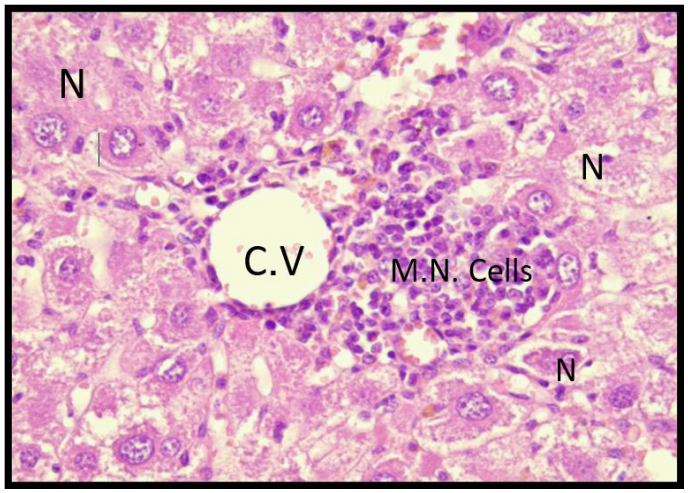

Figure 6: Liver section of mice of C+ve group shows dilated central vein (C.V) with moderate mononuclear cell infiltration (lymphocytes and macrophages) with marked centrelobular necrosis (coagulative N.) × 40H and E stain.

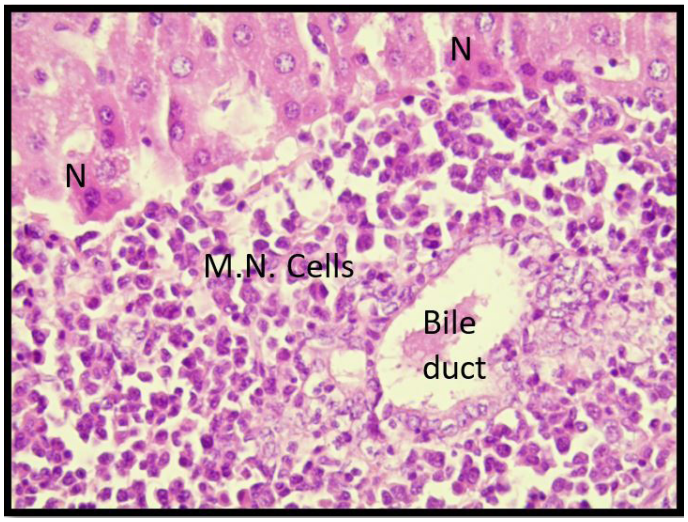

Figure 7: Liver section of mice treated by APAP 700 mg/kgBW and Tempol 20 mg/kgBW orally for one month (G3)shows severe mononuclear cell infiltration) lymphocytes and macrophages (with marked necrotic hepatocytes (coagulative N.)×40 H and E stain.

The APAP group (positive control group) had a substantial increase in ALT and GGT levels, indicating liver injury and cell necrosis, which could be attributed to the development of an APAP toxic metabolite, NAPQI (Azarmehr *et al.*, 2019; Yang *et al.*, 2021; Hameed and Hassan, 2022). The introduction of acetaminophen demonstrated effectiveness in inducing oxidative stress and

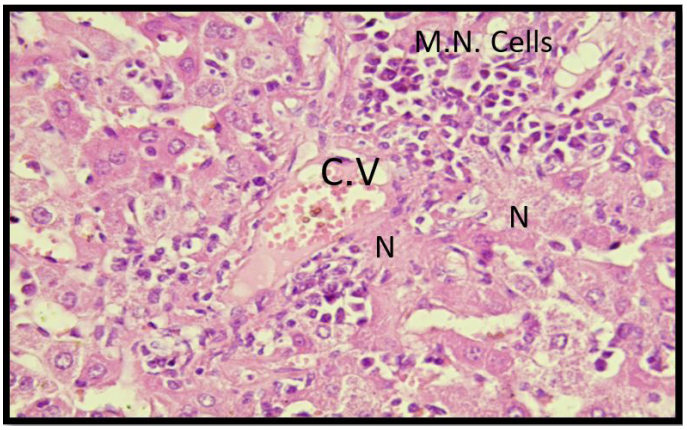

Figure 8: Liver section of mice treated by APAP 700 mg/kgBW and alpha lipolic acid 20 mg/kgBW orally for 30 days (G4) shows central vein (C.V) with moderate mononuclear cell infiltration and mild centrelobular necrosis (coagulative N.) × 40H and E stain.

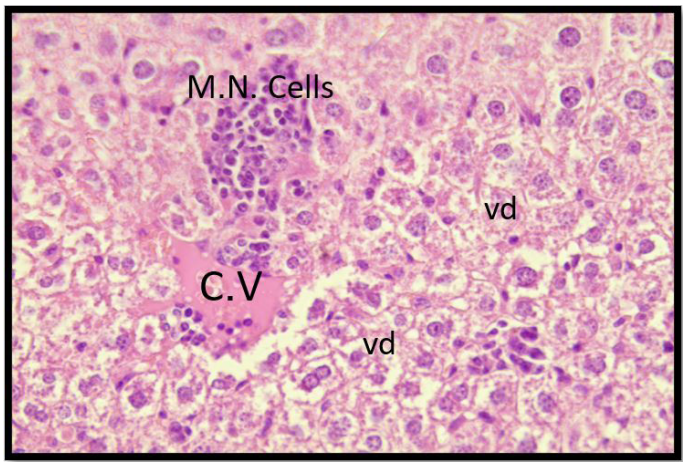

Figure 9: Liver section of mice treated by APAP 700 mg/kgBW and Tempol acid 10 plus alpha lipolic acid 10 mg/kgBW orally for one month (G5) shows mild mononuclear cell infiltration (lymphocytes and macrophages) and (M.N Cell) with moderate vacuolar degeneration × 40 H and E stain.

modifying specific biological markers associated with paracetamol-induced liver injury in rats, achieved through a single injection of paracetamol (500 mg/kg) (Jahan *et al.*, 2023). This is consistent with the findings of the present study on the hepatoprotective effect of nicorandil against acetaminophen-induced oxidative stress (Abd Al-Zahra *et al.*, 2009; Al-Okialy, 2018) and hepatotoxicity in mice, where an elevation in levels of liver enzymes, specifically Alanine Aminotransferase (ALT) and Gamma-Glutamyl Transferase (GGT), was observed. These findings align with the results obtained from the comprehensive investigation and serve as confirmation there of (Govindaraju *et al.*, 2021). A study suggested that mice exposed to APAP exhibited clear signs of liver damage, as indicated by hepatic necrosis and an increase in ALT levels in wild-type C57BL/6 mice (Liao *et al.*, 2023). This is confirmed in the present study, concerning ALT and GGT serum activity, as well as various deleterious histopathological findings in mice dosed with Paracetamol only. In contrast to the decrease in the serum activities of these enzymes in all treated groups (Par+Tempol), (Par + Lipoic), and (Par + ALA + Tempol) along the period of the current study, histopathological findings like necrosis have been observed in mice given a hazardous dose of APAP, 700mg/Kg.BW (positive control group). The ameliorating effect of different agents used in the current study, referring to histopathological patterns of different treated groups (Par+Tempol), (Par + Lipoic), and (Par + ALA + Tempol), shows important improvement in liver histology when necrotic lesions disappeared. ALA has the capability to influence the expression and activities of cytochrome P450 enzymes (P450s), which play a crucial role in the metabolism of APAP and other pharmaceuticals. A research study conducted by (Ewees *et al.*, 2019) has shed light on the impact of APAP-induced liver injury on the expression and activities of various P450s, including CYP2E1, 3A11, 1A2, and 2C29, in the liver of mice in a manner that is dependent on the age of the subjects. The study has put forth the hypothesis that these alterations could potentially affect the effectiveness of drugs that undergo metabolism by P450 enzymes. Conversely, it has been demonstrated that ALA has the ability to inhibit the activity of CYP2E1 and stimulate the expression of CYP1A2 in the liver of mice (Jahan *et al.*, 2023). Bao *et al.* (2020) Consequently, it can be speculated that ALA might possess advantageous properties in terms of safeguarding against and treating APAP-induced liver injury by exerting control over the levels of P450 enzymes. On the contrary, the treated group (Par+ Tempol) showed an increase in the serum activity of GGT, in accordance with the findings of a separate investigation focusing on the impact of Tempol in relation to the renal toxicity induced by cisplatin. It can be concluded that the results obtained from the current study are in alignment with the finding of (Ge *et al.*, 2019) who observed. The observations made during the study on the treated groups, which consisted of (Par + ALA + Tempol), revealed a clear and noticeable decrease in the levels of ALT. This outcome is in accordance with the findings of an ongoing research, which demonstrated that Tempol has the ability to diminish the levels of ALT in CCl4-intoxicated Rats (Jahan *et al.*, 2023). The TD50 of orally administered APAP for mice is estimated at 736 mg/kg BW, derived from the logarithmic equation. However, due to the lack of specific studies on APAP's TD50 value, establishing correlations or comparative analyses with existing literature is currently unfeasible. It's noteworthy that the LD50 of acetaminophen in mice, administered orally, is 1120.28 mg/kg BW, determined with a 24-hour observational period (Aseel, 2021). Alpha-lipoic acid, renowned for its remarkable therapeutic potential, has emerged as a compelling avenue in the field

of hepatotoxicity treatment. Hepatotoxicity encompasses a wide range of liver impairments caused by diverse factors, including the hazardous effects of toxic substances such as Acetaminophen. In the conducted study, rats were administered a daily oral dose of Acetaminophen at 100mg/kg (Mohammed *et al*., 2021). Alpha-lipoic acid (ALA) exhibited antioxidant activity in rats experimentally induced with oxidative stress by H_2O_2 (Eidan, 2017). In a separate study, it was observed that the administration of Tempol resulted in the increase of various antioxidant proteins, such as superoxide dismutase, catalase, and glutathione. Additionally, the phosphorylation of phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt), as well as the protein expression of nuclear factor erythroid 2-related factor (Nrf 2) and heme oxygenase-1 (HO^{-1}), were all significantly elevated due to the treatment with Tempol (Ge *et al*., 2019). These findings strongly indicate that Tempol plays a protective role against APAP-induced hepatotoxicity through the activation of the PI3K/Akt/Nrf2 pathway. Furthermore, it was observed that Tempol treatment led to a decrease in the expression of pro-apoptotic proteins (cleaved caspase-3 and Bax) and an increase in the expression of the anti-apoptotic protein Bcl-2 in the liver. Moreover, the number of apoptotic cells, as determined by TUNEL staining, was significantly reduced following Tempol treatment, suggesting its potential to prevent apoptosis (Zhao *et al*., 2022). Overall, the evidence suggests that Tempol has the ability to restore normal liver function in mice with APAP-induced acute hepatotoxicity by activating the PI3K/Akt/Nrf2 pathway, thereby enhancing the antioxidant response and inhibiting hepatic apoptosis. Studies have provided evidence that lipoic acid exhibits a potential therapeutic benefit in treating the liver structural and chemical aberrations instigated by an overdose of the analgesic drug APAP in male albino mice (Tiiba *et al*., 2022). Besides, studies have revealed that lipoic acid possesses the ability to prevent APAP-induced liver damage by reducing necrosis, inflammation, and oxidative stress in the liver and promoting liver regeneration. This underscores the potential of lipoic acid to be employed as an effective therapeutic agent for treating APAP overdose-induced liver damage. Also, a certain study that is in agreement with histopathological findings in the current study, has found that results of the study indicate that it can mitigate the harm to the liver morphology and ultrastructure induced by hepatotoxicity caused by fluoride (Zhao *et al*., 2022). In a scientific investigation involving rodents that had sustained liver damage due to lipopolysaccharide injection, it was discovered that lipoic acid served to regulate the equilibrium of the oxidant/antioxidant status and avert the occurrence of oxidative stress (Cimen *et al*., 2019; Zalzala and Kareem, 2021; Al-Azzawi and Baraaj, 2016). These results are supported with those of the present study, which found that APAP administration elevated liver biomarker enzymes while lipoic acid and Tempol therapy reduced all of the changed values. The outcomes are reported (Abdel-Zaher *et al*., 2008; Jahan *et al*., 2023). The findings of the study revealed that lipoic acid and Tempol have the potential to mitigate the formation of NO as well as oxidative damage in the liver and kidney. This is made possible through the reduction of lipid peroxidation and glutathione peroxidase (GSH-Px) activity, along with an increase in intracellular GSH depletion. The results of the present study are in alignment with the study providing further evidence that serum ALT activity experiences an elevation when specific medications, such as APAP, are administered. The aforementioned study, which investigated the impact of various drugs on liver function, observed a consistent rise in ALT levels in response to the usage of certain pharmaceuticals, including APAP. Therefore, reinforcing the notion that drug-induced liver injury can manifest through an increase in serum ALT activity (Mehrpour *et al*., 2023). At therapeutic doses, APAP is generally regarded as safe, with no significant adverse effects. However, it is crucial to note that at higher doses such as the dosage rate 700 mg/Kg.BW that was administered to mice subacutely, APAP may lead to centrilobular liver necrosis, a severe condition characterized by the death of liver cells in the centrilobular region (Etemadi *et al*., 2023). It has been proposed that NAPQI, a metabolite that is highly reactive, is responsible for the depletion of glutathione through a reaction known as conjugation, in which it forms a covalent bond with proteins. This suggests that NAPQI plays a crucial role in the regulation of glutathione levels within the body. Additionally, this reactive metabolite has been found to bind to proteins, further illustrating its impact on cellular processes. The depletion of glutathione caused by NAPQI can lead to an increased oxidative stress response, as the detoxification of reactive oxygen and nitrogen species is hindered. This imbalance in the body's antioxidant defenses can result in an elevated level of oxidative stress, which has been implicated in a variety of pathological conditions (Fatemeh, 2023). Furthermore, the perturbation of calcium metabolism has been suggested to be associated with the increased oxidative stress caused by glutathione deficiency. Altogether, these findings highlight the intricate relationship between NAPQI, glutathione, oxidative stress, and calcium metabolism, providing insights into the mechanisms underlying various diseases and potentially paving the way for the development of novel therapeutic strategies (Karthivashan *et al*., 2015).Fulminant hepatic failure (FHF), a severe and life-threatening condition, is frequently characterized by elevated levels of PT and INR, which are indicators of impaired blood clotting. In this particular study, a present a case involving a 36-year-old female patient who experienced FHF as a consequence of an acetaminophen overdose (Yoon *et al*., 2023). When compared to the PT recorded in the positive control group

(APAP) and (Par+Tempol) treated group mice had a considerably longer prothrombin time in contrast to (Par+ ALA) treated mice, Lipoic treated animals in the current study, that showed a decrease in PT. We believe that LA has a more positive effect than Tempol. Moreover, the treatment group, (Par+ ALA +Tempol), had a very close value to the (Par+Lipoic) with a slight difference.

CONCLUSIONS AND RECOMMENDATIONS

The findings of this study underscore the potential of alpha-lipoic acid and tempol in mitigating the subacute toxicity associated with Acetaminophen (APAP) exposure. The observed ameliorative effects suggest a promising confluence of these compounds in counteracting APAP-induced toxicity. However, the precise mechanisms of these effects remain to be elucidated. It is imperative to note that these findings are preliminary and should be interpreted with caution until validated by further research. The potential clinical application of these findings could revolutionize the management of APAP-induced toxicity, underscoring the need for continued research in this area. However, it is important to note that the study was conducted on mice, and further research is needed to determine wether these findings can be applied to humans.

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NOVELTY STATEMENT

This study investigates the hepatoprotective effects of Alpha Lipoic Acid (ALA) and Tempol against subacutely repeated acetaminophen (APAP) exposure in a murine model. The findings reveal a significant increase in body weight and decreased levels of biochemical markers after withdrawal, suggesting the potential of ALA and Tempol in mitigating APAP-induced subacute toxicity. These results contribute to the understanding of novel therapeutic approaches for managing APAP-induced liver injury and emphasize the need for further research in this area.

AUTHOR'S CONTRIBUTION

FMKA-R: Conceptualized and designed the study, analyzed the data, supervised the project, and contributed to the final version of the manuscript. SMHA-R: Performed the experiments and wrote the initial draft of the manuscript. All authors reviewed and approved the final manuscript.

Ethical statemen

This study was approved by the decision of the ethics University of Baghdad, College of Veterinary Medicine Animal Care and Use Committee, with numbered P.G.1247.

Conflict of interest

The authors have declared no conflict of interest.

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