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Original Research Article

Impact of Prolonged Therapeutic Dosage of Acetaminophen in Mice

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Abstract

Background: Acetaminophen (APAP) is the most frequently used medication worldwide, recognized for its longstanding efficacy in managing pain and reducing fever. However, it can lead to serious liver damage, which ranks as the second leading reason for liver transplants globally. Toxicity may occur due to prolonged exposure to high doses or even a single large intake of APAP. Recently, there has been growing concern regarding the potential negative effects associated with the prolonged use of APAP even at standard therapeutic doses.

Methods: In this study, the impact of prolonged therapeutic use of APAP is evaluated by administering a daily dose (equivalent to the therapeutic dose of APAP (40 mg/kg) or lower doses (10 mg/kg or 20 mg/kg) typically used by humans) to mice, and their survival duration and morphological alterations were observed for up to 40 days. Following the survival assessment, mice were anesthetized after a 10-day regimen of APAP (before the mortality caused by a 40 mg/kg dosage) and blood samples were collected to assess liver injury markers, while liver tissues were analysed for morphometric and histological alterations.

Result: Results indicate that prolonged exposure to APAP at therapeutic or sub-therapeutic dose leads to a marked increase in mouse mortality and a considerable decrease in body weight. Additionally, there is significant elevations in liver injury biomarkers in serum, along with alterations in liver morphology and histological architecture.

Conclusion: These results suggest that long-term use of therapeutic or sub-therapeutic doses of APAP may elevate the risk of liver toxicity.

Keywords: Acetaminophen; Therapeutic Dose; Sub-Therapeutic Dose; Prolonged Treatment; Hepatotoxicity.

Introduction

Acetaminophen (Paracetamol/APAP) is the primary choice for alleviating pain and reducing fever [1-3], but it is also a major contributor in causing liver damage among commonly used over the counter medications [4-6]. APAP is considered safe when consumed in doses for up to 4 grams per day or 650 mg for every 4-6 h, and is currently marketed as an analgesic or antipyretic, intended for use no longer than 3 days without medical consultation, but it can also be prescribed for extended periods in chronic conditions such as osteoarthritis or cardiovascular diseases [7-9]. However, concerns regarding its long-term use are significant due to its potential to cause liver damage [8, 9] along with serious side effects such as gastrointestinal bleeding, hypertension, kidney injury and respiratory complications [10-13]. APAP is primarily metabolized in liver through glucuronide and sulfate conjugation, with approximately 90% following this pathway, while less than 5% is eliminated in urine and the remaining 5% is converted into the harmful metabolite N-acetyl-p-benzo-quinone imine (NAPQI) [14, 15]. Excessive NAPQI interacts with cell membranes, leading to oxidative stress and cellular death by depleting glutathione (GSH), an intrinsic antioxidant [15-17]. Hepatic toxicity occurs when the levels of glutathione (GSH) decrease to less than 30% of the basal levels [15-17], and studies show that glutathione depletion can happen even with therapeutic doses of APAP [18, 19]. Additionally, the risk of hepatotoxicity can be influenced by various factors such as age, nutritional status, pre-existing health conditions, alcohol consumption and interaction with other medications [20, 21]. Notably, cases of toxicity have been reported even when these factors are absent in healthy individuals, with an increase in aminotransferase enzymes indicating subclinical liver cell damage, which is associated with additional adverse effects

[22, 23]. Therefore, it is crucial to carefully evaluate APAP dosages when assessing instances of acute liver injury. So, in our study, to check the impact of prolonged therapeutic doses of APAP on mice, we administered APAP orally to Swiss albino mice at a therapeutic dose of 40 mg/kg and sub-therapeutic doses of 20 mg/kg and 10 mg/kg for different groups over a period of 40 days, and monitored their survival rates along with any morphological changes in the mice. Based on these survival outcomes, we subsequently sacrificed the mice from all groups on the 10th day (prior to any death occurring in the 40 mg/kg group) to assess serum biomarkers as well as morphological and histological alterations in the liver. The objective of our current study is to provide experimental evidence regarding the negative effects of APAP, when used therapeutically for prolonged duration.

Materials and Methods

Materials: APAP was sourced from Sigma-Aldrich (St. Louis, MO, USA), hematoxylin was acquired from Merck (Kenilworth, NJ, USA), eosin was purchased from SD Fine Chemicals (Mumbai, MH, India), and biomarker kits for SGOT, SGPT, LDH, and ALP were obtained from Agappe Diagnostics Ltd (Ernakulam, KL, India). All other routine chemicals were procured from Sisco Research Laboratory Pvt. Ltd (Mumbai, MH, India) and SD Fine Chemicals (Mumbai, MH, India).

Mice: Swiss albino mice weighing 20–25 g at 6–8 weeks were obtained from the University of Mysore Central Animal Facility in Mysuru, India. The Department of Studies in Zoology, University of Mysore, Mysuru, approved all animal experiments (UOM/IAEC/08/2021) in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Throughout the experiments, all mice were housed in 12-hour light/dark cycles with adequate ventilation, food and water accessibility, and constant observation.

Effect of prolonged treatment of APAP on mice: To determine the effect of prolonged therapeutic treatment of APAP, mice were divided into four groups (n = 6 each). The first group of animals (Group I) served as control (Received warm saline). The second group (Group II) received a daily dose of APAP (10 mg/kg), third group (Group III) received a daily dose of APAP (20 mg/kg) and the fourth group (Group IV) received a daily dose of APAP (40 mg/kg-this dose was equivalent to the therapeutic dose used in humans) and the survival time and morphological changes were monitored for up to 40 days. The APAP solution was made by dissolving it in warm saline to ensure optimal absorption.

Note: The therapeutic concentration of APAP was determined based on the commonly prescribed dosage of 650 mg/kg every 4 hours, which equates to 650 mg/kg administered four times (total 2.6 g) daily. When compared to a healthy adult weighing 65 kg, this results in a concentration of 40 mg/kg, with half of that being 20 mg/kg. Furthermore, half of that amount is 10 mg/kg, which was utilized in this study.

In a separate experiment, to measure the biomarkers of liver injury we divided mice into 4 groups (n = 6 each). Mice were treated orally with respective daily doses of APAP (10, 20, and 40 mg/kg) in a total volume of 0.8 ml warm saline. The control group received warm saline. After a 10-day treatment period, the mice were anesthetized (i.e., before death induced by a dosage of 40 mg/kg), and blood and liver samples were collected for further biochemical and histological analysis.

Assessment of biomarkers of liver injury: Blood was obtained from the carotid artery of euthanized mice using a 1ml syringe. The serum was isolated from the whole blood and employed for the analysis of specific biomarkers. The levels of SGOT (#11408005), SGPT (#1140900), LDH (# 11407004), and ALP (#11401009) were assessed using the corresponding kits available commercially (Agappe Diagnostics Ltd, Ernakulam, KL, India) and serum enzyme activity was computed following the manufacturer's guidelines (SGOT/SGPT/LDH - 1U = µmole of NADH oxidized/min; ALP - 1U = µmole of p-nitrophenol produced/min).

Histological examination: haematoxylin and eosin staining: Liver samples collected from euthanized mice were fixed in a 10% formalin solution for 24 hours, then dehydrated using ascending concentrations of ethanol and embedded in paraffin. Following this, the samples were sliced into thin sections (5 µm) utilizing a microtome (R. Jung AG, Germany), and the deparaffinised sections were stained with haematoxylin and eosin. The tissue sections were examined under a bright field microscope, and images were captured [24].

Statistical Analysis: Survival significance was assessed using Kaplan–Meier survival analysis. The data were analysed with Graph Pad Prism Version 8.0. Group comparison statistical significance was conducted using a one-way analysis of variance with multiple comparisons. P value of ≤ 0.05 was considered statistically significant for all analyses.

Results

Prolonged administration of both therapeutic and sub-therapeutic doses of APAP results in mortality and reduction of body weight in Swiss albino mice: The safety profile of APAP is a significant factor contributing to its status as one of the most commonly prescribed analgesics [25]; however, the association between standard therapeutic doses of APAP and acute liver failure is controversial [25, 26]. Despite these concerns, APAP is commonly recommended for use during recovery

after surgery and for chronic conditions like osteoarthritis over extended periods [8, 27]. In our study we used a mouse model to offer experimental evidence related to this issue. We examined the impact of long term use of therapeutic APAP by administering doses of 40 mg/kg as well as sub-therapeutic doses of 20 and 10 mg/kg to Swiss albino mice for a duration of 40 days. Our results indicated that all mice died (100%) when given a dose of 40 mg/kg and 20 mg/kg over time in a typical experiment; in the 40 mg/kg group, deaths were recorded on the 12th, 18th, 23rd, 24th, 28th, and 30th days, while in the 20 mg/kg group, deaths were observed on the 18th, 26th, 29th, 30th, 32nd, and 34th days. Furthermore, in the 10 mg/kg group, 2 out of 6 mice died, one on the 28th day and the other on the 34th day within the 40-day period (Figure 1). This confirms that long term use of APAP (at therapeutic doses or lower) results in toxicity. Throughout the survival study, the body weight of the remaining mice in each group was recorded for every 7 days, revealing a significant increase in the control group, while a marked weight loss was noted in the APAP-treated groups, in dose dependent manner. In the control mice, the weight increased significantly from 24.68 ± 0.92 to 29.48 ± 1.28, whereas in the APAP-treated groups, the weight decreased from 26.4 ± 1.08 to 22.5 ± 0.96, from 26.11 ± 1.19 to 22.52 ± 1.26, and from 25.45 ± 0.88 to 18.72 ± 1.61 for the dosages of 10 mg/kg, 20 mg/kg, and 40 mg/kg respectively (Figure 2). This indicates the severe negative effects of APAP at therapeutic levels.

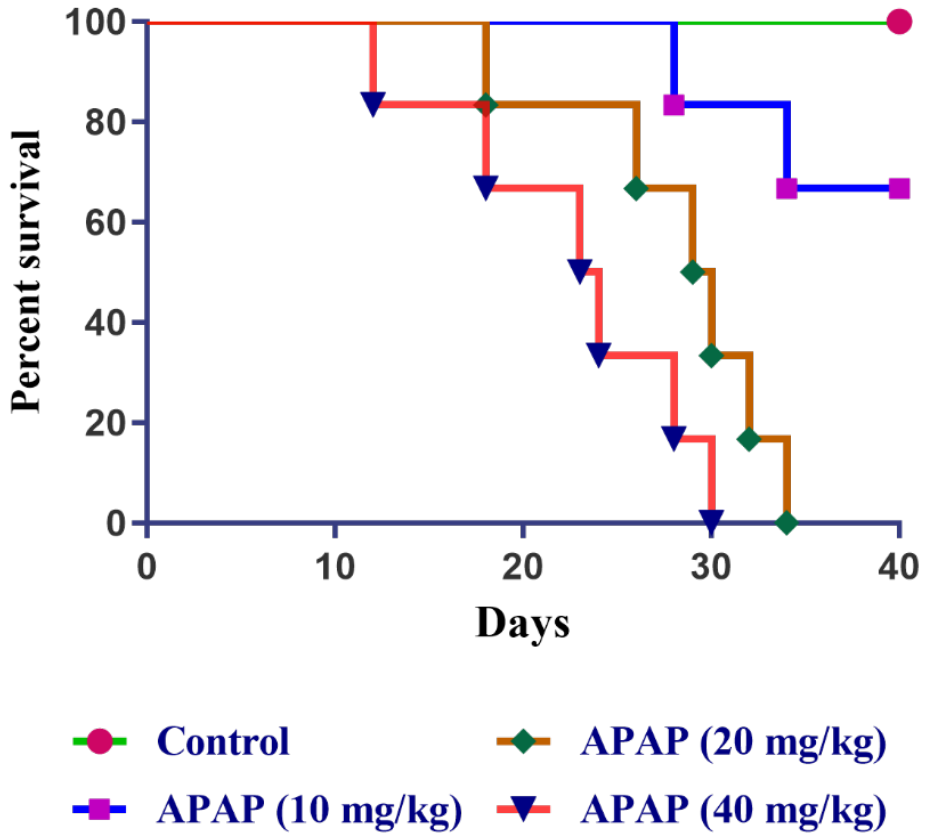

Figure 1: The effect of prolonged therapeutic or sub-therapeutic doses of APAP treatment on Swiss albino mice survival: Swiss albino mice were divided into 4 groups of 6 mice each. Each group received daily oral doses of APAP at concentrations of 10 mg/kg, 20 mg/kg, and 40 mg/kg, respectively. The survival duration of the mice was monitored over a 40-day period, and the outcomes were statistically analysed using the Kaplan–Meier survival method. The survival experiment was repeated, demonstrating consistent toxic effects, with deaths occurring within a comparable timeframe. Figure 1 illustrates the survival duration observed in one of the typical experiment.

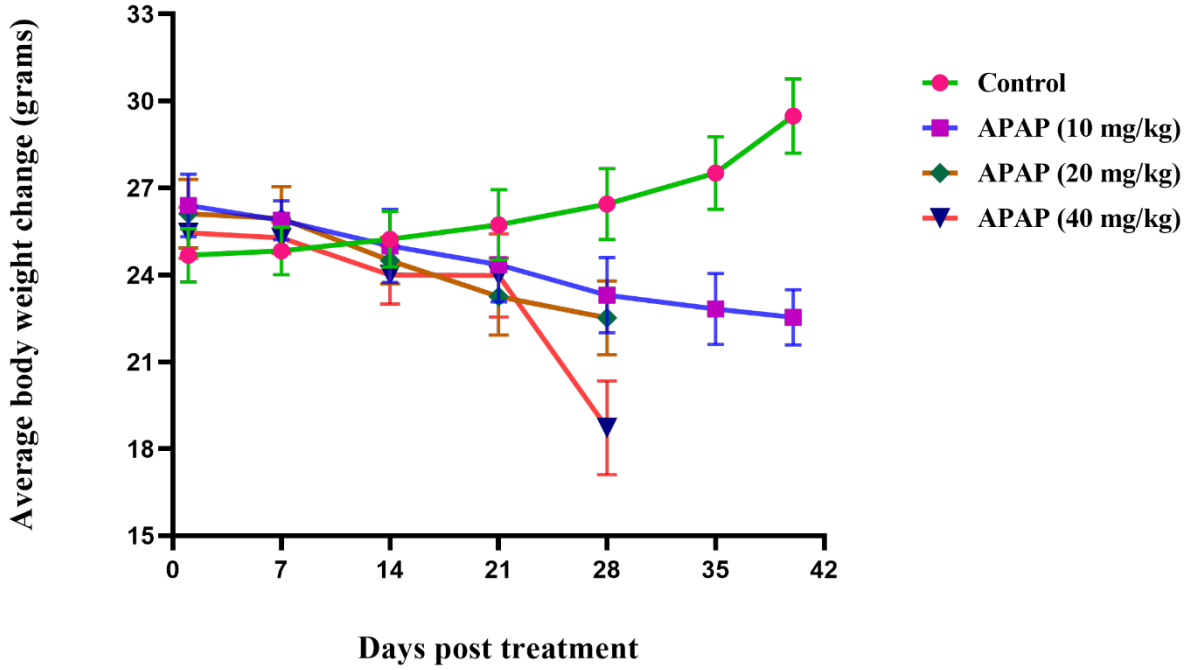

Figure 2: The impact of prolonged therapeutic or sub-therapeutic doses of APAP administration on the body weight of Swiss albino mice: Swiss albino mice were separated into 4 groups, each consisting of 6 mice. Each group was administered daily oral doses of APAP at doses of 10 mg/kg, 20 mg/kg, and 40 mg/kg, respectively. The survival time of the mice was tracked over a 40-day span with their body weight monitored for 7 days, and the plotted results indicated the increase or decrease in body weight of the cumulative number of remaining mice in each group, with the outcomes calculated using the mean and standard deviation.

APAP at therapeutic doses or sub therapeutic doses induced liver damage in mice: Following the outcomes of the survival study, a separate experiment was designed to assess the biochemical and histological variations. Mice received different daily doses of APAP (10, 20, and 40 mg/kg) through oral administration. Following a treatment period of 10 days (before the mortality caused by a 40 mg/kg dosage), the mice were anesthetized, and blood and liver samples were collected for further biochemical and histological examination. We examined markers that signify hepatocellular damage and found a notable dose-dependent rise in serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT). There was a threefold increase at a dosage of 10 mg/kg, a fivefold increase at 20 mg/kg, and approximately a sixfold increase at 40 mg/kg when compared to control groups (Figure 3). Furthermore, levels of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) demonstrated a slight elevation at 10 mg/kg, escalating to nearly twofold at 20 mg/kg, with a threefold increase noted at 40 mg/kg (Figure 3). These results suggest significant hepatocellular injury induced by prolonged use of APAP, Furthermore, our study assessed the effect of a therapeutic dose of APAP on liver morphology and histology, and found that at 40 and 20 mg/kg, there was a moderate increase in morphometric parameters such as liver weight, width, and length in relation to total body weight. However, the effects appeared to be less at lower dosages of 10 mg/kg (Figure 4). Histological examinations of haematoxylin and eosin staining revealed considerable hepatocellular damage and injury at the 40 mg/kg dosage, with minor signs of cellular harm at both the 20 mg/kg and 10 mg/kg doses (Figure 4). This suggests that even APAP doses considered therapeutic or sub-therapeutic could carry a considerable risk of liver toxicity, underscoring the importance of careful dosage management to avoid liver damage.

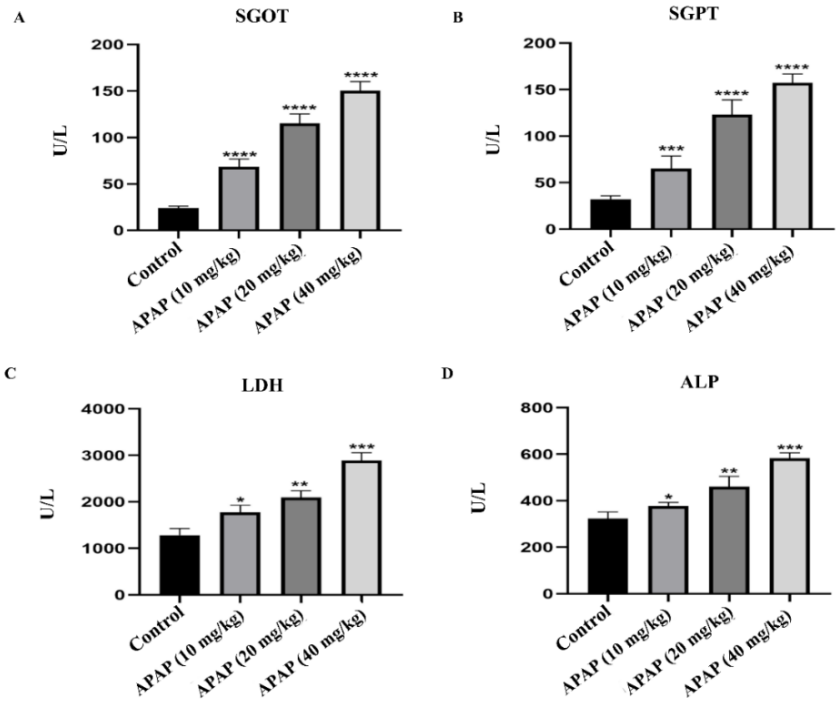

Figure 3: Assessment of liver injury biomarkers in serum when APAP was given at therapeutic or sub therapeutic doses for 10 days: Liver-injury biomarkers, including (a) glutamic-oxaloacetic transaminase (SGOT), (b) glutamic-pyruvate transaminase (SGPT), (c) lactate dehydrogenase (LDH), and (d) alkaline phosphatase (ALP), were assessed in the respective groups. The findings are representative of three indivitual experiments. P values were determined using one-way ANOVA with Tukey's multiple comparison test. *P ≤ 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 in comparison to control mice.

Dosage	Control	APAP (10 mg/kg)	APAP (20 mg/kg)	APAP (40 mg/kg)
Mouse weight (g)	25.37 ± 0.14	24.29 ± 0.76	23.57 ± 0.86	22.59 ± 0.65
Liver weight (g)	1.24 ± 0.004	1.25 ± 0.004	1.26 ± 0.008	1.27 ± 0.008
Liver length (cm)	1.84 ± 0.05	1.86 ± 0.05	1.94 ± 0.05	1.94 ± 0.11
Liver width (cm)	2.52 ± 0.04	2.54 ± 0.05	2.56 ± 0.05	2.58 ± 0.04

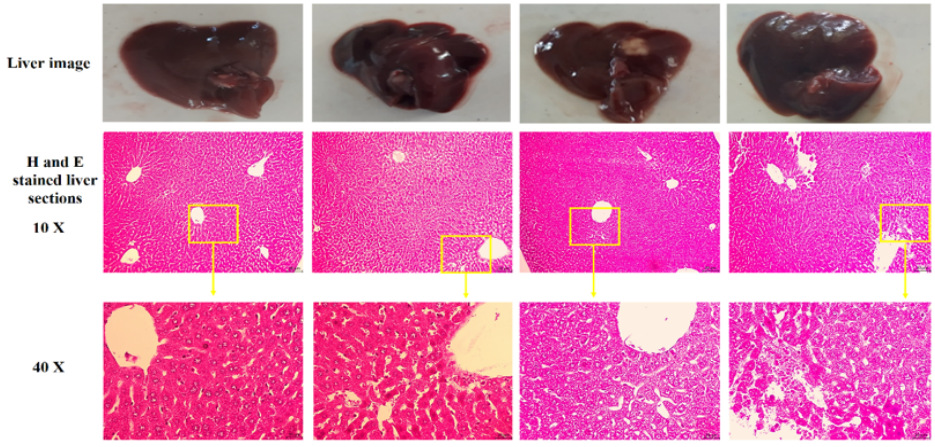

Figure 4: Effect of therapeutic and sub-therapeutic doses of APAP on the morphological and histological integrity of the liver: Mouse liver samples were preserved in a 10% formalin solution for 24 hours, followed by dehydration using increasing concentrations of ethanol before being embedded in paraffin blocks. The samples were then sliced into sections with a thickness of 5 µm, and the deparaffinized sections were stained using hematoxylin and eosin. Representative data of mouse weight and liver dimensions, along with images of the excised liver and liver sections stained with hematoxylin and eosin (magnification of 10X and 40X) are displayed.

Discussion

Numerous studies have indicated that the prolonged therapeutic administration of APAP may significantly contribute to the development of long-term hepatic complications, often associated with various adverse effects [8, 9, 11-13]. Clinical case studies have further supported this; for instance, a 67-year-old man with pre-existing cardiopulmonary conditions suffered significant liver damage after only 3 days of taking APAP at a dosage of 1 to 3 g/day [19]. Similarly, a 45-year-old male with HIV and active hepatitis infections faced acute liver toxicity after ingesting 1g daily for four days [28]. Patients with pre-existing myopathies demonstrate an increased risk of acute liver injury from therapeutic doses of APAP [29]. Individuals who engage in excessive alcohol consumption have a heightened risk of acute liver injury due to lower glutathione levels, when taking APAP at therapeutic doses [25, 30]. Thus, underscoring the critical need for vigilant assessment of its side effects during prescription. In this present study, we examined the long-term impacts of both therapeutic and sub-therapeutic doses of APAP on Swiss albino mice, administering 40 mg/kg, as well as sub-therapeutic doses 20 mg/kg, and 10 mg/kg over a 40-day period. Our results showed a troubling outcome: all mice that received either the 40 mg/kg or 20 mg/kg doses died during the study, while 2 out of 6 mice in the 10 mg/kg group also died (Figure 1).

These findings underscore the toxicological risks of prolonged APAP use, even at doses considered therapeutic or lower. Furthermore, we monitored the body weight of the surviving mice on a weekly basis, revealing a significant increase in the control group, in sharp contrast to the notable weight loss seen in the groups treated with APAP. The observed weight loss was correlated with the dosage given, highlighting the significant adverse effects associated with the use of acetaminophen (APAP) at therapeutic doses (Figure 2). Further, mice administered daily doses of APAP at 10, 20, and 40 mg/kg over a 10-day period, resulted in significant hepatocellular injury, and this was indicated by increased levels of bloodstream biomarkers (Figure 3) and moderate increases in liver morphometric parameters relative to total body weight (Figure 4). Histological analysis using haematoxylin and eosin staining demonstrated marked hepatocellular damage at the 40 mg/kg level, with only minor damage observed at the lower dosages (Figure 4). These findings highlight the potential for liver toxicity even at doses considered therapeutic or sub-therapeutic, emphasizing the necessity for careful management of APAP dosage to mitigate the risk of liver damage.

Our study holds considerable importance when compared to previous studies, as the majority of investigations related to APAP concentrate on the toxicity associated with overdoses while the studies examining extended therapeutic doses are quite sparse. Furthermore, there is a lack of preclinical studies on mice indicating that even therapeutic and sub-therapeutic doses could result in toxicity. In our research, we presented experimental findings of toxicity linked to prolonged use of APAP in mice. The reasons underlying this toxicity are intricate and may be related to a depletion of antioxidants or other contributing elements. Additional research is required to elucidate the mechanisms involved in this scenario.

Conclusion

In summary, we found prolonged use of APAP at therapeutic or sub-therapeutic levels can result in considerable liver damage. Signs of negative impact were noted through multiple assessments, including survival rates, biochemical indicators, morphological evaluations (such as the weight of mice and liver morphometric analysis), and histological alterations in Swiss albino mice exposed to prolonged APAP treatment. These findings underscore the necessity of considering the potential side effects of APAP when prescribing this medication and also to look for molecules that would mimic APAP with lesser side effects

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Author contributions: Gopal Kedihithlu Marathe: Supervision, review & editing, Conceptualization, Methodology, Validation. Mylanayakanahosahalli Chandrashekar Indumathi: Original draft, Investigation, Methodology, Visualization, Software, Data curation, Formal analysis. Kamatam Swetha: Methodology. Thumbala Andanaiah Gagan: Methodology.

Data availability: All data underlying the results are available as part of the article and no additional source data are required.

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