

# Evaluation of hepatotoxicity from excessive acetaminophen: physiological and histological changes

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## SUMMARY

We investigated the effect of acetaminophen (APAP), a commonly used drug world-wide on drug-induced liver damage. APAP causes more drug-induced liver injury than any of the other most prescribed drugs such as statins, isoniazid, and methyldopa. APAP has no significant side effects when taken as prescribed, but if too much is taken, known as overdosing, or if taken with alcohol, it can cause severe liver damage. Although APAP-induced hepatic injury is extensively reported, the exact mechanisms by which it impacts distinct hepatic cell types remain ambiguous. Thus, to further understand this drug-induced liver injury, we reviewed indicators of liver damage using serum and liver tissue in mice treated with APAP. We hypothesized that administering high doses of APAP would result in significant toxicity in mice. By measuring the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in mice treated with APAP, we found that excessive APAP is harmful to the liver. Furthermore, the liver tissue of mice treated with excessive APAP seen under a microscope demonstrated deterioration of normal liver architecture, with the presence of larger vacuoles and damaged sinusoidal structures, indicating considerable hepatotoxicity. Our results show that consuming large amounts of APAP can be detrimental to liver health.

## INTRODUCTION

Acetaminophen (APAP) is a non-opioid analgesic and antipyretic agent used worldwide by those who wish to relieve pain (1). APAP stands out as one of the most widely used drugs globally (2). Compared to ibuprofen, another commonly used pain reliever, APAP absorbs into the body more quickly, allowing patients to experience pain relief sooner (3). However, excessive use of APAP or use in combination with alcohol poses a significant risk of hepatotoxicity—acute or chronic liver damage (4-6).

APAP is metabolized in the liver at typical therapeutic doses, with 60–90% of APAP bound to compounds including glucuronide and sulfate before being excreted in the urine (7). Cytochrome P450 (CYP450) processes the remaining 5–15%, resulting in the formation of a toxic metabolite called N-acetyl-p-benzoquinone imine (NAPQI) (Figure 1) (8). Reactive metabolites such as NAPQI are formed in hepatocytes—the predominant cell type in the liver—and their subsequent reactions with lipids and nucleic acids can damage hepatocytes (9). As a result of the damage to the hepatocytes, the proteins aspartate aminotransferase (AST) and alanine aminotransferase (ALT) leak into the plasma. Thus, increased concentrations of these enzymes

in the bloodstream function as biomarkers for hepatic injury, enabling clinicians to evaluate the extent of liver damage and inflammation due to pharmaceuticals or toxins (10).

There are still some critical unsolved questions regarding APAP-induced liver damage, including the specific biochemical pathways involved and why individuals react differently to the drug. The precise pathways and processes that lead to acute liver injury are unknown. Addressing these knowledge gaps could help us better understand how the drug causes liver damage, discover potential biomarkers of susceptibility, and create effective treatments for such conditions.

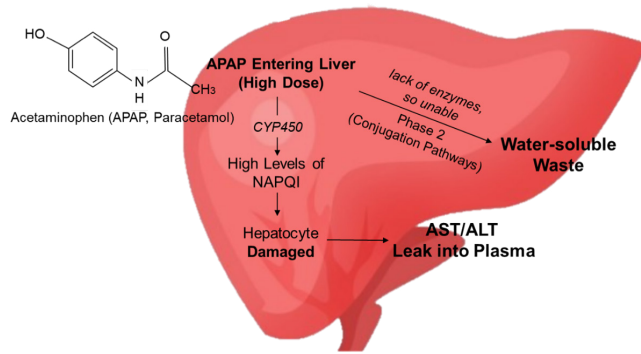
Thus, we conducted experiments to evaluate APAP-induced hepatocyte damage at the serological and histological levels in mice. Based on existing knowledge regarding the toxic effects of high drug doses on the human body, we hypothesized that administering high doses of acetaminophen would result in significant toxicity in mice. Indeed, we noted that escalating dosages of APAP resulted in heightened liver damage. This suggests that liver toxicity is associated with APAP overdose in mice.

## RESULTS

The ALT and AST measurements were conducted as a new experiment using a newly arranged set of mice (Figure 2). The number of mice used is indicated: normal mice ( $n = 4$ ) and 400 mg APAP groups at 4 h ( $n = 3$ ), 6 h ( $n = 6$ ), and 12 h ( $n = 3$ ). The GSH evaluation was performed using the original dataset, and all three mice were included in the analysis.

To evaluate the impact of elevated doses of APAP on hepatotoxicity in mice, we administered APAP at one dosage (400 mg/kg) and monitored ALT and AST levels over a 12 hour period. We selected the dosage of 400 mg/kg based on previous literature where similar dosages effectively induced hepatotoxicity in murine models (11). The typical therapeutic dose for mice is approximately 100 mg/kg; thus, the chosen doses that were substantially higher and therefore expected to produce toxic effects (12). We measured the difference in the serum AST and ALT levels over time when APAP was injected into the abdominal cavity at 400 mg/kg (Figure 2). Serum analysis of ALT and AST levels revealed that mice treated with 400 mg/kg APAP exhibited significantly greater liver injury compared to the control group, which was only injected pure water. Notably, the 400 mg/kg APAP group showed similar levels of hepatic injury across the 0–4 hour time window.

The normal plasma concentrations of ALT and AST in mice are 25–60 U/L and 50–100 U/L, respectively (13). Before injecting APAP, the average serum ALT was 67.5 U/L, and the average serum AST was 161.0 U/L. However, after APAP injection, they both increased. At 4 hours after the 400 mg/kg



**Figure 1: Acetaminophen (APAP) metabolism in liver.** The term "hepatocellular metabolism" refers to the process by which APAP is broken down within liver cells. APAP is primarily metabolized by the enzyme CYP2E1, a subtype of the CYP450 family. The toxic compound NAPQI turns harmless in the presence of glutathione. If there is a lack of enzymes in phase 2 metabolism, meaning an insufficient enzymatic activity to detoxify the high doses of APAP, this overwhelms the glutathione pathway. This is due to an inability of the system to manage excessive NAPQI levels, and it will lead to water soluble waste. AST: aspartate aminotransferase, ALT: alanine transaminase, CYP2E1: Cytochrome P450 2E1, CYP450: Cytochrome P450, NAPQI: N-acetyl-p-benzoquinone.

APAP injection, serum ALT and AST levels were 379.0 U/L and 568.3 U/L, representing 5.6-fold and 3.5-fold increases over baseline, respectively (two-sample t-test,  $p < 0.05$ ). At 6 hours, ALT and AST rose sharply to 7420.0 U/L and 6026.7 U/L, corresponding to 109.9-fold and 37.4-fold increases ( $p < 0.05$ ). By 12 hours, ALT and AST reached 3413.3 U/L and 1740.0 U/L, showing 50.6-fold and 10.8-fold increases over baseline ( $p < 0.05$ ).

A previous study showed that when excessive APAP is metabolized into NAPQI, glutathione decomposes into a toxic substance, which may be the cause of liver damage (14). Thus, we then measured the levels of glutathione in the liver tissue of mice before treatment and 12 hours after treatment with 400 mg/kg the glutathione levels decreased when we

administered 400mg/kg of APAP from 7605 U/L to 2254 U/L (Figure 3).

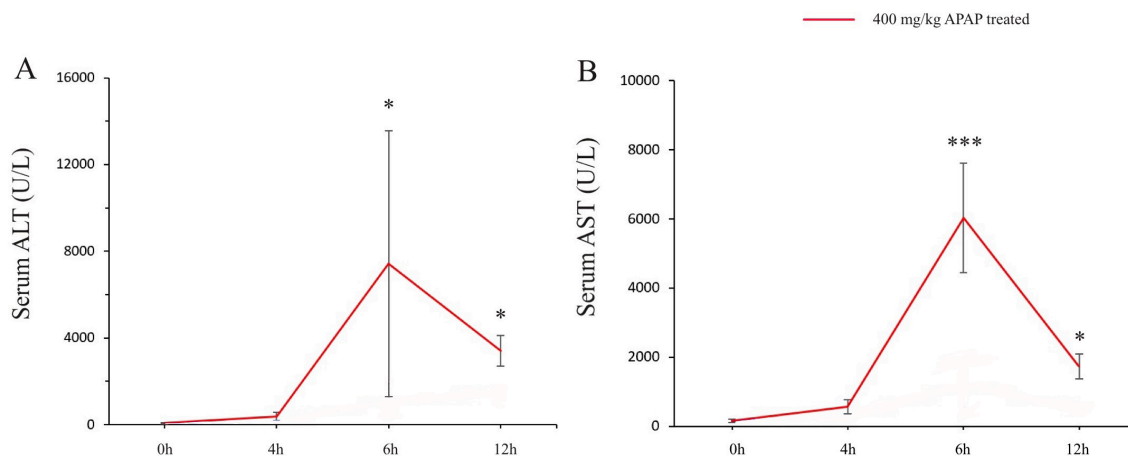
We took images of the liver tissue of the mice under a microscope to visually assess the structural effects of APAP overdose on the liver (Figure 4). Liver tissue from control mice exhibited a typical cellular architecture, characterized by well-organized hepatocytes and consistently arranged nuclei, signifying healthy tissue (15). However, the liver tissue from the 400 mg/kg APAP treated groups were damaged, as seen by the multiple holes in the tissue, abnormal nuclei, and altered color.

## DISCUSSION

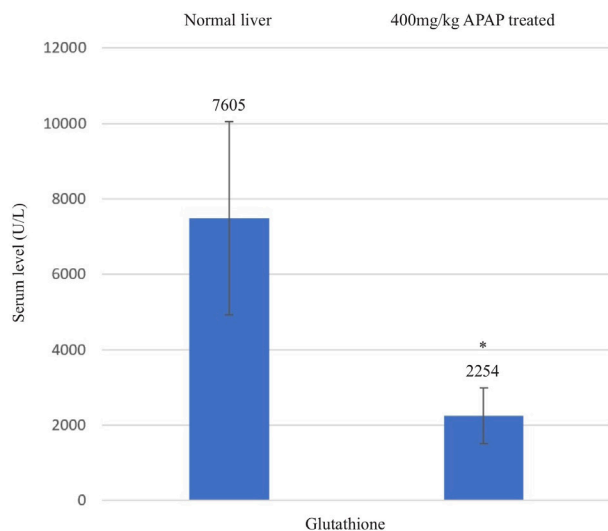
In this study, we evaluated blood ALT and AST levels over time in mice administered large dose, 400 mg/kg, of APAP. Following APAP treatment, we observed a dose-dependent increase in liver enzyme levels (ALT and AST) and histological evidence of hepatotoxicity. The 400 mg/kg group exhibited more severe liver damage than the control group, as indicated by elevated enzyme levels, reduced glutathione, and disrupted hepatic tissue morphology. These results support the hypothesis that excessive APAP metabolism leads to NAPQI accumulation, which depletes glutathione and directly damages hepatocytes (16).

We also found that multiple holes had developed in the mouse liver tissue in the group treated with 400 mg/kg of APAP than those in the control group. This result supports the claim that when too much NAPQI overburdens the glutathione pathway, liver damage occurs as a result. This harmful substance then builds up in the liver, and as a result, the liver tissue is harmed. Thus, we can conclude that APAP overdose-related liver injury occurred in the mice (17). A previous study in mice have shown that APAP hepatotoxicity becomes significantly severe at 300-400 mg/kg, consistent with our findings of enzyme increase and histological damage (18).

A limitation of this study is its use of one particular APAP dosage (400 mg/kg). Assessing further doses, such as therapeutic levels (e.g., 100 mg/kg) or moderate doses (e.g., 300 mg/kg), may clarify the dose-dependent



**Figure 2: Serum ALT and AST levels in mice up to 12 hours after APAP treatment.** A) ALT and B) AST levels were measured in serum samples collected prior to treatment (0 h) and at multiple time points up to 12 hours post-injection. Mice treated with 400 mg/kg APAP showed increases in both ALT and AST levels compared to the control group. Data shown as mean  $\pm$  standard deviation. \*Indicates statistical significance compared to serum levels at 0 h, two-sample t-test,  $p < 0.05$ . AST: aspartate aminotransferase, ALT: alanine aminotransferase.



**Figure 3: Glutathione levels in liver homogenates of mice treated with 400 mg/kg APAP.** Glutathione levels were analyzed from mouse liver tissue samples before or 12 hours after injection with 400 mg/kg APAP to assess glutathione depletion caused by excessive APAP metabolism. Data shown as mean  $\pm$  standard deviation. \*Indicates statistically significant differences (two-sample t-test,  $p < 0.05$ ) compared to the control group. APAP: acetaminophen.

effects of APAP and identify the threshold at which toxicity begins. Additionally, our sample size was relatively small, ( $n = 3-6$  per group), at the 4 h and 12 h time points, and for the glutathione measurements,  $n = 3$ . Because of the limited statistical power, we likely omitted some meaningful differences and may have imprecise estimates for effect size. Using larger cohorts would improve statistical power and better represent inter-individual variation. Another limitation is that we only investigated a single high dose of APAP (400 mg/kg). Although this dose reliably induced hepatotoxicity, it prevented us from establishing a full dose-response curve or

identifying the precise threshold at which toxicity begins. In addition, we only examined early time points up to 12 h after dosing, so we could not assess longer-term outcomes such as recovery, progression to liver failure, or regeneration. Future studies should incorporate a larger range of APAP doses and supplement such issues to establish a detailed threshold at which toxicity emerges. Increasing sample size across all time points would strengthen statistical reliability and reduce uncertainty around effect estimate. If the observation window increases beyond 12h, later phase events can also be accessed and analyzed such as delayed injury progression.

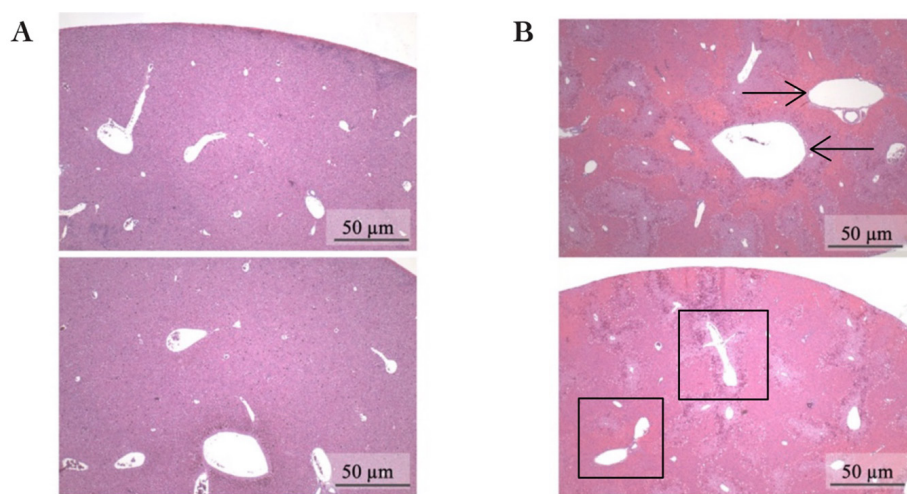
Our study demonstrates that excessively high doses of APAP, one of the most commonly used drugs, can lead to severe hepatotoxicity. Mice break down APAP comparably to humans, allowing us to better understand how overdose causes liver injury (19). In both cases, a dangerous chemical called NAPQI accumulates after consuming too much APAP. This chemical impairs the liver's ability to protect itself, resulting in damage. Studies on mice help us understand how to avoid liver damage by adhering to proper dosage guidelines and being cautious of drug interactions.

## MATERIALS AND METHODS

### ALT/AST assay of mice after APAP injection

All animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee at Chung-Ang University (No. IACUC 22-060-t). Eight-week-old male B6N mice were obtained from KOATECH Co., Ltd. Acetaminophen (Sigma-Aldrich) was dissolved in pure water to prepare the injection solution, which was administered into the abdominal cavity of the mice. In this experiment, 4 mice were administered an APAP-free solution (control), and 12 received 400 mg/kg APAP. Terminal blood samples were collected at 0 (control), 4 ( $n=3$ ), 6 ( $n=6$ ), and 12 hours ( $n=3$ ) post-injection; the eyes of mice were removed to draw terminal blood.

Serum ALT/AST levels in the blood were tested using blood chemistry. Serum extraction was performed, and a



**Figure 4. Histological analysis of liver tissue from control and APAP-treated mice.** Hematoxylin and eosin (H&E) staining shows normal liver architecture in **A**) control mice, while livers from **B**) mice treated with 400 mg/kg APAP display structural abnormalities, including **vacuolation** and disrupted hepatic morphology. Compared to the control **A**) APAP-treated liver **B**) has seen increased eosinophilia, manifested by more pronounced pink staining for hepatocytes, which is consistent with cellular injury. \*Arrows indicate markedly enlarged cytoplasmic vacuoles in hepatocytes. These enlarged vacuoles reflect underlying hepatocyte injury and contribute to the overall disruption of normal hepatic morphology. \*The box highlights irregular, disrupted hepatic architecture in **B**), unlike the clear radial structure seen in **A**.

centrifuge at 10,000 RPM was used to separate the plasma and blood cells. Plasma was collected from the upper layer solution, and the AST and ALT activity was quantitatively evaluated using the Glutathione Assay Kit (Cat. No. 703002, Cayman Chemical) following the manufacturer's instructions.

For data analysis, liver damage was defined based on ALT levels exceeding 180 U/L and AST levels exceeding 300 U/L (based common threshold for indicating liver damage is when enzyme activities are three times higher than the upper limit of normal) (20).

#### H&E staining of liver tissue

After the blood was collected, liver tissue was extracted from two groups: mice that had received 400 mg/kg of APAP and the control group. For hematoxylin and eosin staining, paraffin-embedded tissue was utilized. Glutathione levels in the liver tissue were measured following the manufacturer's instructions using a Glutathione Assay Kit (Cat. No. 703002, Cayman Chemical) (21).

#### Statistical testing

A two-sample t-test was used to calculate p-values and assess the statistical significance of differences in ALT, AST, and GSH levels between the control group and the 400 mg/kg APAP group ( $\alpha = 0.05$ ).

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