

Time-of-day affects acetaminophen lethality despite comparable early blood concentrations: Implications for forensic toxicology

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Abstract

In forensic toxicology, acute drug poisoning diagnosis relies primarily on blood concentration measurements; however, static values cannot capture temporal variations in drug metabolism. To investigate whether administration timing affects the relationship between blood concentration and toxic outcome, acetaminophen (APAP) was used as a model. Male C57BL/6 mice were administered APAP (600 mg/kg, i.p.) at 8:00 AM (AM group) or 8:00 PM (PM group). Blood APAP concentrations at 1–2 hours post-administration showed no significant difference, indicating comparable initial systemic exposure. Nevertheless, 48-hour survival rates differed markedly (80% AM vs. 10% PM). The PM group displayed significantly elevated serum ALT/AST, extensive hepatocellular necrosis, and increased apoptosis. These changes were associated with higher CYP2E1 and lower GCLC expression in the PM group, suggesting enhanced toxic metabolite formation and reduced detoxification capacity, which triggered a secondary inflammatory response. These findings demonstrate that significant differences in clinical outcome can emerge depending on administration timing even with comparable initial blood APAP concentrations. This highlights the limitations of relying solely on quantitative toxicological data and underscores the necessity of incorporating dynamic temporal and metabolic contexts into forensic evaluations.

Introduction

In forensic medicine, diagnosing death due to acute drug poisoning remains both essential and challenging [1, 2, 3]. Often, autopsy findings are limited to nonspecific changes, and quantitative toxicity analyses such as GCMS (Gas Chromatography-Mass Spectrometry) and LC-MS/MS (Liquid Chromatography–Tandem Mass Spectrometry) are considered crucial in determining the cause of death [2, 3]. However, interpreting measured drug concentrations is inherently complex and influenced by numerous pharmacokinetic and pharmacodynamic factors, including metabolism, redistribution, and individual variability. Indeed, postmortem toxicology is known to be affected by processes such as postmortem redistribution and time-dependent changes, complicating the direct correlation between measured concentrations and actual toxicity or mortality [4].

In clinical and experimental pharmacology, it is well known that drug metabolism is not static but dynamically regulated by circadian rhythms [5, 6, 7]. Hepatic metabolic capacity, particularly the expression and activity of cytochrome P450 enzymes, fluctuates throughout the day, affecting the efficacy and toxicity of drugs. Acetaminophen (APAP) is one of the most widely used analgesics and antipyretics in the world, but it is a major cause of drug-induced liver injury and acute liver failure in many countries. Its hepatotoxicity is primarily mediated by N-acetyl-p-benzoquinone imine (NAPQI), a reactive metabolite mainly produced by CYP2E1, which can cause severe liver damage if glutathione detoxification cannot keep up [6, 8, 9].

Importantly, accumulated evidence indicates that APAP toxicity exhibits significant circadian variation (chronotoxicity). Experimental studies have shown that changes in circadian rhythms significantly

modulate APAP-induced liver injury through changes in metabolic and detoxification pathways. Furthermore, CYP2E1 activity and glutathione-dependent detoxification exhibit diurnal variation, leading to time-dependent differences in susceptibility to hepatotoxicity. These findings suggest that even with the same dose or similar blood concentrations, biological outcomes can vary significantly depending on the metabolic state at exposure [5, 10].

Despite these established findings, forensic toxicology does not adequately consider such temporal and biological variations in the interpretation of drug concentrations. In modern society, lifestyle factors such as irregular sleep patterns, alcohol consumption, and smoking can further modulate metabolic enzyme activity, particularly CYP2E1 activity, potentially amplifying the discrepancy between measured concentrations and actual toxicity. Therefore, relying solely on static quantitative values can lead to misinterpretations in forensic judgments.

This study aimed to experimentally demonstrate the discrepancy between blood drug concentrations and toxic outcomes from a forensic perspective, using acetaminophen (APAP) as a model. Focusing on the initial time point where there was no difference in blood concentration, we compared the subsequent progression to survival or fatal liver damage based on differences in administration timing. Furthermore, we investigated the underlying mechanisms by analyzing circadian variations in metabolic activation (CYP2E1), detoxification capacity (GCLC), and downstream inflammatory responses. This study provides new insights into the limitations of conventional toxicological interpretations and emphasizes the importance of incorporating temporal and metabolic contexts in forensic evaluations.

Results

Time-Dependent Differences in Survival and Liver Injury After Acetaminophen Administration

After APAP administration (600 mg/kg), both groups showed signs of toxicity, but survival rates differed significantly depending on the administration time (Figure 1). The AM group showed an 80% survival rate at 48 hours, while the PM group had a survival rate of only 10%, with most deaths occurring within 48 hours (Figure 1). Consistent with this, biochemical and histopathological analyses revealed more severe liver injury in the PM group (Figure 2). Serum ALT and AST levels were significantly higher in the PM group at all measurement time points (Figure 2c, d). Histological examination showed extensive hepatocyte necrosis, hemorrhage, and inflammatory cell infiltration in the PM group, while the AM group showed relatively mild and localized damage (Figure 2a, b). Semi-quantitative scoring further confirmed that the PM group had significantly greater liver injury compared to the AM group (Figure 2e). Taken together, these findings suggest that, even under identical administration conditions, the timing of APAP administration has a decisive impact on survival rates and the severity of acute liver injury.

Enhanced Hepatitis and Apoptosis in the PM Group

After APAP administration, inflammatory cell infiltration and hepatocyte apoptosis were more pronounced in the PM group compared to the AM group (Figure 3). Immunohistochemical analysis

showed that the number of F4/80-positive macrophages and CD3-positive T cells increased over time in both groups, peaking at 24 hours, but these increases were consistently significantly greater in the PM group (Figures 3a-f). Furthermore, ssDNA staining showed a significant increase in apoptotic hepatocytes at 24 hours in the PM group, compared to only a slight change in the AM group (Figures 3g-i). Quantitative analysis confirmed a significant increase in both inflammatory cell infiltration and apoptosis-positive cells in the PM group. These findings suggest that nocturnal APAP administration induces enhanced inflammatory responses and promotes hepatocyte apoptosis, contributing to the worsening of liver damage.

Overinduction of Inflammatory Cytokines and Chemokines in the PM Group

After APAP administration, the expression of inflammatory cytokines and chemokines in liver tissue was significantly elevated in the PM group (Figure 4). At baseline (0 hours) and initial time (10 hours), there was no significant difference between the AM and PM groups in mRNA expression levels of cytokines (Il1b, Il6, Tnfa, Ifng) or chemokines (Ccl3, Cxcl1, Cxcl9) (Figure 4a-e, f-h). However, at 24 hours post-administration, the PM group showed a significantly increased expression of all measured cytokines and chemokines compared to the AM group (Figure 4). In particular, the significant increase in pro-inflammatory cytokines and leukocyte-mobilizing chemokines was consistent with the enhanced inflammatory cell infiltration observed histologically (Figure 3). These results suggest that nocturnal APAP administration likely amplified a secondary inflammatory response, contributing to the exacerbation of liver damage observed in the PM group.

Serum APAP concentrations were similar, and there was no effect on glucuronidation-related gene expression

There were no significant differences in serum APAP concentrations or glucuronidation-related gene expression between the morning and afternoon administration groups (Figure 5). Serum APAP concentrations 1- and 2-hours post-administration were similar in both groups, indicating similar initial systemic exposure (Figure 5a). Furthermore, there were no significant differences in mRNA expression levels of enzymes involved in UDP-glucuronide synthesis (including Ugp2 and Ugdh) between the morning and afternoon administration groups (Figures 5b, c). These results suggest that differences in APAP absorption or variations in glucuronidation-mediated detoxification are not the cause of the differences in toxicity observed between the two groups. Rather, these results support the idea that time-dependent differences in downstream metabolic activation and detoxification pathways are the cause of the differences in results.

Time-Dependent Imbalance Between Metabolic Activation and Detoxification Pathways

Genetic expression analysis of the liver revealed a significant imbalance between the metabolic activation and detoxification pathways depending on the administration time (Figure 6). The mRNA expression level of Cyp2e1, the major enzyme that in vivo activates APAP to the toxic metabolite NAPQI, was significantly higher in the PM group compared to the AM group (Figure 6a). Conversely, the

expression level of Gclc, the rate-limiting enzyme in glutathione (GSH) synthesis and a crucial component of detoxification capacity, was significantly lower in the PM group (Figure 6b). These contradictory changes suggest that nocturnal administration is associated with increased production of toxic metabolites and decreased detoxification capacity. A schematic diagram (Figure 6c) illustrates the transition to a toxic-promoting metabolic state in the PM group. Taken together, these findings suggest that circadian fluctuations in liver enzyme expression cause metabolic imbalance, making the PM group more susceptible to severe APAP-induced liver injury.

Discussion

Acetaminophen (APAP) is one of the most widely used analgesics and antipyretics worldwide and is generally considered safe at therapeutic doses [12, 13]. However, overdoses remain a major cause of drug-induced liver injury and acute liver failure globally. The hepatotoxicity of APAP is primarily mediated by N-acetyl-p-benzoquinone imine (NAPQI), a reactive metabolite produced by cytochrome P450 enzymes, particularly CYP2E1. When glutathione (GSH) is depleted, NAPQI binds to cellular proteins, leading to mitochondrial dysfunction, oxidative stress, and ultimately hepatocyte death [14, 15, 16, 17].

The most noteworthy finding of this study is the marked discrepancy between blood APAP concentrations and clinical outcomes. In mice, the time to reach peak blood concentration (T_{max}) following intraperitoneal APAP administration is generally within 30 to 60 minutes [18]. Thus, our sampling at 1 and 2 hours post-administration effectively captured the initial peak systemic exposure (C_{max}) and the early elimination phase, demonstrating that initial systemic drug exposure, absorption kinetics, and bioavailability were comparable regardless of the administration timing. Furthermore, the absence of significant differences in hepatic expression of UDP-glucuronosyltransferase-related genes (*Ugdh* and *Ugp2*) indicates that major non-toxic clearance pathways were similarly functional in both groups. These findings consistently suggest that blood APAP concentrations were unlikely to diverge markedly after 2 hours. Importantly, these data clearly decouple systemic pharmacokinetics (PK) from intracellular pharmacodynamics/toxicodynamics (PD). Despite equivalent initial systemic drug burdens, the intracellular metabolic fate of APAP was driven in drastically opposing directions by the time-of-day-dependent balance between bioactivation (CYP2E1) and detoxification (GCLC/GSH), ultimately resulting in a dramatic divergence in 48-hour survival rates (80% vs. 10%). This fundamental divergence between static circulating concentrations and dynamic organ-level toxicity challenges the conventional assumption in forensic toxicology that initial blood drug concentrations serve as a reliable, direct surrogate marker for toxicity and lethality. While postmortem toxicological interpretation is inherently complex due to confounders such as metabolism, postmortem redistribution, and individual variability [4], our findings highlight the inherent limitations of relying solely on static quantitative data and underscore the critical necessity of integrating dynamic metabolic contexts and temporal factors into forensic evaluations.

A key mechanistic explanation for this contradiction lies in the time-of-day-dependent regulation of hepatic metabolism. The liver exhibits strong diurnal variations in gene expression related to xenobiotic

metabolism, detoxification, and inflammatory responses. In particular, CYP2E1 activity and the GSH-dependent detoxification pathway are known to fluctuate on a 24-hour cycle, leading to time-dependent differences in susceptibility to APAP-induced hepatotoxicity. In this study, the PM group showed increased CYP2E1 expression and decreased GCLC expression, suggesting enhanced *in vivo* activation of APAP and decreased detoxification capacity. This imbalance is thought to have led to excessive accumulation of NAPQI and increased vulnerability to hepatocyte damage [7, 10].

Importantly, this metabolic abnormality appears to cause downstream amplification of the inflammatory response. APAP-induced liver injury is now understood to involve not only direct toxicity to hepatocytes but also a secondary sterile inflammatory response mediated by cytokines, chemokines, and immune cell infiltration. In this study, the PM group showed significant upregulation of inflammatory cytokines and chemokines at 24 hours, accompanied by increased macrophage and T cell infiltration. This excessive inflammatory response is thought to have contributed to the progression from initial metabolic dysfunction to widespread hepatic necrosis and organ failure [19].

These findings have critical and practical implications for forensic toxicology. Traditionally, toxicological evaluations have heavily relied on standardized reference concentration ranges—such as therapeutic, toxic, and lethal thresholds—derived from controlled clinical settings. However, such static reference values fail to capture the dynamic biological processes, including circadian phase, metabolic enzyme fluctuations, and environmental modifiers, that dictate *in vivo* drug toxicity. As demonstrated in this study, identical initial drug concentrations can yield vastly disparate clinical outcomes depending on the temporal metabolic state at exposure, underscoring that sole reliance on static concentration values carries an inherent risk of misinterpreting the cause of death. Accordingly, our results advocate for two essential diagnostic strategies in forensic death investigations: First, in borderline cases where postmortem blood drug concentrations fall within a gray zone between therapeutic and toxic thresholds, determining the estimated time of drug ingestion relative to the individual's circadian context—including lifestyle factors such as shift work or alcohol-induced CYP2E1 state—becomes indispensable. A blood concentration that might otherwise be considered non-lethal can indeed trigger fatal toxicity if exposure occurs during a metabolic phase characterized by impaired detoxification capacity. Second, our findings highlight the necessity of transitioning from single-parameter quantitative evaluations to an integrated, multi-marker diagnostic approach. In routine autopsy practice, quantitative blood measurements should be systematically integrated with histopathological scoring of hepatocellular necrosis, immunohistochemical assessment of inflammatory infiltration and apoptosis (e.g., F4/80 and ssDNA), and molecular profiling of metabolic/detoxification status (or APAP-protein adducts). Combining these complementary parameters will overcome the limitations of static drug concentrations, providing a far more objective and robust foundation for forensic causality assessment.

Several potential limitations must be considered when extrapolating these findings to human forensic scenarios. First, rodents are nocturnal animals whose circadian activity, metabolic cycles, and feeding rhythms are phase-shifted relative to diurnal humans [20, 21]. Consequently, the time-of-day differences observed at 8:00 AM and 8:00 PM in mice cannot be directly translated to identical wall-clock times in

human clinical or forensic settings. Nevertheless, human hepatocytes and liver tissue are also known to exhibit robust autonomous circadian rhythms in the expression of xenobiotic-metabolizing enzymes—including CYP2E1—and in cellular antioxidant capacity [7]. Rather than aiming to define specific vulnerable time windows in humans, the primary objective and strength of this study provide a proof-of-concept: even when initial systemic drug exposure (blood concentration) is equivalent, biological lethality can vary dramatically depending on the metabolic state dictated by the circadian phase. Therefore, in forensic interpretations of drug-induced deaths, relying solely on static postmortem blood concentrations is insufficient; dynamic, context-dependent variables, such as the temporal metabolic status at the time of exposure, must be carefully integrated. Secondly, real-world cases involve more complex factors that may further modulate CYP2E1 activity and GSH availability, such as concomitant medications, chronic alcohol consumption, and disease status [22, 23]. For example, chronic alcohol consumption is known to induce CYP2E1 expression, thereby increasing APAP toxicity, but it also contributes to oxidative stress and reduced detoxification capacity. Third, blood APAP concentrations were measured only at early post-administration time points (1 and 2 hours). While these sampling points effectively reflect initial absorption, peak systemic exposure ($C_{\max}$), and early elimination dynamics in mice, full pharmacokinetic profiling—including systemic clearance (CL), half-life ($t_{1/2}$), and total area under the concentration-time curve (AUC)—was not determined. Although a more comprehensive pharmacokinetic evaluation across later time points remains a subject for future study, the equivalent early blood concentrations firmly demonstrate that differences in initial systemic drug burden do not account for the drastic divergence in lethality.

Finally, several limitations of this study should be noted. We evaluated APAP toxicity at only two specific time points: 8:00 AM and 8:00 PM. Although these time points clearly demonstrated marked time-of-day-dependent divergences in lethality and metabolic enzyme expression, they do not provide a continuous 24-hour circadian profile. Future studies incorporating multiple sampling time points across the 24-hour cycle will be valuable to precisely delineate the continuous circadian kinetics of this metabolic discrepancy. Although time-of-day-dependent variations in APAP hepatotoxicity have been reported in previous animal studies, the novelty and forensic significance of the present study lie in demonstrating that, despite comparable early circulating APAP concentrations (1–2 h post-administration, representing initial peak exposure and early clearance), subsequent outcomes diverged dramatically, resulting in 48-hour survival rates of 80% versus 10%. In conclusion, our findings demonstrate that even with comparable blood drug concentrations, dynamic temporal factors and the underlying metabolic context can dictate profoundly different toxicological outcomes. This underscores the fundamental limitation of relying solely on static quantitative toxicological data in forensic practice and highlights the crucial need for a more comprehensive, dynamic approach that integrates temporal factors, metabolic status, and individual background parameters when evaluating drug-related deaths.

Materials and Methods

Reagents and Antibodies

Acetaminophen was purchased from Sigma-Aldrich (A7085, St. Louis, MO). The following primary antibodies were used in this study: rat anti-mouse F4/80 monoclonal antibody (MABF1968, Dainippon Pharmaceutical, Osaka, Japan), rat anti-mouse CD3 monoclonal antibody (17A2, Dainippon Pharmaceutical) and rabbit anti-single-stranded DNA (ssDNA) polyclonal antibody (MAB3868, Sigma-Aldrich, Inc. St. Louis, MO).

Mice

Pathogen-free male C57BL/6 mice, 8-12 weeks old, were obtained from SLC (Shizuoka, Japan). During the experiment, all mice were individually housed under specific pathogen-free conditions and on a 12-hour light-dark cycle (lights on at 8 AM, lights off at 8 PM). All animal experiments were approved by the Animal Experiment Committee of Wakayama Medical University (Approved No. 746). All methods were performed in accordance with relevant guidelines and regulations including the ARRIVE 2.0 reporting guidelines. We also referred to the American Veterinary Medical Association's (AVMA) Guidelines for Animal Euthanasia (2020) for comprehensive guidance on veterinary best practices for animal anesthesia and euthanasia. Mice were euthanized by cervical dislocation under deep anesthesia with isoflurane (3%, 0.3 L/min) using the Animal Anesthesia System SN-487 (Shinano Seisakusho, Tokyo, Japan).

Preparation of an APAP-Induced Liver Injury Model

Mice were fasted with free access to water for 24 hours prior to APAP administration. The APAP solution was prepared by dissolving it in phosphate-buffered saline (PBS, pH 7.2) and warmed to 37°C immediately before administration. Mice were divided into two groups: an AM group (administered at 8:00 AM) and a PM group (administered at 8:00 PM). APAP (600 mg/kg) was administered intraperitoneally to both groups. After administration, the survival rate and clinical course of each group were observed.

Serum Biochemical Analysis

Blood samples were collected from mice at 2, 6, 10, 24, and 48 hours after APAP administration, and serum was obtained by centrifugation. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using an automated biochemical analyzer (Fuji Drychem 7000V, Fujifilm Medical, Tokyo).

Histopathological and Immunohistochemical Analysis

Mice were euthanized at 10 and 24 h after APAP administration, and their livers were removed. The collected tissue was fixed in 10% formalin buffered with PBS (pH 7.2) and then embedded in paraffin according to standard procedures. 6 µm thick sections were stained with hematoxylin and eosin (HE). The degree of hepatocyte necrosis was evaluated using a five-point scale under blind conditions, excluding information from the experimental group: 0: no necrosis, 1+: mild centrilobular necrosis, 2+: severe necrosis limited to the central lobule, 3+: extensive necrosis extending from the central lobule to

the portal area, 4+: extremely extensive necrosis throughout the entire liver. For immunohistochemical staining, rat anti-mouse F4/80 antibody (macrophages), rat anti-mouse CD3 antibody (T cells), and rabbit anti-ssDNA antibody (apoptotic cells) were used. Infiltrating inflammatory cells and apoptosis-positive cells were counted in 10 randomly selected high-power fields (HPFs) at 400x magnification, and the average value was calculated.

Total RNA Extraction and Real-Time RT-PCR Analysis

Total RNA extraction from liver tissue was performed using ISOGEN II (Nippon Gene, Toyama) according to the product protocol. 1 µg of the obtained total RNA was reverse transcribed using PrimeScript RT Reagent Kit (Takara Bio, Otsu) and random primers in a total of 20 µL of reaction system at 37°C for 15 minutes to synthesize cDNA. Real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio) and gene-specific primer sets on a Thermal Cycler Dice Real Time System II (Takara Bio). The expression levels of each target gene were corrected using the expression level of Gapdh as an internal standard and calculated as relative values to the mean of the control group.

Measurement of serum APAP concentration by GC-MS

Blood samples were collected at 1 and 2 hours after APAP administration, and serum APAP concentrations were measured using an Agilent 7890B gas chromatograph and 5977A mass spectrometer (Agilent Technologies, Santa Clara, California, USA). Target components were extracted from cardiac blood (0.05 mL) by the QuEChERS method and acetylated derivatized with acetic anhydride [11]. The resulting extract was evaporated to dryness under a nitrogen stream at room temperature and dissolved in 100 µL of GC-MS redissolution solvent (1-chlorobutane/isopropanol, 3:1). 2 µL of this sample solution was injected into a GC-MS instrument and subjected to analysis.

Statistical analysis

Data are expressed as the mean and standard error (SE). Survival curves were analyzed using the log-rank test. Differences among groups were evaluated by one-factor analysis of variance, followed by Scheffe's F test for post-hoc comparisons. Histological scores were analyzed using the Mann-Whitney U test. A P-value of < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01).

Declarations

Ethics

All animal experiments were approved by the Committee on Animal Care and Use at Wakayama Medical University (approval number 746), and all methods were performed in accordance with relevant regulations and guidelines including the ARRIVE guideline.

Author contribution

Y. I. and T. K. formulated the hypothesis and designed the project; H. Y. and Y. I. performed the main experiments; Y. K., A. I., and Y. I. provided technical assistance and discussion; M.N., A.I., Y.K., M.O., S.P., W.Z., and M.H. helped with some experimental procedures; A. K. and N. I. oversaw the experiments; H. Y. and T. K. participated in writing the manuscript.

Data availability

The authors declare that all data are available in the article file and, available from the authors upon reasonable request.

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Figures

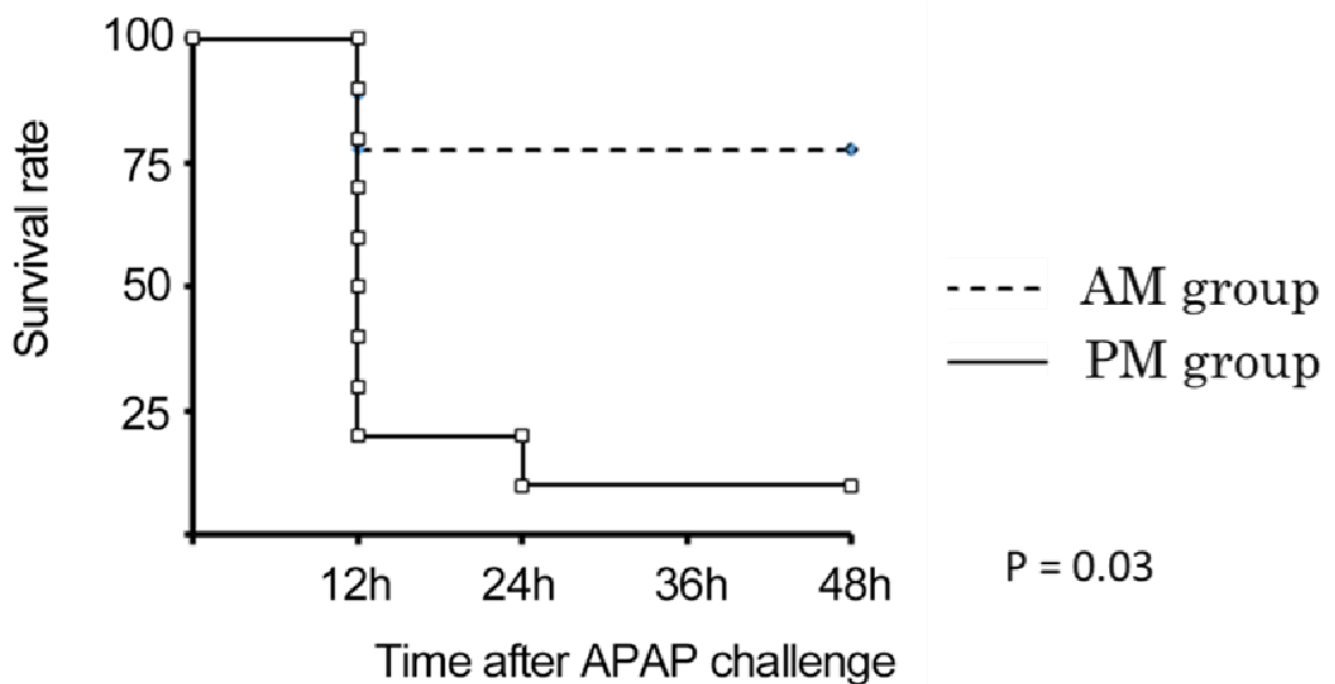


Figure 1

Time-dependent differences in survival following acetaminophen administration. Mice in the AM group (administered at 8:00 AM) and PM group (administered at 8:00 PM) were intraperitoneally injected with acetaminophen (600 mg/kg). Survival was monitored at 12, 24, 36, and 48 hours after administration. The PM group showed a significantly lower survival rate compared to the AM group over the observation period. Statistical significance between groups was determined using the log-rank test ($P = 0.03$). Each group included 10 animals.

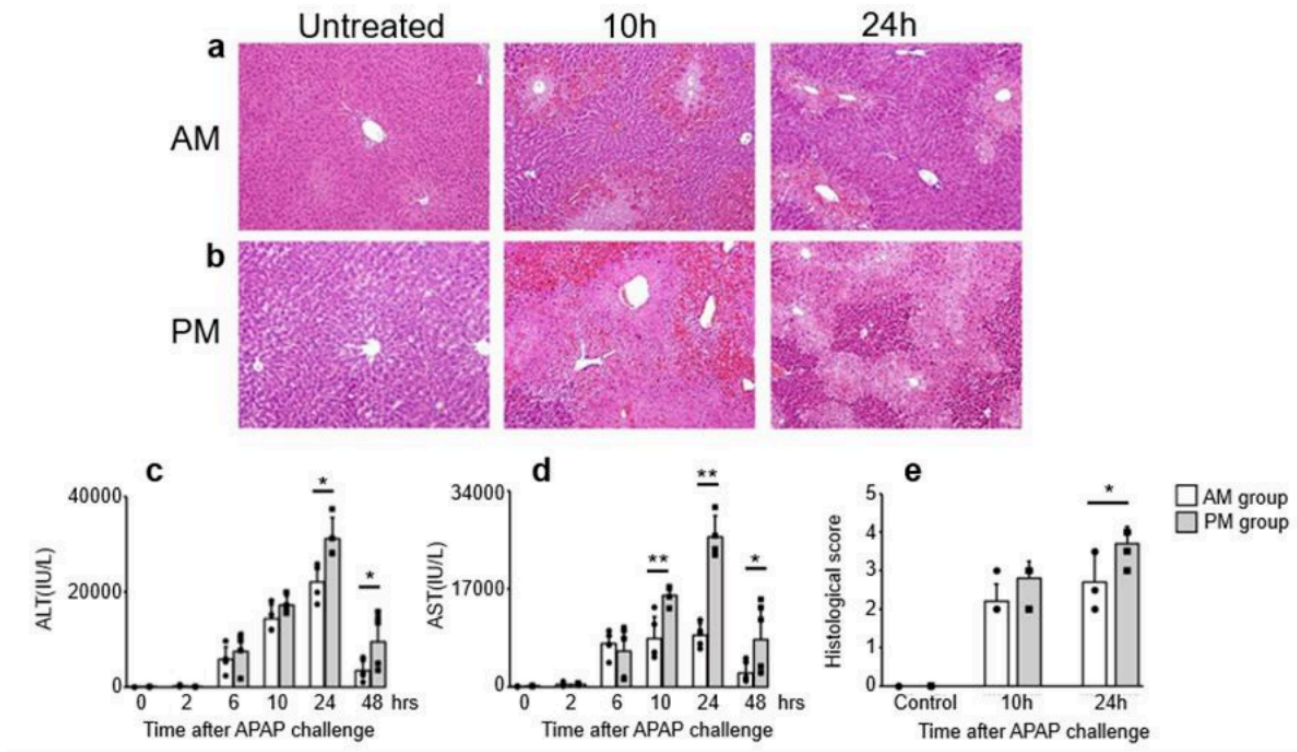


Figure 2

Evaluation of acetaminophen-induced liver injury in the morning and afternoon administration groups. (a, b) Representative hematoxylin-eosin (H&E) stained liver tissue sections (original magnification $\times 400$) from the morning administration group (a) and the afternoon administration group (b). The afternoon administration group showed more extensive hepatocyte necrosis, hemorrhage, and inflammatory cell infiltration compared to the morning administration group. (c, d) Time course of serum alanine aminotransferase (ALT) (c) and aspartate aminotransferase (AST) (d) levels at 2, 6, 10, 24, and 48 hours after acetaminophen administration. The afternoon administration group showed significantly higher enzyme levels and more severe liver injury. (e) Semi-quantitative histological score of liver injury as described in "Materials and Methods". The afternoon administration group showed a significantly higher injury score than the morning administration group. Data are shown as mean $\pm$ standard error ($n=5$ for each group). The open-circle bar graphs represent the morning group, and the filled-in bar graphs represent the afternoon group. * $P < 0.05$, ** $P < 0.01$.

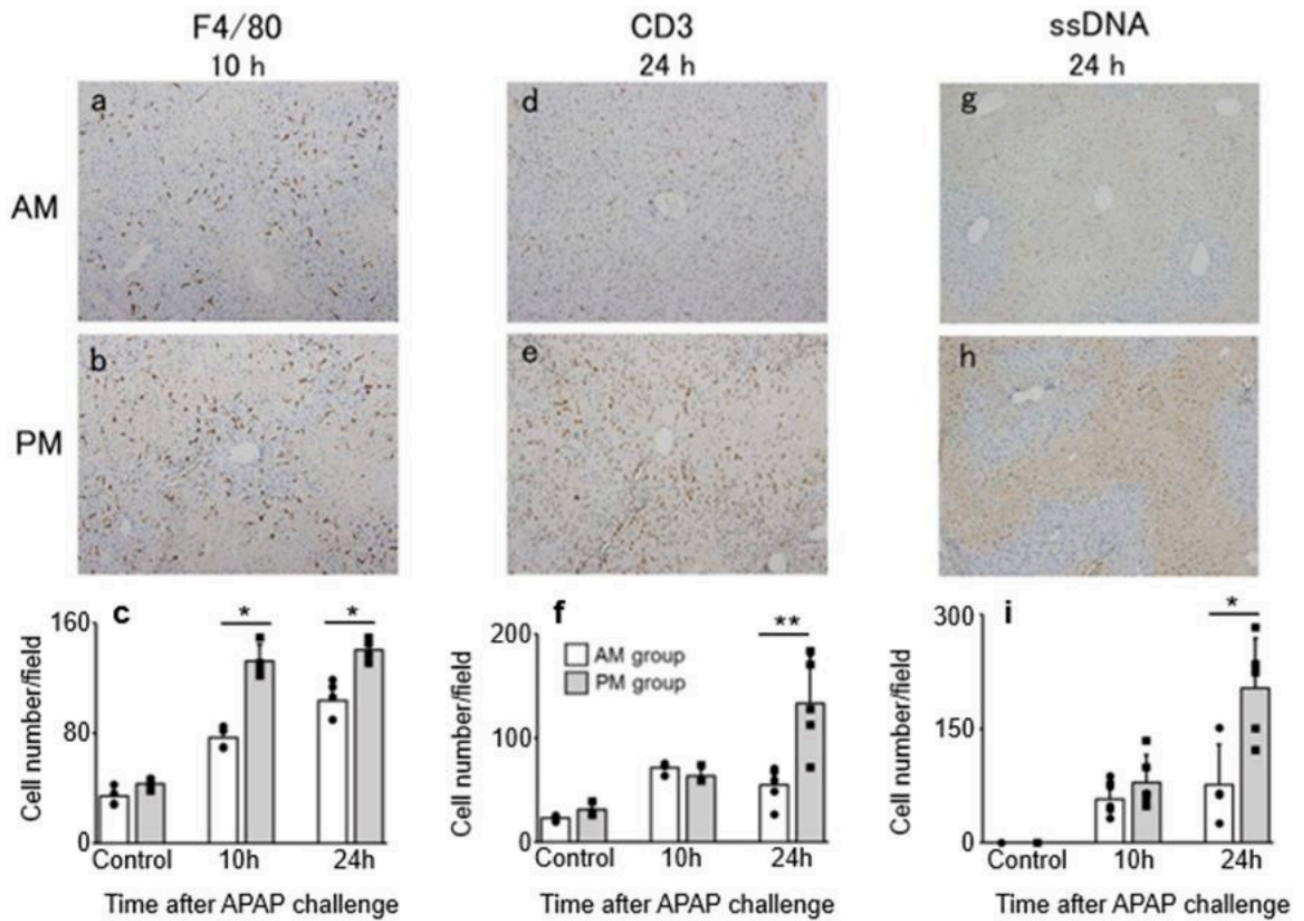


Figure 3

Enhanced inflammatory cell infiltration and hepatocyte apoptosis in AM and PM groups. (a, b) Representative F4/80 immunostaining images (macrophages) at 10 hours; (c) Quantitative analysis. (d, e) Representative CD3 immunostaining images (T cells) at 24 hours; (f) Quantitative analysis. (g, h) Representative ssDNA immunostaining images (apoptotic cells) at 24 hours; (i) Quantitative analysis. Inflammatory cell infiltration and apoptosis were significantly increased in the PM group compared to the AM group. Data are shown mean $\pm$ standard error (n=5). *P < 0.05, **P < 0.01.

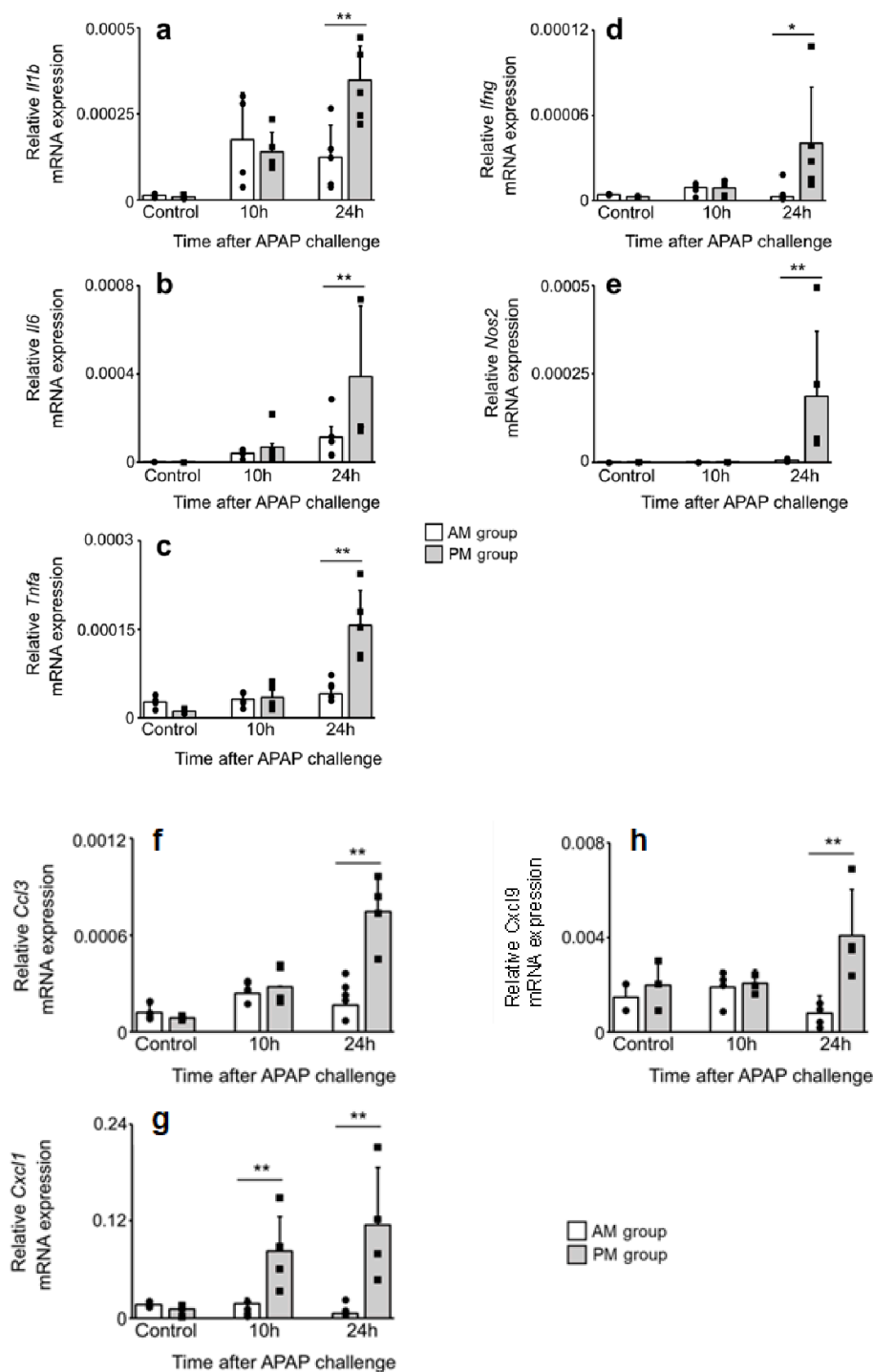


Figure 4

Time-dependent induction of inflammatory cytokines and chemokines in the liver. Hepatic inflammatory cytokine mRNA expression at 10 and 24 hours after APAP administration: (a) *Il1b*, (b) *Il6*, (c) *Tnfa*, (d) *Ifng*, (e) *Nos2*. Hepatic chemokine mRNA expression at 10 and 24 hours after APAP administration: (f) *Ccl3*, (g) *Cxcl1*, (h) *Cxcl9*. In the PM group, cytokine and chemokine expression increased significantly at

24 hours compared to the AM group. Data are shown as mean $\pm$ standard error (n=5). *P < 0.05, **P < 0.01.

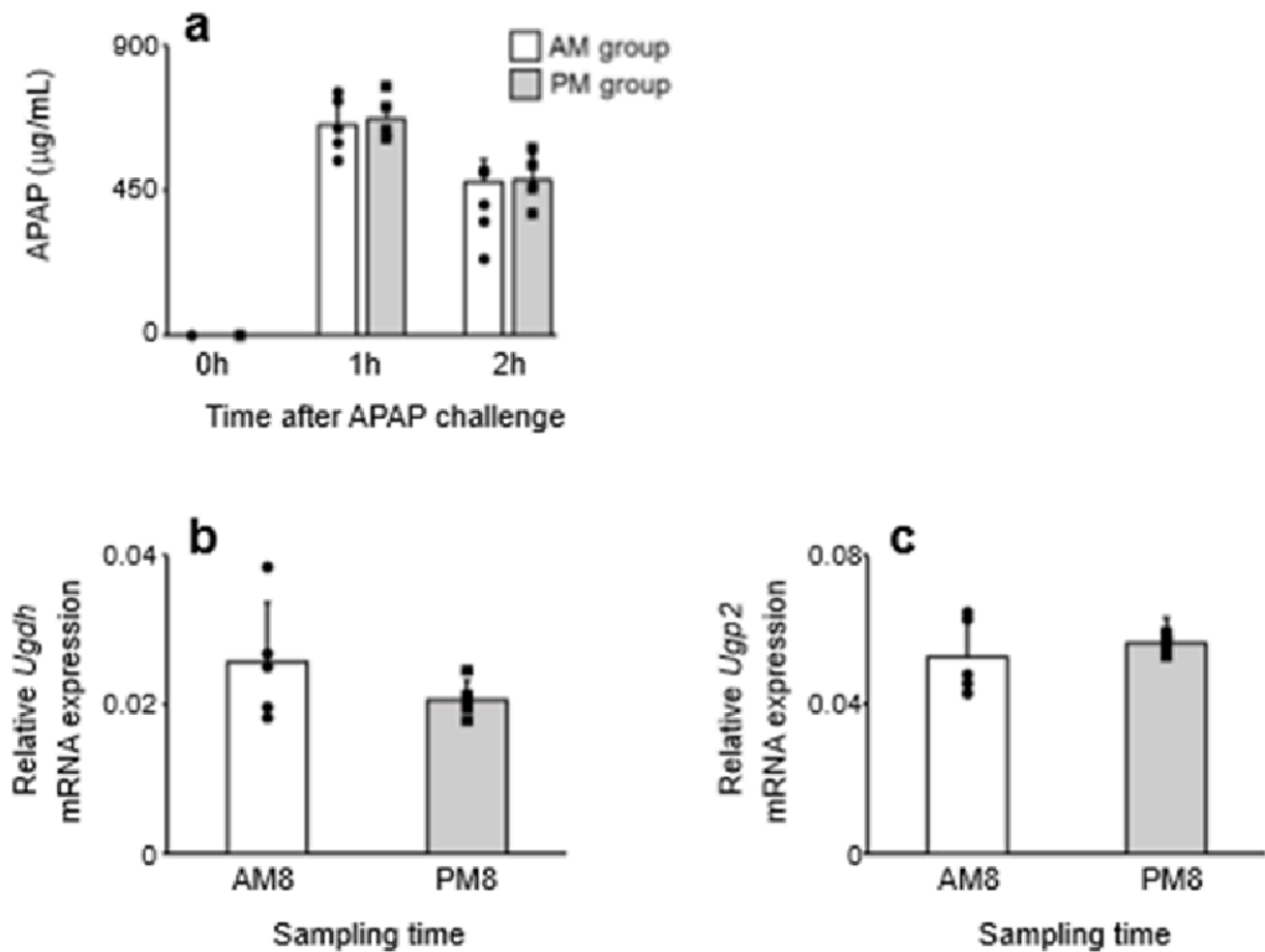


Figure 5

Comparable blood acetaminophen concentrations and glucuronidation-related gene expression. (a) Blood APAP concentrations at 1 and 2 hours after administration in AM and PM groups. (b, c) Hepatic mRNA expression of glucuronidation-related enzymes: (b) *Ugdh* and (c) *Ugp2*. No significant differences were observed between the AM and PM groups in either blood APAP concentrations or gene expression levels. Data are presented as mean $\pm$ SE (n = 5).

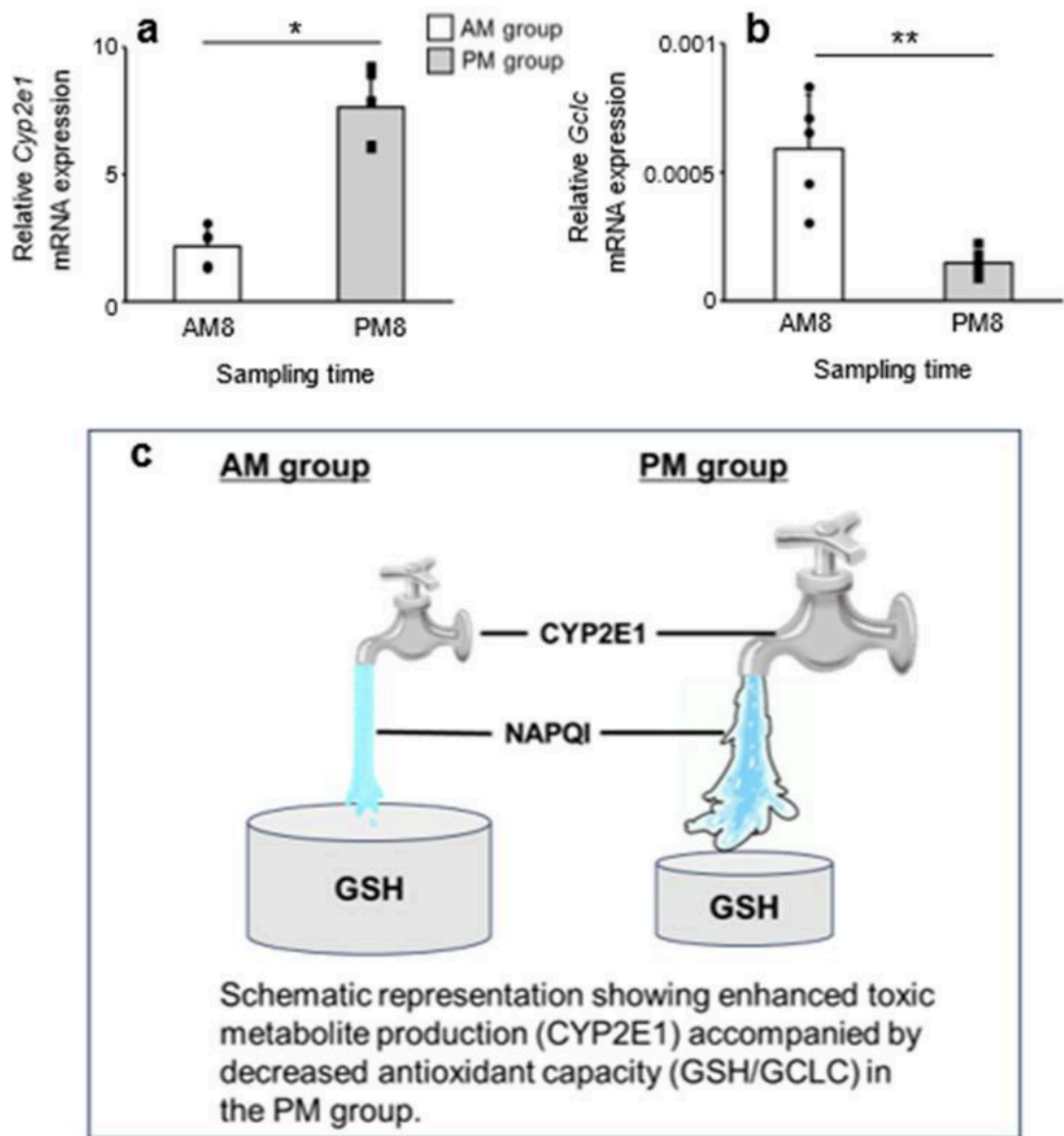


Figure 6

Time-dependent imbalance between metabolic activation and detoxification pathways. (a, b) mRNA expression of (a) *Cyp2e1* and (b) *Gclc* in the liver of the AM and PM groups. (c) Schematic diagram summarizing the metabolic balance between the production of toxic metabolites and detoxification. In the PM group, *Cyp2e1* expression is increased and *Gclc* expression is decreased, indicating enhanced

metabolic activation and decreased detoxification capacity. Data are shown as mean $\pm$ standard error (n=5). *P < 0.05, **P < 0.01.