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ORIGINAL ARTICLE

# Effect of Nutrition on Drug-Induced Liver Injury: Insights from a High-Fat Diet Mouse Model

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## ABSTRACT

**Objectives:** Literature suggests that a high-fat diet (HFD) potentially increases the risk of chemical/drug-induced toxicity after an acute overdose. Drug/chemical-induced hepatotoxicity has been well studied, and the mechanism that regulates this toxicity has been extensively examined using different experimental animal models. Our study focuses on drug-induced hepatotoxicity in HFD-fed female Balb/C mice. This study addresses the effect of nutrition on the magnitude of acetaminophen (APAP)-induced hepatotoxicity at different time intervals.

**Materials and Methods:** Female Balb/C mice, after the weaning period separated into two different groups, normal diet (ND) and HFD receiving groups; after 15 weeks, they were dosed with a single dose (300 mg/kg *per os* (*p.o.*) of APAP. Blood samples were collected at different time intervals (0, 6 and 24 hours), and liver samples were collected at the end time point. Liver injury parameters [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], antioxidant assay (sodium dismutase, glutathione, and catalase), and histopathology study were conducted. Pharmacokinetic (PK) analysis was done using the RP-HPLC system and Phoenix WinNonlin 8.3 software.

**Results:** APAP-induced liver injury decreased AST and ALT in the HFD group compared with the ND group at 6 and 24 hours ($p < 0.01$ and $p < 0.001$), respectively. Antioxidant enzyme levels remained constant in the HFD group, whereas histopathology showed remarkable changes. The PK's of APAP in HFD indicate lower plasma concentrations of APAP ($p < 0.05$), with two-fold higher clearance and volume of distribution.

**Conclusion:** HFD significantly reduced susceptibility to APAP-mediated liver injury in Balb/C mice compared with ND mice. Our study mimics the clinical scenario where the same dose of the drug is prescribed to the normal and obese population. Our results suggest the potential need for dose titration to assess an individual's nutritional state in a clinical scenario.

**Keywords:** Acetaminophen, high-fat diet, liver injury, nutrition, pharmacokinetics, stage-II toxicity

## INTRODUCTION

Nutrition plays an important role in drug kinetics and influences the efficacy or toxicity of a molecule.[1,2] Both fasting and malnutrition are risk factors for acetaminophen (APAP)-induced hepatotoxicity in healthy individuals.[3] In contrast, a high-fat diet (HFD) may increase the risk of chemical/drug-induced toxicity following an acute overdose. Short-term HFD causes changes in the liver and may alter the activity of hepatic drug-metabolizing enzymes. Such a change in enzyme expression or activity may increase APAP-induced hepatotoxicity.[4]

The liver plays a pivotal role in numerous processes such as food and drug biotransformation, protein synthesis, detoxification, and the generation of enzymes essential for digestion.[5] During these processes, both toxic chemicals and drug overdose may cause drug-induced liver injury (DILI), and hepatocytes become the primary target.[6] The classical hepatotoxic chemicals include alcohol, carbon tetrachloride, anticancer drugs, anti-inflammatory drugs, and analgesics.[7,8] During the process of DILI, hepatocytes play various roles in inflammatory and fibrotic processes.[8] The inflammatory response initiated by a

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damaged hepatocyte accelerates the injury process, leading to tissue damage. Early toxic injury (toxicity) also depends upon innate immune activation, and APAP-induced hepatotoxicity is one of the best examples of this type of injury.[9]

APAP is also referred to as paracetamol and is used over the counter as an efficient pain reliever. Although APAP is typically considered a safe medicine, an overdose can cause immediate liver damage or failure.[10] Depending on the body mechanism, the maximum advised dose might cause mild or moderate hepatotoxicity, resembling non-alcoholic fatty liver disorders (NAFLD), even in a healthy individual.[10,11] At therapeutic doses, 90% of APAP is metabolized without toxicity through glucuronidation and sulfation and is eliminated through the kidney. The remaining 10%, however, is metabolized in phase I, where CYP2E1 and CYP450 enzymes convert small amounts of APAP into toxic N-acetyl-p-benzoquinone imine (NAPQI), which disrupts the immune system and causes oxidative stress, lipid peroxidation, and eventually liver injury.[8,12]

Both the risk and severity of APAP-induced hepatotoxicity are increased by other factors such as alcohol, fasting, undernutrition, and diet.[4] Diet is the most significant environmental factor linked to the prevalence of drug or chemical toxicity.[13,14] Current lifestyle modifications have encouraged a large increase in the use of high-energy diets such as HFD.[14] HFD's have substantially higher fat than what is typically consumed. HFD increases the risk of developing numerous metabolic and cardiovascular complications and drug-mediated toxicity.[14] Recently, studies combining APAP and HFD have been conducted clinically but require more in-depth mechanistic research queries using experimental animals.[4,15,16] HFD increases the expression of CYP 2E1 in C57BL/C mice, explaining APAP-mediated NAFLD susceptibility.[17] Most overweight and obese individuals suffer from fatty liver disease; however, symptom-free fatty liver patients may unknowingly take a higher dose of APAP. Recent research suggests that oxidative stress is critical for the onset of NAFLD, causing energy depletion, liver cell destruction, and accumulation of fatty acids in hepatocytes. Oxidative stress is also a major factor in the etiology of APAP-induced toxicity.[18]

The present study is a time-course experiment demonstrating the role of nutrition (HFD and 18% protein diet) on a single dose of APAP-mediated liver injury. Furthermore, biomarkers of liver injury and histopathology data were correlated with the pharmacokinetic (PK) profile of APAP.

## MATERIALS AND METHODS

### *Chemicals*

APAP from Ce-Chem Pharmaceuticals Pvt. Ltd. 4th phase, #336, 9th Cross Rd, Ganapathy Nagar, Phase 3, Peenya, Bengaluru, Karnataka 560 058, India. Methanol was obtained from HIMEDIA (cat#AS061), ethyl acetate was acquired from RANKEM (cat#LTR/RANK30200), and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits were obtained from Aspen Laboratories Pvt. Ltd.

### *Animal study*

Female 3-week-old Balb/C mice (n= 24) were housed at NUCARE, NGSM Institute of Pharmaceutical Sciences, Paneer campus, Mangalore, Karnataka, India. Animals (n= 6) were provided free access to food and water under controlled temperature (22 °C) and humidity (50%) with a 12:12, light: dark cycle. Mice were randomly assigned into two groups (n= 6 each). 1) Normal diet group (ND, 18% protein) fed with ND and 2) high-fat diet group (HFD) fed with HFD. Dietary compositions of HFD[19] are listed in Table 1. After 15 weeks, mice were treated with a single dose of APAP-300 mg/kg; *per os* (*p.o.*). Blood samples were collected at different time intervals

**Table 1. Dietary compositions of ND and HFD**

| Serial number | Ingredients (ND, 18% protein) | % | Serial number | Ingredients (HFD) | % |
|---|---|---|---|---|---|
| 1 | Wheat flour | 56.2 | 1 | Milk casein | 24.50 |
| 2 | DCP (rock base) | 1.8 | 2 | Egg white | 5.00 |
| 3 | Calcite powder | 1.0 | 3 | L-cystine | 0.43 |
| 4 | LAF mix | 1.0 | 4 | Powdered beef tallow | 15.88 |
| 5 | Linseed | 5.0 | 5 | Safflower oil (high oleic acid) | 20.00 |
| 6 | Maize gluten | 5.0 | 6 | Crystalline cellulose | 5.50 |
| 7 | Roasted gram flour | 25.0 | 7 | Maltodextrin | 8.25 |
| 8 | Skimmed milk powder | 5.0 | 8 | Lactose | 6.93 |
| | | | 9 | Sucrose | 6.75 |
| | | | 10 | AIN93 vitamin mix | 1.40 |
| | | | 1 | AIN93G mineral mix | 5.00 |
| | | | 12 | Choline bitartrate | 0.36 |
| | | | 13 | Tertiary butylhydroquinone | 0.00 |

ND: Normal diet, HFD: High-fat diet, DCP: Dicalcium phosphate

(0, 6 and 24 hours) using isoflurane anesthesia through the retro-orbital sinus. The choice of time points mentioned (0, 6, and 24 hours) for blood collection after APAP administration is a common approach in PK and pharmacodynamic studies. These time points allow for capturing the immediate (0 hour), short-term (6 hours), and long-term (24 hours) effects of the drug. Collecting blood samples at 24 hours allows researchers to assess liver function markers and investigate delayed toxic effects, if any.[20] After the 24th hour of collection of blood samples, the animals were euthanized and dissected, and liver samples were removed. Part of the fresh liver sample was stored at -20 ˚C, and the other part of the tissue sample was stored in 10% formalin for histopathology studies. Separate sets of animals (n= 6) were taken for PK study and blood samples were collected at 0, 0.5, 1.0, 2.0, and 4.0 hours time intervals after APAP treatment.

*Ethical clearance*

All animal experiments were performed according to institutional guidelines for the care and use of laboratory animals as approved by the IAEC, under the committe for control and supervision of experiments on animals (CCSEA). In accordance with the Institutional Animal Ethics Committee of NGSM Institute of Pharmaceutical Science (approval number: NGSMIPS/IAEC/DEC-2020/2021, date: 29.11.2020).

*Measurement of AST and ALT*

Blood samples were collected at 0, 6 and 24 hours after a single dose of APAP 300 mg/kg *p.o.*, serum separated and stored at -20 ˚C until further analysis. Serum AST and ALT were measured using commercially available kits (Aspen Laboratories Pvt. Ltd.) as per the manual instruction by semi-auto analyzer model: Star 21+ from a rapid diagnostic group of companies.

*Measurement of hepatic antioxidant enzymes [sodium dismutase (SOD), glutathione (GSH) and catalase]*

Fresh liver samples were used to prepare 5% tissue homogenate using 0.25 M phosphate buffer, centrifuged at 10000 rpm for 20 minutes, and the supernatant separated. Antioxidant assays, catalase,[21] SOD[22] and GSH[23,24] were measured as per the respective protocol using an ultraviolet (UV) spectroquant prove 600 analyzer from Merck.

*Histopathological examination of liver tissue*

Liver tissue samples stored in 10% formalin were used to make paraffin-embedded sections. The sections were used to prepare slides, stained using hematoxylin and eosin (H&E) dyes, and observed under various magnifications.

*PK study*

This study was conducted to examine the role of nutrition in the absorption, distribution, metabolism and elimination (ADME) profile of APAP in mice. After weaning, female Balb/C mice were separated into two groups receiving ND and HFD. After 15 weeks, both groups were treated with APAP (300 mg/kg *p.o.*) and blood samples (100 µL) were drawn from the retro orbital sinus under the influence of isoflurane anesthesia at 0, 0.5, 1.0, 2.0 and 4.0 hour time intervals (from a separate set of animals), centrifuged (3000 rpm for 10 min) and stored at -20 ˚C until analysis. The liquid-liquid extraction method was used to extract APAP from plasma. Briefly, to a 50 µL plasma sample, 1.5 mL ethyl acetate was added, vortexed for 5 min and centrifuged at 3000 rpm for 5 min, supernatant separated and vacuum dried. To the dried residue, 200 µL of mobile phase was added and vortexed. 10 µL of aliquot was injected into the HPLC system.

*PK sample analysis*

Sample analysis was performed using a Waters RP-HPLC system (Model-1525 separation module and model 2998, photodiode array detector) and a C18 column (Waters SPHERISORB 5 m, ODS 1, 4.6*150 mm) as described in the literature.[2,25] A60:40 *v/v* methanol: water solution was used as the mobile phase (filtered through a 0.45 m nylon syringe membrane filter). The injection volume was 10 µL, and the effluent was monitored with a UV detector at a flow rate of 1 mL/min at 254 nm.

*PK parameter calculation*

We employed Phenix WinNonlin 8.3 software to conduct a non-compartment analysis to analyze the time profiles of plasma concentrations versus time data obtained from each mouse. Maximum plasma concentrations ($C_{max}$) and time to reach maximum plasma concentrations ($t_{max}$) were calculated directly from individual plasma concentration-time curves. The areas under the plasma concentrations area under the curve $(AUC)_{0-4}$, $AUC_{0-\infty}$were estimated. Drug elimination half-life ($t_{1/2}$), obvious total body clearance or oral clearance CL/F, and volume of distribution $V_Z$/F were calculated and interpreted.

*Statistical analysis*

Data are presented as mean ± SEM. Graph pad prism 8.0.1 software was used to analyze the statistical difference between groups using the Student's t-test. Also, One-Way analysis of variance with Newman-Keuls post hoc test. The level of statistical significance was considered at $p < 0.05$.

## RESULTS

*Biochemical analysis data*

Both ND and HFD groups had similar levels of ALT at 0 hour, suggesting no nutritional role at 0 hour time point. The challenge with a single dose of APAP (300 mg/kg; *p.o.*) increased ALT level by 6th hour in both ND and HFD ($p < 0.05$ and $p < 0.01$, respectively ) as compared to 0 hour time point. Surprisingly, injury was further increased in the ND group ($p < 0.001$). In contrast, injury was regressed ($p < 0.001$) in the HFD group at 24th hour compared to ND group (Figure 1A). AST estimation (Figure 1B) follows a similar trend and strengthens the ALT data.

*Antioxidant assay*

SOD, catalase, and GSH in fresh liver samples were estimated at the end of the study. HFD decreased antioxidant enzyme levels of SOD, catalase, and GSH (Figure 2). APAP challenge significantly ($p < 0.01$) decreased antioxidant levels in the ND group, but not in the HFD group.

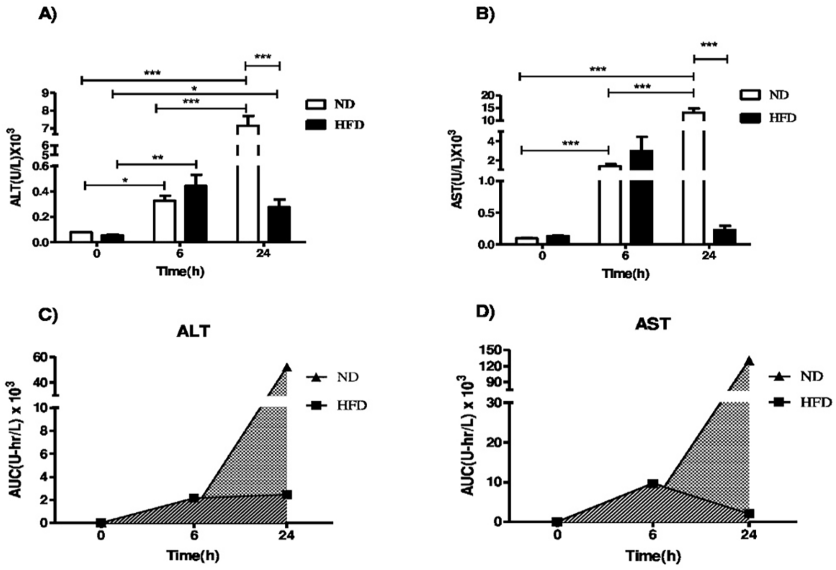


**Figure 1.** Serum ALT, AST levels in APAP-treated mice. (A) ALT, (B) AST levels, C) and D) AUC graphs of ALT and AST of APAP (300 mg/kg *p.o.*) treated mice at various time points. ND and HFD. ND mice received ND and HFD mice received HFD. Data are presented as mean ± SEM, n= 6. Statistical analysis was performed by t-test analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, APAP: Acetaminophen, AUC: Area under the curve, ND: Normal diet, HFD: High-fat diet, SEM: Standard error of the mean

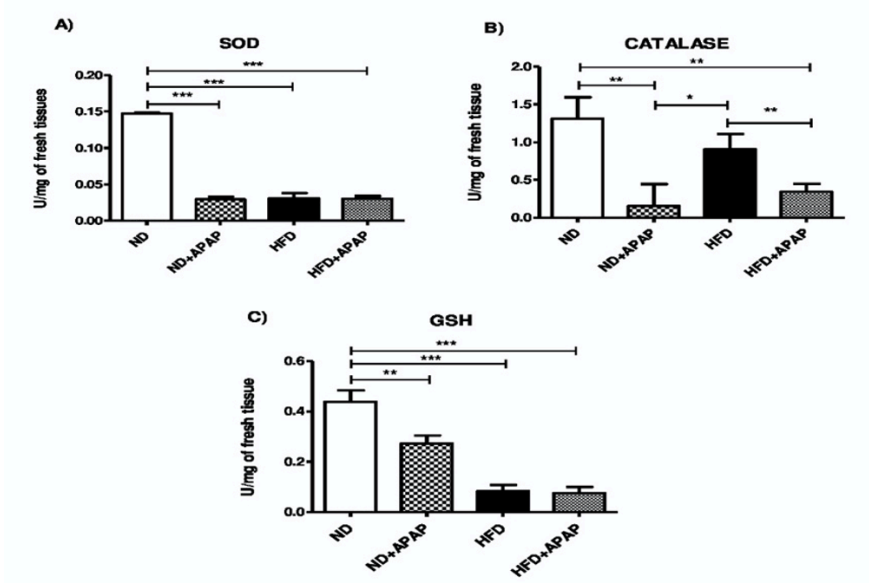


**Figure 2.** Effect of HFD on cellular antioxidants (A) SOD, (B) catalase, (C) GSH in the liver. ND and HFD of control and 24-hour time point after APAP 300 mg/kg *p.o.* treatment. ND mice received ND and HFD mice received HFD. ND + APAP, ND receiving group treated with APAP (300 mg/kg *p.o.*) and HFD + APAP, HFD group treated with APAP (300 mg/kg *p.o.*). Data are presented as mean ± SEM, n= 6. Statistical analysis was performed using One-Way ANOVA with Newman-Keuls post hoc test. Statistical significance is considered *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

SOD: Sodium dismutase, GSH: Glutathione, ND: Normal diet, HFD: High-fat diet, APAP: Acetaminophen, SEM: Standard error of the mean, ANOVA: Analysis of variance

*Histopathological examination of liver tissue*

H&E staining of HFD liver tissue showed obvious fat accumulation in the form of droplets, a characteristic feature of HFD-consuming mice.[26] Livers of ND animals showed minimal or no changes (Figure 3A), whereas those from animals fed with HFD showed severe fatty infiltration in the liver (Figure 3C, 3D). APAP administration caused extensive centrilobular injury with apoptotic cells in the livers of the ND group (Figure 3B). On the other hand, there were few inflammatory cells in the centrilobular regions of the liver from APAP-treated animals fed HFD. However, fat accumulation remained similar to that in the HFD-fed group (Figure 3C-F), confirming less liver injury in the HFD group.

*PK's study*

PK results were obtained from ND and HFD animals for 300 mg/kg APAP. PK study was designed to investigate the ADME profile of APAP in plasma samples from mice given ND and HFD. On the 15th week of the study, all of the mice (ND and HFD) were given single-dose APAP (300 mg/kg, *p.o.*). The average APAP retention time was 3,407 min. Peak area and APAP retention duration in ND and HFD were 3,409 and 3,409 min, respectively. HFD- receiving mice had lower plasma concentrations (37,141± 22.98, $p < 0.05$) than ND (74.38 ± 18.63). In contrast, APAP clearance and volume of distribution were two-fold higher in HFD-treated mice. As a result, the systemic exposure parameters ($C_{max}$ and AUC) in HFD groups are lower than in ND groups (Table 2). Plasma concentrations of APAP in HFD and ND are in a ratio of 1:2.

## DISCUSSION

Dietary protein and fat influence drug metabolism, altering drug toxicity and therapeutic response.[27] Clinical research examines the effects of diets, malnutrition, diet restriction, and high-fat on the PK of drugs.[3,28] Evaluating drug toxicity and PK in the clinical setting is difficult because of high cost, time, volunteer unavailability, *etc*. This study aimed to analyze the effect of HFD on APAP-mediated toxicity and PK profile in a mouse model.

APAP is chiefly metabolized by the liver, where 90% is renally eliminated after sulfide or glucuronide conjugation. The remaining 10% is metabolized *via* CYP2E1, generating the toxic metabolite NAPQI.[4] At therapeutic doses, APAP generates NAPQI in quantities that conjugate with cellular GSH without producing toxicity on hepatocytes. However, toxic doses of APAP deplete cellular GSH, and NAPQI covalently binds with the sulfhydryl group of many proteins to form APAP protein adducts in hepatocytes, leading to mitochondrial dysfunction and necrosis.[29-31] As expected, a single dose of APAP (300 mg/kg, *p.o.*) caused liver injury as reflected by elevated liver injury biomarkers (ALT and AST) in the ND and HFD groups, confirming hepatotoxicity as early as 6 hours after APAP challenge. This increase in ALT and AST is comparable in both the ND and HFD groups, suggesting similar bioactivation-mediated liver injury (stage I toxicity).[32-34] Extensive liver injury in the ND group (reflected by high AST and ALT at 24 hours) confirms greater susceptibility to APAP toxicity (stage-II toxicity). In sharp contrast, liver injury regressed in HFD-receiving animals, underlining the toxicodynamics (progression V regression of injury) of dietary fat. The HFD group probably encounters stage-II toxicity much before the 24th hour time point. Nevertheless, early publications have documented that liver progression of injury continues even after 24 hours and peaks at 36-48 hours after toxin challenge.[35] The difference in the timing of maximal injury depends on the nature of the molecule and its dose.[36] In the present study, HFD-receiving mice experienced regression of liver injury by 24 hours itself; thereby suggesting that recovery will be complete by 48 hours. Differences in liver injury between ND and HFD are reflected in the graphs showing AUC for AST and ALT (Figure 1C, 1D). However, we could not collect blood samples after the 24 hour time point. Liver histopathology provides additional evidence supporting the difference in injury (Figure 3, 4).

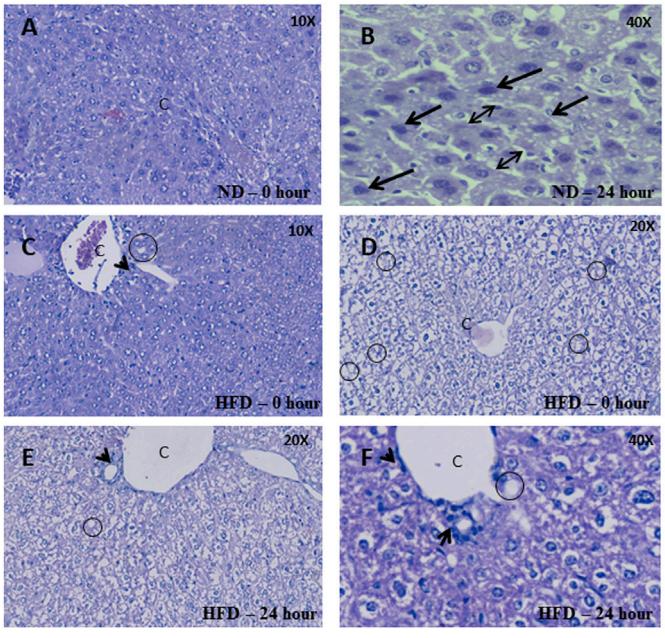


**Figure 3.** Liver histology of mice (A) control ND group 10. (B) 40X ND + APAP treated group, at 24 hours (C) 10 and (D) 20X HFD control group, (E) 20 and (F) 40X HFD + APAP. ND and HFD n= 6, of control and 24-hour time point after APAP 300 mg/kg *p.o.* treatment in Balb/C mice representative gures were stained with H&E. Arrows indicate apoptotic cells, arrowheads indicate inammatory cell clusters, circles indicate small fat droplets and double-headed arrows indicate sinusoids in hepatocytes

ND: Normal diet, HFD:High-fat diet, APAP: Acetaminophen, H&E: Hematoxylin and eosin

Different animal models have been used to study the impact of NAFLD on APAP toxicity. Many conflicting reports imply that HFD either increases or decreases hepatotoxicity.[2,18,37] Clinical research appears to discount the impact of HFD on APAP toxicity[4,15] which correlates with the current study's assertion that HFD suffers little or no toxicity compared with ND. There are conflicting reports in which some models reflected greater injury in the HFD group at 300 mg/kg doses of APAP, and others showed similar or lower toxicity. Multiple reports suggest a decrease in CYP2E1 enzyme activity in the HFD group.[37] The difference between previous reports and the present study could be due to differences in animal species, dose of APAP, route of administration, time points of blood sampling, *etc*. Recently, Achterbergh et al.[3] reported that short-term fasting increases APAP toxicity. However, healthy subjects did not experience APAP toxicity after an HFD, demonstrating the importance of diet in APAP-induced toxicity.

We evaluated the PK difference in APAP between the ND and HFD groups to examine the role of blood levels of APAP in liver injury. The initial absorption of APAP is more rapid in the ND group than in the HFD group. Likewise, ND had greater $C_{max}$ and AUC than HFD. Furthermore, APAP was eliminated faster in the HFD group, explaining the lower plasma concentration

(Table 2). Similar liver injury in the ND and HFD groups at 6 hours (Figure 1) was possible because CYP2E1 enzyme levels were similar in both groups, generating similar bioactivation-mediated liver injury.

*Study limitations*

Further research is needed to understand the molecular mechanisms underlying the protective effects of a high-fat diet against APAP-induced liver injury, and caution should be exercised when applying these findings to obese patients without human studies.

## CONCLUSION

The present study has demonstrated that HFD protects mice from APAP-mediated liver injury. Additionally, the ADME profile is considerably different in the HFD group than in the ND group. The reason for lower stage II injury in the HFD group needs further investigation. Our findings indicate that obese patients may respond differently to APAP efficacy or toxicity because of altered drug kinetics. Therefore, such victims may be treated differently from normal ones. A detailed mechanistic study is essential at the molecular level to understand the effects of nutritional status on stage-II toxicity. The outcomes of such a study will help in decision-making while treating APAP overdose victims.

**Table 2. Plasma PK parameters of APAP (300 mg/kg, *p.o.*) in ND and HFD receiving Balb/C mice (n= 6)**

| Serial number | Parametersa | Unit | ND | HFD |
|---|---|---|---|---|
| 1 | $C_{max}$ | µg/mL | 74.38 ± 18.63 | 37.141 ± 22.99* |
| 2 | $AUC_{0-4}$ | h* µg/mL | 77.37 ± 17.26 | 37.558 ± 28.89* |
| 3 | $AUC_{0-\infty}$ | h* µg/mL | 79.41 ± 16.71 | 39.328 ± 28.52* |
| 4 | $t_{max}$ | H | 0.58 ± 0.20 | 0.500 ± 0.00 |
| 5 | $t_{1/2}$ | H | 0.65 ± 0.18 | 0.737 ± 0.69 |
| 6 | CL/F | mL/h | 111.90 ± 25.58 | 238.788 ± 142.83 |
| 7 | $V_z$/F | L | 102.25 ± 23.30 | 295.662 ± 178.76 |

[a]Parameters values are expressed as mean ± SEM, * $p < 0.05$, when compared with the ND group, $C_{max}$: Maximum plasma concentration, $AUC_{0-4}$: Area under the drug concentration-time curve from time zero to the time of the last measurable concentration, $AUC_{0-\infty}$: Area under the drug concentration-time curve from time zero to infinity, $t_{max}$: Times to achieve maximum plasma concentrations, $t_{1/2}$: Elimination half-life period of the drug, CL/F: Apparent total body clearance or oral clearance, $V_z$/F: Volume of distribution, PK: Pharmacokinetics, APAP: Acetaminophen, ND: Normal diet, HFD: High-fat diet, AUC: Area under the curve, SEM: Standard error of the mean

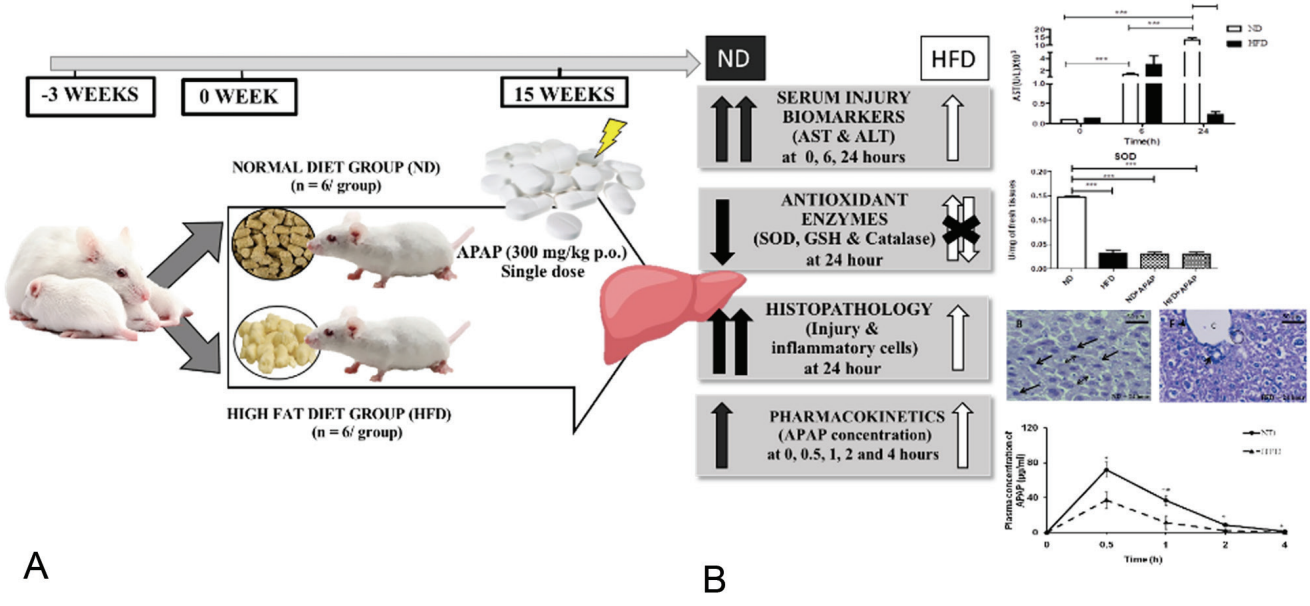


**Figure 4.** Graphical abstract representing animal experimental methodology. Female Balb/C mice after the weaning period were separated (0 week of the study period) into two different groups: A) ND and B) HFD receiving groups. After 15 weeks, they were administered a single dose (300 mg/kg *p.o.*) of APAP. Blood samples were collected at different time intervals (0, 6, and 24 hours), and liver samples were collected at the end time point. Liver injury parameters (ALT and AST), SOD assay (catalase and GSH), and histopathology study were conducted. Aseparate set of animals (n= 6, each group) was used for PK analysis

ND: Normal diet, HFD: High-fat diet, APAP: Acetaminophen, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, SOD: Sodium dismutase, PK: Pharmacokinetics

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*Ethics*

**Ethics Committee Approval:** All animal experiments were performed according to institutional guidelines for the care and use of laboratory animals as approved by the IAEC. In accordance with the Institutional Animal Ethics Committee of NGSM Institute of Pharmaceutical Science (approval number: NGSMIPS/IAEC/DEC-2020/2021, date: 29.11.2020).

**Informed Consent:** Not necessary.

*Authorship Contributions*

Concept: M.B., Design: M.B., V.D'S., M.S., Data Collection or Processing: V.D'S., M.S., Analysis or Interpretation: M.B.S., V.K., V.D'S., Literature Search: V.D'S., Writing: M.B., V.D'S., V.A., M.R.J.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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