Effect of nutrition on drug-induced liver injury: insights from a high-fat diet mouse model

Short Title in English: Effect of HFD on paracetamol-induced hepatotoxicity

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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 is 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 APAP-induced hepatotoxicity at different time intervals.

Materials and Methods: Female Balb/c mice after weaning period separated into two different groups normal diet (ND) & high fat diet (HFD) receiving groups, after 15 weeks they were dosed with single dose (300 mg/kg, p.o.) of acetaminophen (APAP). Blood samples were collected at different time intervals (0, 6 & 24hour), liver sample at the end time point. Liver injury parameters (ALT and AST), antioxidant assay (SOD, GSH & Catalase), and histopathology study was conducted. Pharmacokinetic analysis was done using RP-HPLC system and Phoenix WinNonlin 8.3 software.

Results: APAP-induced liver injury decreased AST, ALT in HFD group compared to normal diet (ND) group. Antioxidant enzyme levels remained constant in HFD group, while histopathology showed remarkable changes. Pharmacokinetics of APAP in HFD indicates, it had lower plasma concentrations of APAP, with two-fold higher clearance and volume of distribution.

Conclusion: HFD reduces susceptibility to APAP-mediated liver injury in Balb/C mice compared to those on ND. 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 an individual's nutritional state in a clinical scenario.

Keywords: Acetaminophen, High-fat diet, Liver injury, Nutrition, Pharmacokinetics, Stage-II toxicity

Highlights:
1. Liver injury is low and not progressive in HFD mice compared to ND mice.
2. APAP concentration is higher in the ND group compared to the HFD group.
3. HFD (nutrition) plays a vital role in APAP kinetics and dynamics.

Introduction

Nutrition plays an important role in drug kinetics and thereby 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 cause changes in the liver and may change 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, anti-cancer drugs, anti-inflammatory drugs, and analgesics.[7,8] During the process of DILI, hepatocytes play various roles in inflammatory and fibrotic processes.[8] Inflammatory response initiated by a 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 kind of injury.[9]

APAP is, also referred to as paracetamol, used over the counter as an efficient pain reliever. Even though 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 eliminated through the kidney. The remaining 10%, however, is metabolized in phase I, where CYP2E1 and CYP450 enzymes convert the small amount of APAP into the 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[4,15,16] but require more in-depth mechanistic research queries using experimental animals. HFD increases the expression of CYP 2E1 in C57BL/C mice[17] explaining APAP-mediated NAFLD susceptibility. Most overweight and obese individuals suffer from fatty liver disease, but 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 a time-course experiment demonstrating the role of nutrition (HFD and 18% protein diet) on a single dose of APAP-mediated liver injury in. Further, biomarkers of liver injury and histopathology data correlated with the pharmacokinetic profile of APAP to clarify the significance of a victim's nutritional status.

MATERIALS AND METHODOLOGY

Chemicals

Acetaminophen from Ce-Chem Pharmaceuticals Pvt. Ltd. 4th phase, #336, 9th Cross Rd, Ganapathy Nagar, Phase 3, Peenya, Bengaluru, Karnataka – 560 058, Methanol obtained from HIMEDIA (Cat # AS061), Ethyl acetate acquired from RANKEM (Cat # LTR/RANK30200), AST, ALT kits from Aspen Laboratories Pvt. Ltd.

Animal study

Female Balb/C mice (n=24) 3 weeks old were procured from Nitte Centre for Animal Research and Experimentation [NUCARE], NGSM Institute of Pharmaceutical Sciences, Paneer campus, Mangaluru, Karnataka, India. Animals were housed in the NUCARE department with 6 animals in each cage and provided free access for food and water under controlled temperature (22^0 C) & 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 normal diet and 2) high-fat diet group (HFD) fed high with fat diet. Dietary compositions of HFD[19] are mentioned in Table 1. After 15 weeks, mice were treated with a single dose of acetaminophen (APAP) - 300 mg/kg; p.o. Blood samples were collected at different time intervals (0, 6 & 24hour) using isoflurane anesthesia through retro-orbital sinus. The choice of time points mentioned (0, 6, and 24 h) for blood collection after APAP administration is a common approach in pharmacokinetic and pharmacodynamic studies. These time points allow for capturing the immediate (0 h), short-term (6 h), and long-term (24 h) effects of the drug. Collecting blood samples at 24 h allows researchers to assess liver function markers and investigate delayed toxic effects, if any.[20] After 24th hour collection of blood samples, animals were euthanized and dissected and liver samples were removed. Part of fresh liver sample was stored at -20 ^{0}C, also other part of tissue sample was stored in 10% formalin for histopathology studies. Separate set of animals

were taken for pharmacokinetic study (n = 6) in each group (ND & HFD) and blood samples were collected at 0, 0.5, 1.0, 2.0 & 4.0hour time intervals after APAP treatment.

Ethical clearance

Animal experiments were all performed according to institutional guidelines for the care and use of laboratory animals as approved by IAEC. In accordance with the guidelines of the CCSEA (Committee for the Control and Supervision of Experiments on Animals), with approval number NGSMIPS/IAEC/DEC-2020/2021.

Measurement of AST & ALT

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

Measurement of hepatic antioxidant enzymes (SOD, GSH & Catalase)

Fresh liver samples were used to prepare 5% tissue homogenate was prepared using 0.25M phosphate buffer, centrifuged at 10000 rpm for 20 minutes and the supernatant separated. Antioxidants assays, catalase,[21] Sodium Dismutase (SOD)[22] Glutathione (GSH)[23,24] was carried as per the respective protocols using UV spectroquant trove 600 analyzer from MERCK.

Histopathology examination of liver tissue

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

Pharmacokinetic study

Study was performed to examine the role of nutrition on the ADME profile of paracetamol 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 (300mg/kg p.o.) and blood samples(100µl) were drawn from the retro orbital sinus using slight isoflurane anesthesia at 0, 0.5, 1.0, 2.0 & 4.0 hour time intervals (from separate set of animals), centrifuged (3000 rpm for 10 min) and stored at -20°C until analysis. Liquid- liquid extraction method was used to extract APAP from plasma. Briefly, to 50µl plasma sample, 1.5ml ethyl acetate was added, vortexed for 5 min and centrifuged at 3000 rpm for 5min, supernatant separated and vacuum dried. To the dried residue, 200µl of mobile phase was added and vortexed. 10µl of aliquot was injected into the HPLC system at Nitte university center for science education and research (NUCSER).

Pharmacokinetic 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] A 60:40 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 10µL, and the effluent was monitored with a UV detector at a flow rate of 1 mL/min at 254 nm.

Pharmacokinetic parameter calculation

We employed Phoenix WinNonlin 8.3 software to conduct a non-compartment analysis to analyze the time profiles of plasma concentrations versus time data obtained from in 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 AUC_{0-4}, $AUC_{0-\infty}$ were estimated. Drug's elimination half-life ($t_{1/2}$), apparent total body clearance or oral clearance CL/F, and volume of distribution V_Z/F, were calculated and interpreted.

Statistics

Data were 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 ANOVA 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 hours, suggesting minimal nutritional to begin with in both 18% protein and HFD. Challenge with single dose of APAP (300 mg/kg; p.o.) increased ALT level by 6th hour in both ND and HFD. Surprisingly, injury was further increased in ND group and injury regressed in HFD group 24th hour (Figure 1A). AST estimation (Figure 1B) follows similar trend and strengthens ALT data.

Antioxidant assay

SOD, Catalase and GSH in fresh liver sample were estimated at the end of the study. HFD decreases antioxidant enzyme levels of SOD, catalase, and GSH (Figure 2). APAP challenge significantly decreased antioxidant levels in ND group, not altered in HFD.

Histopathology examination of liver tissue

The H&E staining of HFD liver tissue showed obvious fat accumulation in the form of droplets, a characteristic feature of HFD diet consuming mice.[26] Livers of ND animals showed minimal or no changes (Figure 3A), while those from animals fed with HFD show severe fatty infiltration in the liver (Figure 3C, 3D). APAP administration caused extensive centrilobular injury with apoptic cells in the livers of 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 with HFD. However fat accumulation remained similar to HFD fed group (Figure 3C, 3D, 3E, 3F), confirming less liver injury in HFD group.

Pharmacokinetics study

Pharmacokinetic (PK) results obtained from ND and HFD animals for 300 mg/kg APAP. PK study was designed to investigate ADME profile (absorption, distribution, metabolism and elimination) 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 found to be 3.407 minutes. 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) 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 the ratio of 1:2.

DISCUSSION

Dietary protein and fat influence drug metabolism, altering drug toxicity and therapeutic response.[27] Clinical research has 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. The present study was 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% APAP 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 conjugates with cellular GSH without producing toxicity on hepatocytes. However, toxic doses of APAP depletes 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.[29,30,31] As expected, a single dose of APAP (300mg/kg, p.o.) caused liver injury as reflected by elevated liver injury biomarkers (ALT and AST) in ND and HFD groups, confirming hepatotoxicity as early as 6hours after APAP challenge. This increase in ALT and AST is comparable in both ND and HFD groups suggesting similar bioactivation-mediated liver injury (stage 1 toxicity).[32,33,34] Extensive liver injury in the ND group (reflected by high AST and ALT at 24hours) confirms greater susceptibility to APAP toxicity (stage II toxicity). In sharp contrast, liver injury regressed in HFD-receiving animals underlining the toxicodynamics (progression Vs regression of injury) of dietary fat. 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 24hours, and peaks at 36 - 48hours 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 24hour itself; thereby suggesting that recovery will be complete by 48hour. Differences in liver injury between ND & HFD are reflected in the graphs showing AUC for AST and ALT (Figure 1C, 1D). However, we could not collect blood samples after the 24hour time point. Liver histopathology provides additional evidence supporting the difference in injury (Figure 3).

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 to ND. There are conflicting reports in which some models reflected greater injury in the HFD group at 300 mg/kg or higher doses of APAP, and some 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 on account of differences in animal species, dose of APAP, route of administration, time points of blood sampling etc. Recently, Achterbergh et al. (2019)[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 of APAP between ND and HFD groups, to examine the role of blood levels of APAP in liver injury. Initial absorption of APAP is more rapid in ND than HFD group. Likewise, ND had greater C_{max} and AUC than HFD. Further, APAP was eliminated faster in the HFD group, explaining the lower plasma concentration (Table 2). Similar liver injury in ND and HFD groups at 6hour (Figure 1) was possibly 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 taken 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 compared to 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 due to altered drug kinetics. Therefore, such victims may be treated differently than normal ones. 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.

ACKNOWLEDGEMENTS

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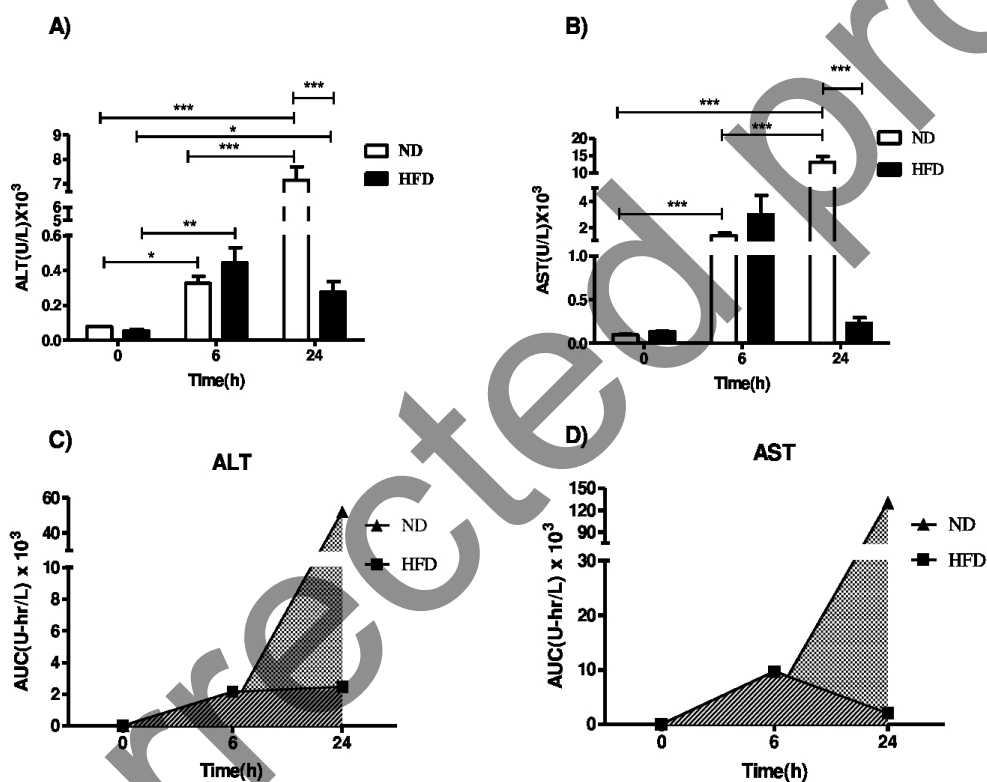
Figure 1. Serum ALT, AST levels in APAP-treated mice. (A) ALT & (B) AST levels C) & D) AUC graphs of ALT & AST of APAP (300 mg/kg p.o.) treated mice at various time points. Normal diet (ND) & High-fat diet (HFD). ND mice received normal diet & HFD mice received high fat diet. Data are presented as Mean ± SEM, n=6. Statistical analysis was performed by t-test analysis, *p<0.05, **p<0.01, ***p<0.001.
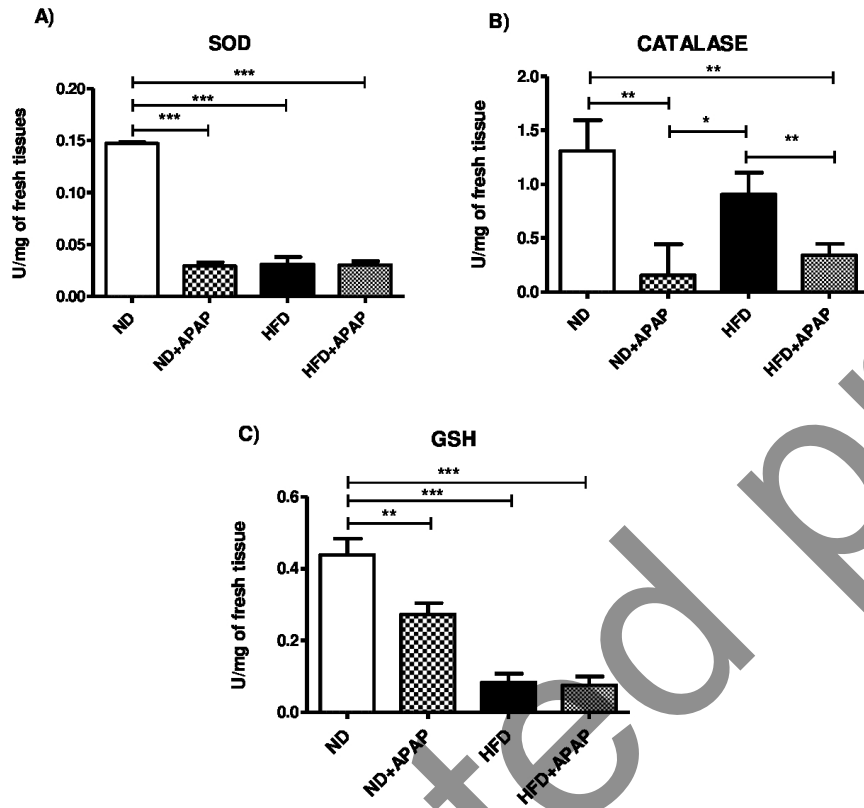
Figure 2. Effect of HFD on cellular antioxidants (A) SOD, (B) Catalase, (C) GSH, of liver.Normal diet (ND) &
High-fat diet (HFD) of control & 24h time point after APAP 300mg/kg p.o. treatment. ND mice received normal
diet & HFD mice received high fat diet. ND +APAP, Normal diet receiving group treated with APAP (300 mg/kg
p.o.)& HFD+APAP, high fat diet group treated with APAP (300 mg/kg p.o.). Data are presented as Mean ± SEM,
n=6. Statistical analysis was performed by one way ANOVA with Newman-Keuls post hoc test. Statistical
significance is considered as *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Liver histology of mice (A) Control ND group 10X. (B) 40X ND+APAP treated group, at 24h (C) 10X & (D) 20X HFD control group, (E) 20X & (F) 40X HFD+APAP. Normal diet (ND) & high-fat diet (HFD) n=6, of control & 24h time point after APAP 300mg/kg p.o. treatment in Balb/C mice Representative figures were stained with H&E. Arrows indicate apoptic cells and arrow heads indicate inflammatory cell clusters, circles indicate small fat droplets, double headed arrows indicate sinusoids in hepatocytes.
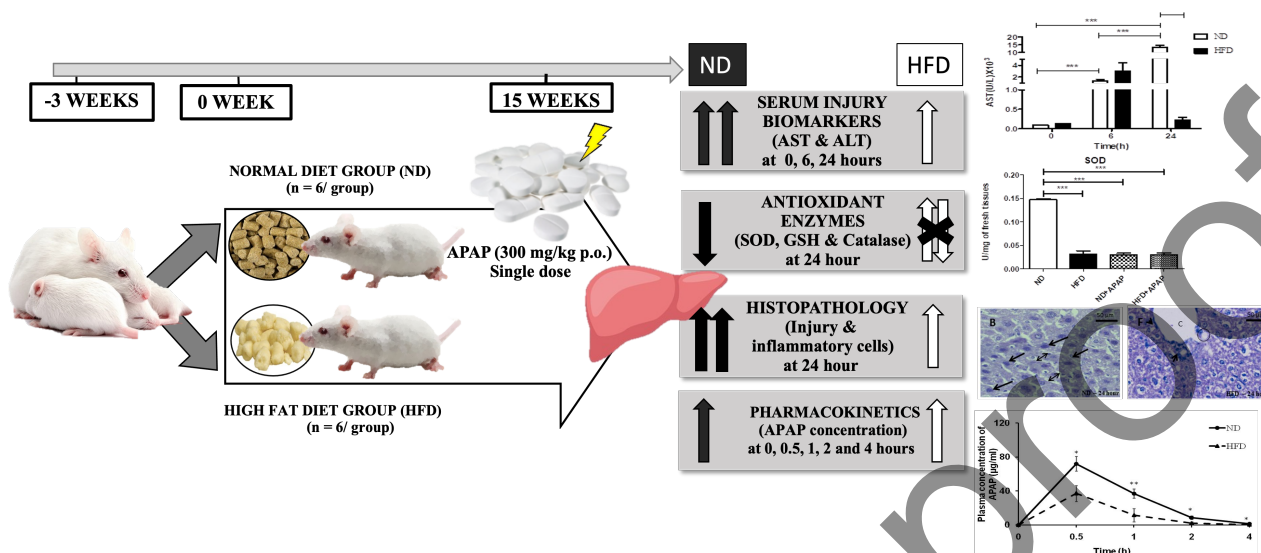
Figure 4. Graphical abstract representing animal experimental methodology. Female Balb/c mice after the weaning period were separated (0 week of the study period) into two different groups: a) Normal diet (ND) and b) High-fat diet (HFD) receiving groups. After 15 weeks, they were dosed with a single dose (300 mg/kg p.o.) of acetaminophen (APAP). Blood samples were collected at different time intervals (0, 6, and 24 hours), and liver samples were at the end time point. Liver injury parameters (ALT and AST), antioxidants assay (SOD, Catalase & GSH), and histopathology study was conducted. A separate set of animals (n = 6, each group) was taken for pharmacokinetic analysis.

Table 1: Dietary compositions of ND & HFD

S. No.	Ingredients (ND, 18% protein)	%	S. No.	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
			11	AIN93G mineral mix	5.00
			12	Choline bitartrate	0.36

13	Tertiary butylhydroquinone	0.00

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

Sl.no.	Parameters[a]	Unit	ND	HFD
1.	C_{max}	μg/mL	74.38 ± 18.63	$37.141 \pm 22.99^*$
2.	AUC_{0-4}	h*μg/mL	77.37 ± 17.26	$37.558 \pm 28.89^*$
3.	$AUC_{0-\infty}$	h*μg/mL	79.41 ± 16.71	$39.328 \pm 28.52^*$
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 ± 378.76

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** - the apparent total body clearance or oral clearance &V_Z/F -volume of distribution.[a]Parameters values are expressed as Mean ± SEM.* $p < 0.05$, when compared to the normal diet(ND) group.