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Hepatic toll of keto: unveiling the inflammatory and structural consequences of ketogenic diet in rats

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Abstract

Background The ketogenic diet (KD) has been used as a therapeutic diet for a range of diseases such as epilepsy, obesity, and cancer. However, it may cause some adverse effects that are not well known. This study aimed to assess the possible impact of the KD on liver structure and function, as well as hepatic inflammatory markers.

Methods Ninety male rats were randomly divided into two groups: the normal diet group consumed a standard rat chow, and the KD group consumed a diet composed of 90% fat, 8% protein, and 2% carbohydrates for 30 days. The serum levels of lipid profile (cholesterol and triglyceride), liver enzymes, hepatic levels of inflammatory markers, and steatosis grading were evaluated and compared between the two groups.

Results The serum cholesterol and alanine transaminase (ALT) levels in the KD group were significantly higher than in the normal diet group. However, there were no significant differences between groups in serum triglyceride and aspartate transaminase (AST) levels. Hepatic inflammatory markers, interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), both were higher in the KD group compared to the normal diet group. In the liver biopsy, the degree of steatosis was significantly higher in the KD group compared to the normal diet group.

Conclusion The KD may cause hepatic adverse effects by inducing steatosis and inflammation.

Keywords Ketogenic diet, Liver function, Hepatic inflammation, Steatosis, Alanin transaminase (ALT)

Introduction

Ketogenic diet (KD) is a therapeutic diet used to treat of medication-refractory epilepsy since the 1920s. It is consisted of 4:1 or 3:1 g of fat to carbohydrate plus protein, so that 90% of calorie intake is from fat [1]. Several studies indicated therapeutic potential effects of the KD on obesity, respiratory distress, breast cancer, and polycystic ovary syndrome as a supplementary therapy. It is also beneficial in treatment of some neurological diseases like multiple sclerosis, Parkinson disease, Alzheimer disease, and migraine [2–5]. Although the anti-seizure mechanisms of the KD were not fully understood, elevated ketone bodies and polyunsaturated fatty acids (PUFAs)

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are the possible underlying mechanism of anti-seizure effects of the KD [6].

Despite beneficial therapeutic effects of the KD on some chronic disease, it may cause some adverse effects like dyslipidemia, kidney stones, hyperuricemia, lethargy, and gastrointestinal complications including gastroesophageal reflux, constipation, fatty diarrhea, abdominal pain, vomiting, and hunger [7, 8]. Moreover, metabolic effects on liver incurred by the KD have only been preliminarily characterized and there is some discrepancy in the KD effects on hepatic structure and function. Although increased level of liver enzymes, serum inflammatory markers and hepatic steatosis have been reported in some studies [9–12], the KD showed anti-steatogenic and anti-inflammatory effects in other studies [13, 14].

Given the conflicting evidence regarding the effects of the KD on liver structure and function, further research is needed to clarify the impact of this diet on hepatic health. Understanding the potential hepatic adverse effects, including steatosis and inflammation, is crucial in assessing the long-term safety of the KD, particularly when used as a therapeutic intervention. This study aims to address these gaps by evaluating the effects of the KD on liver enzyme levels, inflammatory markers, and histopathological changes in liver tissue.

Methods and materials

Animals

Male Wistar rats ($n = 20$) (Pasteur Institute of Iran, Tehran, Iran) weighing 180–240 g, 12-week-old age were used for experiment. The rats were housed two in a cage under controlled temperature (22 ± 2 °C), humidity (60% ± 5%), and lighting (12:12-h light-dark cycle). The access to food and water was free.

Experimental protocols

All rats were acclimated for two weeks in the laboratory before starting the experiment with ad libitum access to standard pellets and water. Following this stage, 20 weight-matched rats were randomly divided into two groups: (1) normal diet (ND); and (2) KD. The sample size was calculated by G-power software to be 10 rats per group, using an α error probability of 0.05 and statistical power (1− β error probability) of 80%. This sample size ensures adequate statistical power while adhering to ethical guidelines by minimizing animal use. Similar studies investigating the effects of the KD on liver function have used comparable group sizes (8–12 rats per group), supporting the validity of our approach [15–18]. Two groups received diets for 30 days. The normal diet was a standard rat chow and the KD resembled the classic KD, meaning that the ratio of fat to carbohydrate plus protein was four to one. The macronutrient composition of diets was shown in Table 1. The ND formulation (3.8 Kcal/g) derived 10% of its calories from fat, 10% from protein, and 80% from carbohydrates. Its fatty acid profile was 19% saturated, 24% monounsaturated, and 57% polyunsaturated. The KD was a 6.7 Kcal/g formulation (6.7 Kcal/g) consisting of 90% fat (mutton tallow and cocoa butter), 8% protein, and 2% carbohydrates. Its fatty acid distribution was 64% saturated, 26% monounsaturated, and 10% polyunsaturated. The macronutrient composition and fat percentage of the diets were assessed by a reference laboratory. After confirmation of the composition of diets, the high-fat food was prepared weekly and stored in a -20 °C freezer. The rats were offered the foods (ND and KD) daily ad *libitum*.

Table 1 Macronutrient composition of diets

Macronutrients	Normal Diet (ND)	Ketogenic diet (KD)
Carbohydrate (g%/Kcal%)	77/80	3.4/2
Protein (g%/Kcal%)	10/10	13.6/8
Fat (g%/Kcal%)	4/10	67/90
Fatty acid distribution		
Saturated fat (%)	19	64
Monounsaturated fat (%)	24	26
Polyunsaturated fat (%)	57	10
Energy density (Kcal/g)	3.8	6.7

The rats were weighed and anesthetized with ketamine 10% (60 mg/kg) and xylazine (10 mg/kg) (Bremer Pharma GmbH, Germany) following 30 days of treatments. The blood samples were withdrawn and the sera were separated by centrifugation at 3000 g (unit of gravity) for 15 min and stored at -80 °C. Then, the animals were killed by a single lethal dose of ketamine (200 mg/kg) and the livers were excised. The right lobes of the liver tissues were fixed in 10% neutral buffered formalin and were kept for histology assays. The left lobes were stored at -80 °C for biochemical analysis.

Biochemical measurements

The serum levels of triglyceride (TG), cholesterol (Chol), alanine transaminase (ALT), and aspartate transaminase (AST) were measured colorimetrically using Pars-Azemoon kits (Tehran, Iran). The serum ketosis marker, β-hydroxybutyrate (BHB) levels, were assessed by a commercially available kit (Randox Laboratories Ltd, UK). An automated biochemical analyzer (Roche Cobas Mira) was used for measurements.

Hepatic inflammatory markers measurements

Tissue lysates were prepared from the collected liver samples using phosphate-buffered saline (PBS) and butylated hydroxytoluene to prevent oxidation [19]. The hepatic levels of interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were measured using the commercial DuoSet ELISA Development System kits (R&D System, USA) according to the manufacturer's instructions.

Histology

The liver structure was evaluated by hematoxylin and eosin (H & E) staining and histological methods. The liver specimens were collected and fixed in 10% buffered formalin and then embedded in paraffin. Tissue stained with H & E using standard methods and it was observed by pathologist who was blind to the allocation sequence of rats. Steatosis was scored using Brunt et al. [20] staging with a four grades scoring system from 0 to 3: grade 0, no fat in the liver; grade 1, up to 33%; grade 2, 33–66%; and grade 3, >66% of hepatocytes containing lipid vacuoles.

Statistical analysis

The Shapiro-Wilk test was initially conducted to assess the normality of variables. All variables were normally distributed and thus were presented as mean ± standard deviation (SD) except BHB and steatosis score that were presented as median and 25-75% percentiles. The independent samples t-test was used to compare the parameters with normal distribution between the study groups. Additionally, Levene's test was used to check for variance equality, ensuring the correct application of the t-test. Mann-Whitney U test was used to compared BHB and steatosis score between the groups. Pearson's correlation coefficient and Spearman's correlation coefficient were employed to determine the correlations between the evaluated parameters where appropriate. A *p*-value less than 0.05 ($p<0.05$) was considered statistically significant. The analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). G-power was used to determine the sample size and effect-size values.

Results

Body weight, lipid profile, ketone body, and liver enzymes

At the outset of the study, the rats were matched for weight across both groups (ND: 230.30 ± 6.90, g; KD: 232.11 ± 9.10, g; $p=0.629$). However, post-intervention, the KD group exhibited significantly ($p<0.001$, effect size = 2.57) lower mean body weights (204.44 ± 25.99, g) compared to the ND group (263.50 ± 19.39, g). Table 2 presents the measured serum parameters, including Chol, TG, BHB, AST, and ALT levels, for the study groups. Rats on the KD showed significantly ($p<0.001$, effects size = 2.16) elevated serum Chol levels (95.33 ± 18.60, mg/dL) compared to those on the ND (59.40 ± 14.44, mg/dL). In contrast, TG levels did not differ significantly between the groups (ND: 86.90 ± 58.49; KD: 119.11 ± 42.88, mg/dL; $p=0.193$, effects size = 0.62). The KD group demonstrated markedly ($p<0.001$, effects size = 2.27) higher serum BHB concentrations [1.46 (0.90–1.52), mmol/L] than the ND group [0.03 (0.02–0.05), mmol/L].

Table 2 Comparison of lipid profile, liver enzymes and BHB levels in two groups of rats

Variables	Normal diet ($n=10$)	Ketogenic diet ($n=9$)	*p*-value*	Effect Size
Chol (mg/dL)	59.40 ± 14.44	95.33 ± 18.60	<0.001*	2.16
TG (mg/dL)	86.90 ± 58.49	119.11 ± 42.88	0.193	0.62
BHB (mmol/L)	0.03 (0.02–0.05)	1.46 (0.90–1.52)	<0.001**	2.27
AST (IU/L)	168.00 ± 30.85	176.44 ± 39.72	0.609	0.23
ALT (IU/L)	34.00 ± 5.60	65.00 ± 16.77	<0.001*	2.48

ALT, alanine transaminase; AST, aspartate transaminase; BHB: β-hydroxybutyrate; Chol, cholesterol; TG, triglyceride

*Statistically Significant ($p<0.05$), compared by independent samples t-test

**Statistically Significant ($p<0.05$), compared by Mann-Whitney U test

Regarding liver function enzymes, serum ALT levels were significantly ($p<0.001$, effects size = 2.48) higher in the KD group (65.00 ± 16.77, IU/L) compared to the ND group (34.00 ± 5.60, IU/L). However, serum AST levels showed no significant difference between the groups (ND: 168.00 ± 30.85; KD: 176.44 ± 39.72, mg/dL; $p=0.609$, effects size = 0.23).

Hepatic inflammatory markers

The concentrations of hepatic inflammatory markers are illustrated in Fig. 1 (a & b). The levels of IL-6 in the KD group (545.68 ± 66.88, pg/mL) were significantly higher ($p=0.005$, effect size = 1.47) than in the ND group (300.62 ± 38.29, pg/mL). Similarly, TNF-α concentrations in KD-fed rats (65.70 ± 11.58, pg/mL) were significantly greater ($p=0.002$, effect size = 1.66) compared to ND-fed rats (47.25 ± 10.52, pg/mL).

Histology of hepatic steatosis

Histological examination of liver sections from rats on the KD revealed the presence of macrovesicular steatosis, characterized by both small and large lipid droplets (indicated by arrows). In contrast, liver tissues of rats fed the ND showed no signs of steatosis (Fig. 2a & b). As illustrated in Fig. 2c, the average steatosis score was significantly elevated in the KD group [2.00 (1.00–2.00)] compared to the ND group [0.00 (0.00–0.25)].

Correlations of the evaluated parameters

The possible correlations among the measured parameters were also evaluated. As shown in the Fig. 3 (a-h), there were positive correlations between serum Chol levels with BHB ($r=0.461$, $p=0.047$), IL6 ($r=0.751$, $p<0.001$), TNF-α ($r=0.711$, $p=0.001$), ALT ($r=0.588$, $p=0.008$) levels, and steatosis score ($r=0.866$, $p<0.001$). The levels of BHB were positively correlated with TNF-α ($r=0.513$, $p=0.025$), and ALT ($r=0.500$, $p=0.029$) levels. The IL6 levels were positively correlated with TNF-α ($r=0.806$, $p<0.001$), ALT ($r=0.550$, $p=0.015$) levels, and steatosis score ($r=0.761$, $p<0.001$). The levels of TNF-α were positively correlated with ALT levels ($r=0.561$, $p=0.013$), and steatosis score ($r=0.695$, $p=0.001$). There

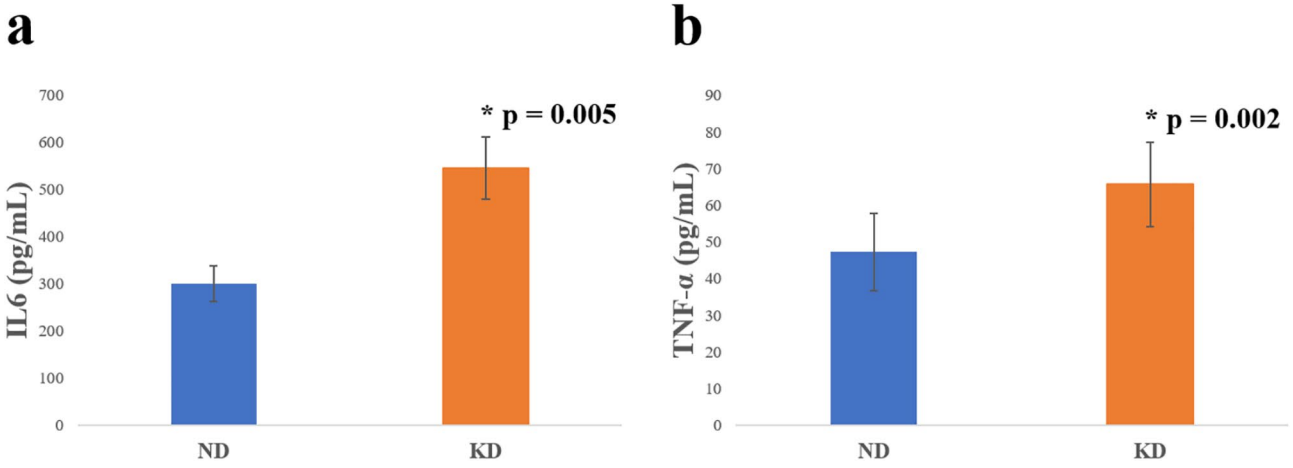

Fig. 1 The levels of interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) in the rat liver tissue samples. The levels of IL-6 (**a**) and TNF-α (**b**) in the liver tissue samples of the rats that received KD (545.68 ± 66.88, 65.70 ± 11.58; pg/mL; respectively) were significantly ($p = 0.005$, $p = 0.002$; respectively) higher than those in the rats received ND (300.62 ± 38.29, 47.25 ± 10.52; pg/mL; respectively)

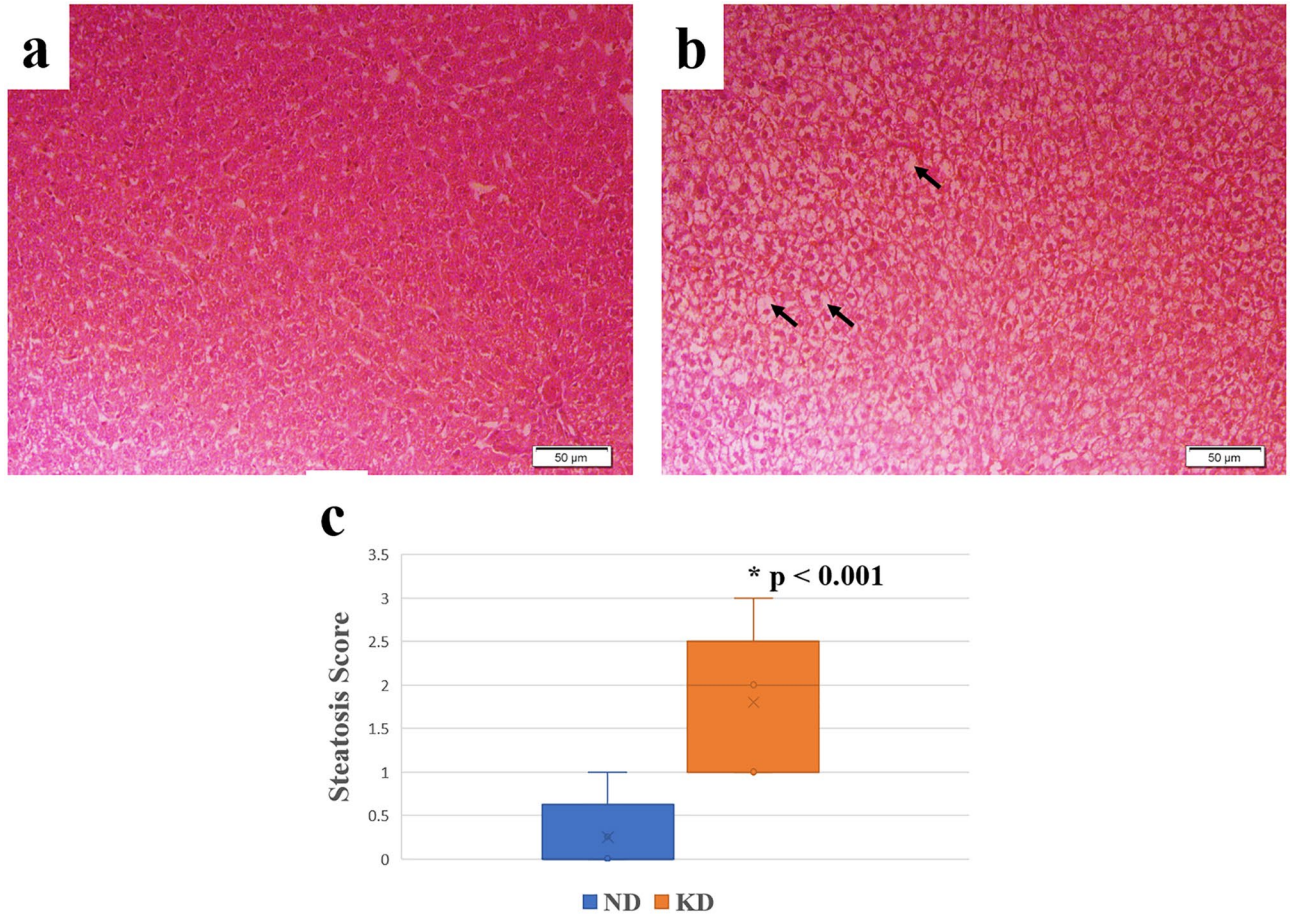

Fig. 2 Histology of hepatic steatosis. (**a**) The liver tissues of the rats fed the normal diet (ND) exhibited no evidence of steatosis. (**b**) The liver sections from the rats fed the ketogenic diet (KD), showed small and large droplet macrovesicular steatosis (arrows). (**c**) The steatosis score in the KD group [2.00 (1.00–2.00)] was significantly higher than in the ND group [0.00 (0.00–0.25)]

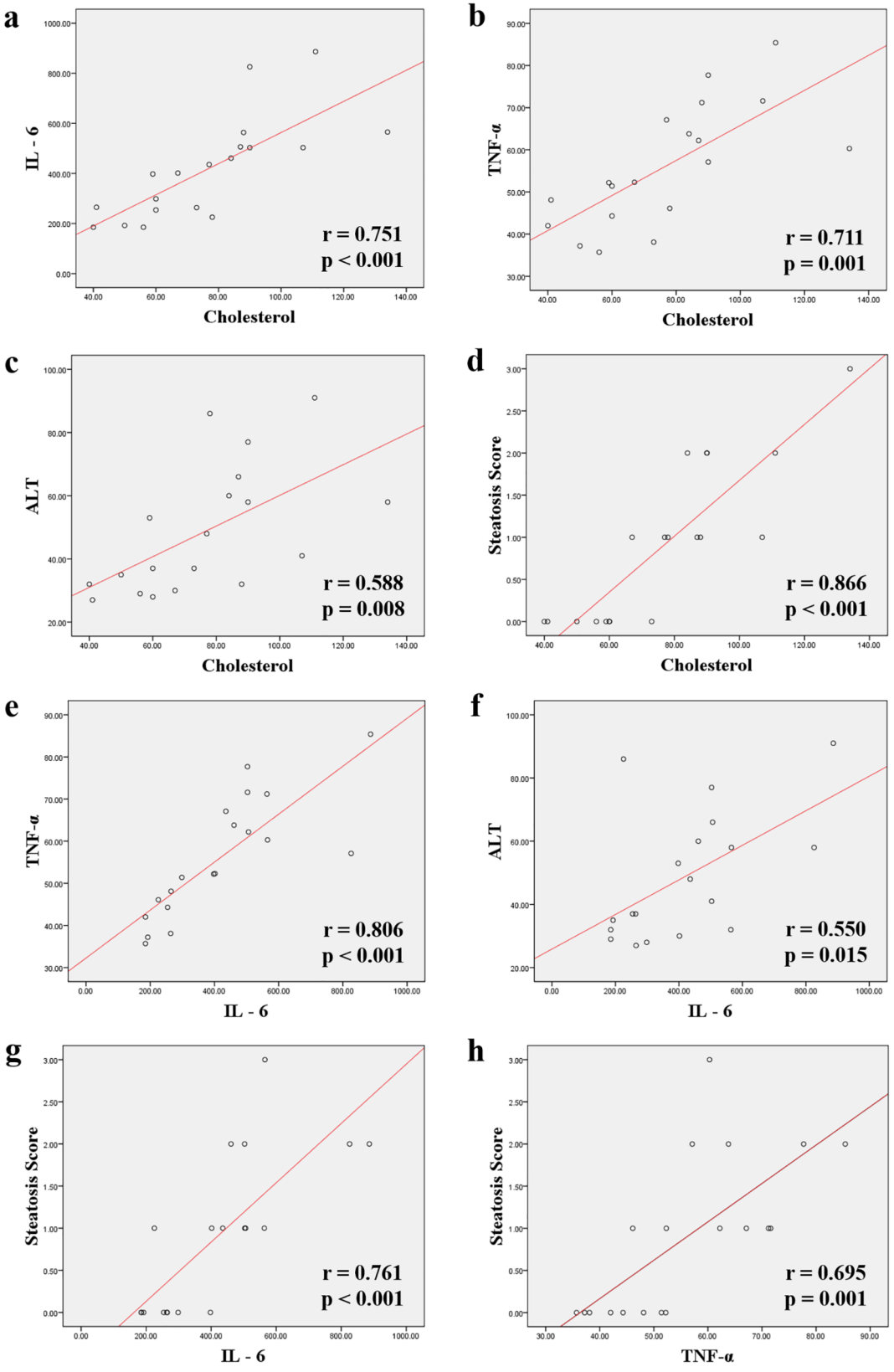

Fig. 3 Correlations of the evaluated parameters. There were positive correlations between serum Cholesterol (Chol) levels with (**a**) IL6 ($r = 0.751$, $p < 0.001$), (**b**) TNF-α ($r = 0.711$, $p = 0.001$), (**c**) ALT ($r = 0.588$, $p = 0.008$) levels, and (**d**) steatosis score ($r = 0.866$, $p < 0.001$). The IL6 levels were positively correlated with (**e**) TNF-α ($r = 0.806$, $p < 0.001$), (**f**) ALT ($r = 0.550$, $p = 0.015$) levels, and (**g**) steatosis score ($r = 0.761$, $p < 0.001$). (**h**) The levels of TNF-α were positively correlated with steatosis score ($r = 0.695$, $p = 0.001$)

was also a significant positive correlation between ALT levels and steatosis score ($r = 0.651$, $p = 0.003$).

Discussion

The present study aimed to evaluate the potential adverse effects of the KD on liver function, specifically focusing on hepatic inflammation and lipid accumulation. The KD, characterized by its high-fat content, is known to significantly affect lipid metabolism, which can have downstream effects on liver health. Beyond its therapeutic applications in neurological diseases and cancer, the KD has recently gained traction as a weight loss strategy for obesity management [21, 22]. Consistent with previously published results [9, 10, 23], rats maintained on a KD exhibited decreased weight compared to the ND group. KDs are effective for weight loss, as evidenced by both short-term and long-term studies [24–28]. Despite strong evidence supporting KDs for weight-loss therapy, different theories exist regarding their exact mechanisms. The weight-loss effects are attributed to factors like appetite reduction, hormonal regulation, increased fat metabolism, and higher metabolic efficiency [29–31].

Our findings demonstrated a significant increase in serum Chol levels in the KD group, while TG levels remained unchanged. This aligns with the known impact of high-fat diets, but the inconsistent results in the literature suggest that the exact effects of the KD on lipid profiles may depend on various factors, including diet composition and duration. The scientific literature presents conflicting findings regarding the KD's impact on lipid metabolism. While some investigations have noted significant increases in both TG and Chol levels [11, 12], others have reported no substantial changes or even decreases in these indicators [10, 16, 32]. For example, research by Bielohuby et al. showed elevated TG concentrations but no notable differences in Chol when comparing two distinct KD formulations [16]. In contrast, Holland et al.'s study revealed an unexpected reduction in both TG and Chol in KD-fed rats compared to those on Western or standard diets [32]. These varying outcomes might be explained by differences in the specific proportions of macronutrients and the types of fats employed across studies, highlighting the need for additional research to elucidate how these factors affect lipid profiles in the context of the KD.

A high intake of dietary fat, particularly saturated fat, can lead to excessive fat accumulation in the liver, promoting hepatic steatosis and inflammation. In the present study, we observed a significant increase in hepatic inflammatory markers, specifically TNF-α and IL-6, along with notable signs of hepatic steatosis in the KD group. These findings are consistent with several studies that have reported increased liver inflammation and fat accumulation in response to high-fat diets, including the KD [9, 11, 12, 33–35]. However, not all studies agree on the inflammatory effects of the KD. While some have documented pro-inflammatory responses in the liver, others have reported anti-inflammatory or anti-steatogenic effects [13, 36]. For example, Douris et al. found that both short-term (8 weeks) and long-term (80 weeks) consumption of the KD led to hepatic steatosis and increased expression of pro-inflammatory markers [12]. Similarly, another study associated the KD with systemic inflammation and hepatic steatosis after 22 weeks [11]. Asrih et al. found increased liver inflammation and lipid accumulation in mice fed a KD, but a decrease in inflammation in white adipose tissue (WAT) after 4 weeks on the diet, compared to standard chow [33]. Additionally, research by Carmiel-Haggai et al. showed that obese rats on a high-fat diet for 8 weeks developed liver injury and steatohepatitis, along with elevated TNF-α levels [34]. Wang et al. demonstrated a clear link between elevated serum fatty acids and liver lipid content, which was associated with heightened non-alcoholic fatty liver disease (NAFLD) activity scores, liver inflammation, and injury [35]. Also, Garbow et al. observed hepatic lipid accumulation and steatosis in rats on the KD for 12 weeks without significant changes in TNF-α and IL-6 levels [9]. In contrast, Jani et al. reported anti-steatogenic effects of the KD in rats after 8 weeks [13], and Liu et al. demonstrated the anti-inflammatory effects of a calorie-restricted KD in rats after 30 days [36]. The contrasting outcomes highlight the complexity of the KD's effects on different tissues and organs, suggesting that diet composition, duration, and caloric intake play crucial roles in determining its impact.

In addition to the inflammatory response, our study also found that serum ALT levels, a marker of liver injury, were significantly elevated in the KD group. This increase in ALT may be linked to the lipid accumulation and inflammation observed in the hepatic tissue. Several studies have similarly reported elevated ALT levels in response to the KD, further supporting the potential hepatotoxic effects of the diet [9, 12, 37]. Douris et al. observed increased ALT levels in both short-term (8 weeks) and long-term (80 weeks) KD-fed mice [12], also Garbow et al. found elevated ALT in KD-fed mice when compared to those on a Western or standard diet [9]. Notably, Ellenbroek et al. and Jornayvaz et al. reported significant increases in both ALT and AST in mice fed a classic KD [10, 11].

However, some studies have reported no significant changes or even reductions in serum liver enzyme levels in response to the KD. For example, Arsyad et al. found no significant changes in ALT and AST levels in rats fed a KD for 60 days [38], and Holland et al. reported decreased ALT levels in rats on a KD with a different macronutrient composition (20% protein, 10%

carbohydrate, and 70% fat) compared with the western diet and standard diet [32]. These discrepancies may be due to differences in diet composition, duration, and fat sources used in the studies, further underscoring the importance of considering these factors when interpreting the KD's effects on liver function.

A significant factor that may account for the discrepancies observed in various studies is the specific formulation of the KD, particularly with respect to macronutrient ratios and caloric restriction. Our investigation employed the classic KD, characterized by a composition of 90% fat, 8% protein, and 2% carbohydrates, which is consistent with numerous other studies [9, 11, 12, 33, 34]. In contrast, Jani et al. implemented a diet comprising 80% fat and 20% protein, with carbohydrates entirely excluded [13]. Additionally, Liu et al. restricted caloric intake to 20% of the required levels, although the macronutrient ratios were comparable to those of the classic KD [36].

Research indicates that rats necessitate a minimum protein intake of 12–14% for optimal growth and physiological function [39, 40]; however, most KD formulations, including the one utilized in our study, contain less than 10% protein. Diets with elevated protein levels, such as the 20% protein diet employed by Jani et al. and Holland et al. have demonstrated more beneficial effects on lipid metabolism and liver enzyme activity [13, 32].

Another critical consideration that may significantly influence the outcomes associated with the KD is the source of fat utilized in the study. The primary fat source in the present study were mutton tallow and cocoa butter. Both sources are high in saturated fatty acids (52–64%) [41–45]. Saturated fats are recognized for their potential to elevate serum cholesterol levels, which may lead to lipid accumulation in the liver and subsequent inflammatory responses [46].

Furthermore, the duration of KD administration is an important aspect to consider. Our study was confined to a 30-day timeframe; however, longitudinal studies have indicated that the effects of the KD may change over time. For example, Douris et al. observed that both short-term and long-term KD consumption resulted in hepatic steatosis and heightened liver inflammation [12], while Garbow et al. reported a continued increase in liver enzyme levels and lipid accumulation after 12 weeks on the KD [9]. These findings imply that the duration of KD administration may be a crucial determinant of its long-term effects on liver health.

Limitation

This study had several limitations that should be addressed in future research. The short duration of the experiment (30 days) may not have been sufficient to fully capture the long-term effects of the KD on liver function. The lack of quantitative assessment of hepatic lipids and glycogen contents could be considered study limitations. Future studies should address this to gain valuable biochemical and metabolic insights into liver fat accumulation. Cocoa butter and mutton tallow were applied as fat sources in the present study. Future studies should explore the effects of different fat sources, such as vegetable oils, and varying macronutrient ratios to better understand how these factors influence the KD's impact on liver health. Moreover, a broader range of inflammatory markers and liver injury biomarkers should be evaluated to provide a more comprehensive understanding of the KD's effects. Lastly, the potential adverse effects of the KD on other organs, such as the kidneys and heart, should also be investigated.

Conclusion

In summary, our study provides a detailed evaluation of the effects of KD on liver function, focusing on hepatic inflammation, lipid accumulation, and liver enzyme levels. A KD consisting of 90% fat, 8% protein, and 2% carbohydrates may elevate serum cholesterol, induce hepatic steatosis and inflammation, and increase ALT levels, suggesting potential liver injury. Further research is needed to explore the long-term effects of the KD, as well as the influence of different diet compositions and fat sources on liver health. It seems crucial that the liver function of the individuals under KD be monitored regularly for optimal clinical management.

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Author contributions

Khatereh Rezazadeh: Conceptualization, Methodology, Data Collection, Formal Analysis, Writing– Original Draft, Visualization. Mohammad Barzegar: Methodology, Data Collection, Writing– Review & Editing. Erfan Nezamdoost: Data Collection, Formal Analysis, Writing– Review & Editing. Maryam Shoaran: Data Collection, Validation, Writing– Review & Editing. Mehran Mesgari Abbasi: Data Collection, Validation, Writing– Review & Editing. Babollah Ghasemi: Supervision, Writing– Review & Editing. Solmaz Madadi: Supervision, Writing– Review & Editing. Sina Raeisi: Supervision, Project Administration, Writing– Review & Editing.

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Data availability

The data are available from the correspond author upon reasonable request.

Declarations

Ethics approval and consent to participate

All procedures were according to the NIH Guidelines for the Care and Use of Animals and have been approved by the research ethics committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1400.917).

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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