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Effect of Ketogenic diet on hepatic tissue and the role of fibroblast growth factor 21 signaling in obese and non-obese rats

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Abstract

Background: Ketogenic diet (KD) is a high fat, moderate protein, and very low carbohydrate diet. Initially developed as an alternate therapy for refractory epilepsy, various adaptations of the KD have since gained popularity for obesity management due to their strong effects on appetite suppression and weight reduction.

Aim: This study was designed to find to what extent KD can affect the hepatic tissue in obese and non-obese experimental animals through modulating the secretion and response to FGF21.

Material and Methods: In our study, sixty male albino rats were used and randomly assigned to 4 equal groups: a control group, a normal-calorie diet followed by ketogenic diet group, a high-fat diet (HFD)-induced obesity group, and a HFD followed by ketogenic diet group. The following parameters were assessed in all group: liver enzymes, total lipid profile, blood glucose level, serum FGF21 and TNF- α levels. Furthermore, malondialdehyde (MDA), hydroxyproline, triacylglycerol levels and β klotho gene expression were assessed in liver tissue homogenate by colorimetric assays. Hepatic tissue was subjected to histopathological analysis.

Results: The ketogenic diet showed a significant decrease in FGF21, TNF- α , hydroxyproline, and MDA levels, along with a notable upregulation of the β klotho gene.

Conclusion: These results suggest that the KD has therapeutic potential for NAFLD and may serve as a promising strategy for its management.

Keywords: Ketogenic diet, FGF21, TNF- α , MDA, β klotho

Introduction

Obesity is a global health issue that has reached pandemic proportions with a steady rising prevalence across every age, sex and race. It is linked to a higher risk of several metabolic comorbidities, psychosocial problems and functional limitations. The pathogenesis of obesity is a complex multifactorial issue. It isn't just lipid accumulation and involves interactions among many cell types [1].

The ketogenic diet (KD) is a high-fat, adequate protein and low-carbohydrate diet, aiming to facilitate weight reduction. It induces ketosis, in which the body shifts to using fat rather than carbohydrates as its main source of energy [2].

The main objectives of KD consumption are to improve metabolic health and reduce total body fat. Recent study suggests potential advantages in lowering the risk of some illnesses, such as hyperlipidemia, heart disease, and cancer [3]. According to some researchs, KD results in dyslipidemia, a pro-inflammatory condition that contributes to the development of hepatic steatosis, cardiomyocyte apoptosis and cardiac fibrosis [4].

Fibroblast growth factor 21 (FGF21), a hormone that regulates lipid metabolism, and ketogenesis. It is produced mainly in the liver and then secreted into the bloodstream. It is also produced by extrahepatic tissues, including skeletal muscle and white and brown adipose tissues [5]. Numerous stimuli, including starvation and ketogenic diet can trigger the production of FGF21 which in turn encourages cellular adaptation. FGF21 exerts its effects on target cells through fibroblast growth factor receptors and β -Klotho, a transmembrane protein that is an essential for FGF21 signaling [6].

Beta klotho (KLB) functions as a mandatory coreceptor within the FGFR signaling pathway, enabling the activity of the endocrine fibroblast growth factors FGF19 and FGF21 [7]. The limited expression of transmembrane β -Klotho primarily in the liver, pancreas, and adipose tissue corresponds to the major sites of FGF21 action. As a result, KLB, rather than the broadly distributed FGF receptors, is responsible for conferring tissue-specific responsiveness to FGF21 [8].

Tumor necrosis factor- α (TNF- α), a pleiotropic immune cytokine produced mainly by innate and adaptive immune system cells. It is involved in numerous regulatory and inflammatory processes and has critical role in the development of autoimmune and inflammatory diseases [9]. TNF- α exerts a wide range of effects through its two receptors, TNF receptor 1 and 2 (TNFR1 and 2), each activating distinct downstream signaling pathways. It regulates both acute and chronic inflammation and, under certain conditions, can induce cell death [10].

Materials and Methods

Chemicals

All chemical substances utilized in this research, unless otherwise specified, were supplied by Sigma Aldrich (St. Louis, USA).

Experimental animals

In this research, sixty male albino rats weighing 130-150 g were used. These rats were sourced from the experimental animal colony at Tanta University. They were housed in wire mesh cages, allowed a two-week acclimation period, and provided with standard rat feed and water.

Experimental design

After that, the rats were grouped into 4 equal groups with fifteen rats per group. Group I was fed a normal chow diet for 16 weeks. Group II (normal chow diet followed by ketogenic diet): This group was fed a standard diet for 8 weeks followed by ketogenic diet (KD) for 8 weeks. Group III (HFD-induced obesity group): This group was fed house prepared HFD for 16 weeks. Group IV (HFD followed by ketogenic diet): This group was fed house prepared HFD for 8 weeks followed by KD for 8 weeks. With an approval code NO (35204/01/22), the experiment was performed in accordance with the guidelines of Tanta's institutional Research Ethics Committee (REC).

Blood and tissue sampling

After an overnight fast at the end of the study, the rats were anesthetized with ether, and blood and liver tissues were collected.

Blood sampling

Blood was withdrawn through cardiac puncture. Sera were obtained by centrifuging the samples at 4 °C for 20 minutes at 3000 rpm after being clotted at room temperature, in turn, aliquoted and maintained at -80°C for biochemical assays

Hepatic tissue sampling

Following blood collection, the thorax and abdomen were opened, the liver was removed, and any remaining material

was cleaned with ice-cold saline. Filter paper pieces were used to wipe away the extra saline. Liver samples were chilled, weighed, and divided. Left liver lobes were fixed in 10% formalin for histopathological examination, while right lobes were stored at -80 °C for biochemical analyses. For MDA and hydroxyproline assays, liver tissues were homogenized (1:5 w/v) in buffer or saline, centrifuged, and the resulting supernatants preserved at -80 °C. For triacylglycerol determination, 200 mg liver fragments were homogenized in propan-2-ol, shaken, the centrifuged, and the supernatant stored. Another liver portion was foil-wrapped and stored at -80 °C for PCR analysis.

Biochemical assays

The serum levels of glucose, lipid profile, as well as hepatic enzymes activities, were determined using commercially available kits (Cat. Nos. GL 13 20, CH 12 20, TR 20 30, CH 12 30, AL1031 (45), AS1031 (45), respectively; Bio-diagnostic, Giza, Egypt). The concentrations of low-density lipoprotein cholesterol (LDL-c) were calculated as follows: $LDL-c = TC - (TG/5) - HDL$

Serum FGF21 and TNF- α levels assay

Serum levels of FGF21 and TNF- α were determined with ELISA kits obtained from Sun Red Biotechnology (Shanghai, China; Catalogue numbers 201-11-4127 and 201-11-0765, respectively).

Assessment of hepatic redox status

Hepatic malondialdehyde (MDA) level was measured colorimetrically using a Biodiagnostic Company kit (Egypt; Cat. No. MD 25 29), based on the detection of thiobarbituric acid-reactive substances at 534 nm.

Assessment of hepatic triacylglycerol and hydroxyproline levels

Hepatic triacylglycerol level was assessed colorimetrically using a commercial enzymatic kit from BIOMED Diagnostics, Germany., Germany. The hepatic hydroxyproline content was measured by oxidizing hydroxyproline to pyrrole, followed by reaction with p-dimethylaminobenzaldehyde to yield a red product. The color intensity was determined colorimetrically at a wavelength of 555 nm.

Quantitative real time-PCR analysis of beta klotho

Total RNA was isolated from frozen liver tissue by utilizing the PureLink® RNA Mini Kit. RNA purity, concentration, and integrity were assessed with a NanoDrop spectrophotometer. cDNA synthesis (first-strand) was carried out using the FastGene® 55-Scriptase Kit, and subsequently amplified with the SensiFAST™ SYBR® Lo-ROX Kit. Gene expression was analyzed using GAPDH as the reference gene, with primer sequences listed in Table 1. qPCR was carried out with an initial activation at 95 °C for 2 min, followed by 40 amplification cycles (95 °C for 5 s, 60 °C for 10 s, 72 °C for 20 s), ended by a melting curve from 72 °C to 95 °C. Amplification and data collection were conducted on the StepOnePlus RT-PCR System. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression levels, with GAPDH serving as the reference gene.

Table 1: Shows the primer sequences used in the analysis

Gene	Forward primer (5'to 3')	Reverse primer (5'to 3')
β klotho	CTCTGAAAGCCTACGTGTTGG	TAGAAACGAGATGAAGGCCAG
GAPDH	AGGGCTGCCTTCTCTTGTGAC	TGGGTAGAATCATACTGGAACATGTAG

Histopathological examination of liver tissue

The hepatic tissue samples from all groups were preserved at room temperature for twenty-four hours in 10% neutral buffered formalin. The tissues were then processed through standard histological procedures, involving dehydration, clearing, infiltration, and embedding in paraffin blocks that were sectioned at five micrometres thickness by a microtome. The resulting sections were subsequently stained with H&E. Then, these stained sections were examined and imaged by a light microscope (Olympus, Japan).

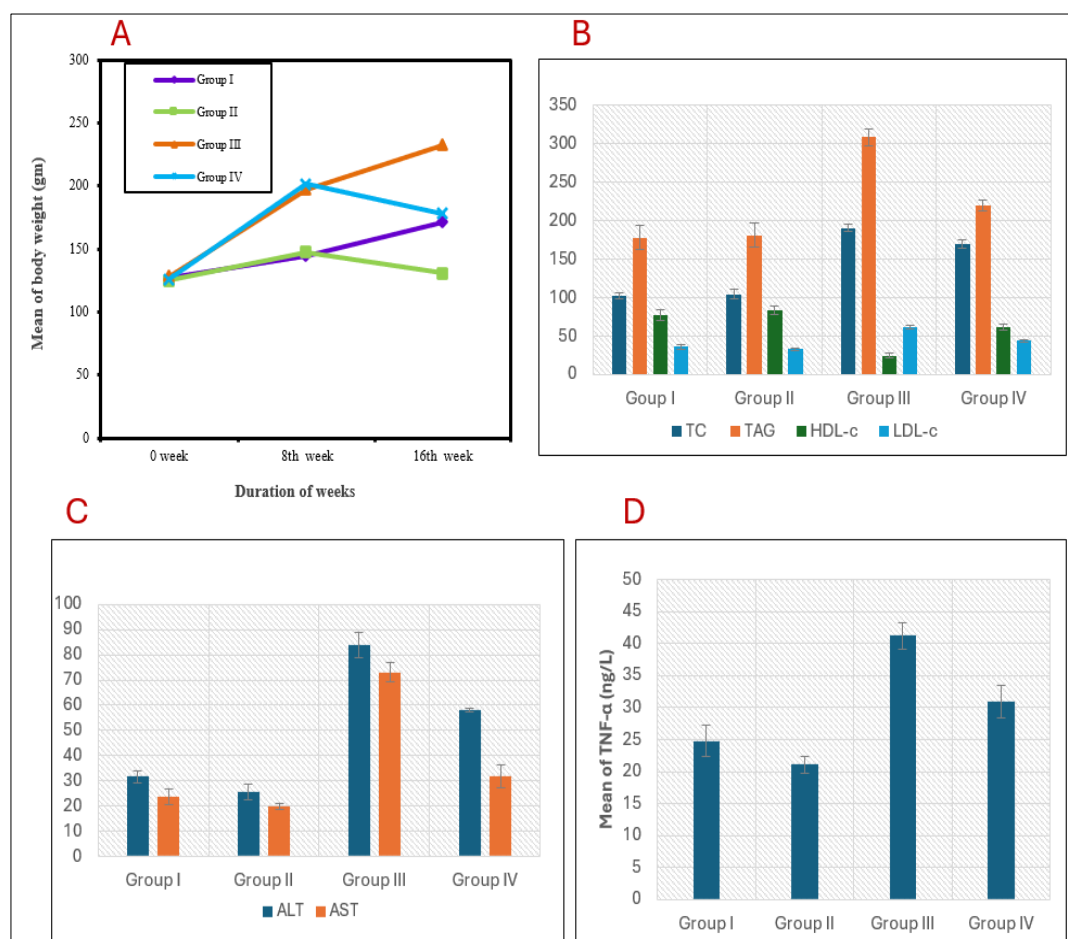
Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD) and p-values. Differences among groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered significant.

The Effect of KD on Body Weight, Lipid Profile, Liver Enzymes and Serum TNF- α Level:

By the 8th week of the study, body weight differed significantly among the groups. Body weight of groups III and IV was markedly increased than the normal-calorie diet groups. At the 16th week, the end of the study, both ketogenic diet groups (Groups II and IV) demonstrated a marked decrease in body weight than group I and III, respectively ($P < 0.05$), as illustrated in Figure 1A.

Group III rats exhibited marked elevation in serum glucose, TG, TC, LDL-c, liver enzymes activity (ALT and AST) and TNF- α levels when compared with the other studied groups. However, group III exhibited marked decrease in HDL-c levels compared with the other studied groups. The HFD followed by ketogenic diet group demonstrated a significant improvement in serum glucose, lipid profile and liver enzymes, as well as TNF- α levels, compared with the HFD induced obesity group, as presented in Figure 1B, 1C and 1D.

**Fig 1:** Effect of KD on Body Weight, Lipid Profile, Liver Enzymes and Serum TNF- α Level

The Effect of KD on oxidative stress, hepatic steatosis and fibrosis markers

Results showed significant elevations in hepatic MDA, TAG and hydroxyproline levels in induced group III in comparison with other studied groups. The KD had a strong

antioxidant, antisteatotic and antifibrotic effects as indicated by low MDA level, decreased TAG and hydroxyproline levels when compared to the HFD-induced obesity as presented in Table 2.

Table 2: Effect of KD on serum glucose, oxidative stress, hepatic steatosis and fibrosis marker levels

Parameters/ Groups	Group I (n=15)	Group II (n=15)	Group III (n=15)	Group IV (n=15)
Glucose (mg/dl)	92.27 ± 4.94 ^{c,d}	84.80 ± 5.26 ^{c,d}	157.54 ± 3.27 ^{a,b,d}	112.18 ± 3.93 ^{a,b,c}
MDA (nmol/mg tissue protein)	4.97 ± 1.30 ^{b,c,d}	2.68 ± 0.54 ^{a,c,d}	26.94 ± 4.25 ^{a,b,d}	10.67 ± 1.86 ^{a,b,c}
Hydroxyproline (µg/mg tissue protein)	49.15 ± 3.43 ^{b,c,d}	42.44 ± 3.14 ^{a,c,d}	94.22 ± 3.49 ^{a,b,d}	74.87 ± 3.41 ^{a,b,c}
Hepatic triacylglycerol (mg/ mg tissue protein)	4.72 ± 0.59 ^{b,c,d}	3.70 ± 0.31 ^{a,c,d}	10.29 ± 1.2 ^{a,b,d}	7.6 ± 0.81 ^{a,b,c}

Values are expressed as mean ± standard deviation (SD). Significant differences among the groups were determined at $P < 0.05$ (*n denotes the number of rats per group) using one-way ANOVA followed by Tukey's post hoc test. The study included the following experimental groups: Control (Group I), normal chow diet followed by ketogenic diet (Group II), HFD-induced obesity (Group III), and HFD followed by ketogenic diet (Group IV). Abbreviation: MDA, malondialdehyde.

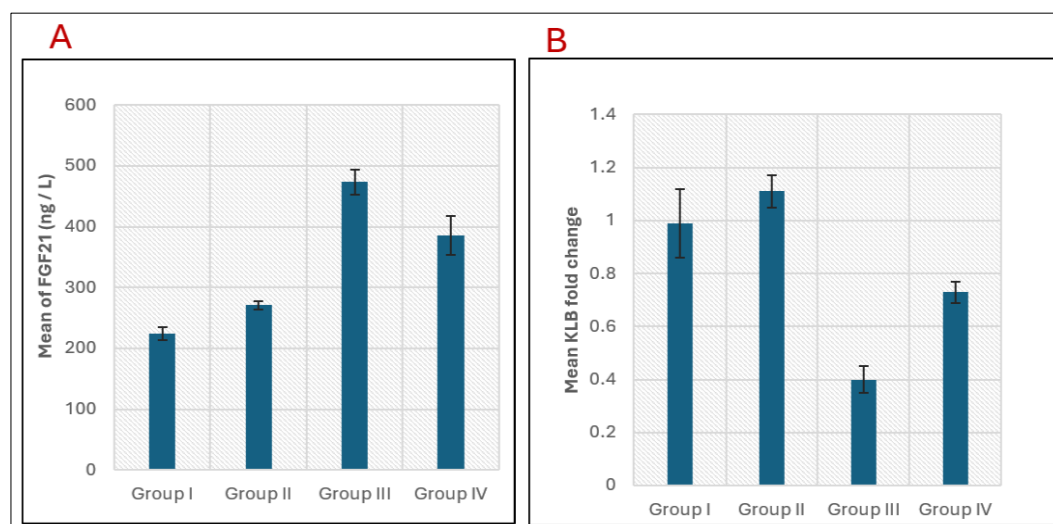
The Effect of KD on Serum Levels of FGF21

As shown in Figure 2A, the HFD-induced obesity group exhibited a significant elevation in serum FGF21 levels

compared to the other groups. Group II, which received a normal chow diet followed by a ketogenic diet, also showed a significant increase in FGF21 levels compared to the control group (Group I). Moreover, serum FGF21 levels were significantly reduced in the HFD followed by ketogenic diet group (Group IV) relative to the HFD-induced obesity group (Group III).

The Effect of KD on Beta klotho expression

Hepatic KLB gene expression was markedly decreased in group III relative to the control group. Conversely, administration of KD resulted in a significant increase in KLB expression (Figure 2B).

**Fig 2:** Effect of KD on Serum Levels of FGF21 and Hepatic Beta Klotho Expression

Histopathological results of liver tissue

Hematoxylin & Eosin-stained sections of livers from the control and normal chow followed by ketogenic diet groups showed normal liver tissue, hepatocytes are normal polygonal cells with single central nucleus, no steatosis and no inflammatory infiltrate (H&E 200X) [Figure 3A & B]. In the HFD induced obesity group, sections showed macrovesicular steatosis, ballooned hepatocytes (intracytoplasmic fat droplet displacing the nucleus to the periphery), and lobular inflammation (H&E, 200X) [Figure 3C]. Sections from the HFD followed by ketogenic diet group demonstrated minimal steatosis (hepatocyte with eosinophilic cytoplasm, small infrequent fat droplets, small central nucleus) and minimal inflammation (H&E, 200X) [Figure 3D].

Discussion: Obesity is an increasing serious public health problem around the world. It is a complex, multifactorial

condition that has significant consequences for health. With the prevalence of obesity continuing to rise, considerable attention has been focused on identifying effective management strategies. Among these, Low-carbohydrate diets have been shown to be more effective for weight loss than low-fat diets, especially in overweight but otherwise healthy individuals. The ketogenic was used to manage obesity and related metabolic disorders [11]. However, while KD has beneficial effects on weight reduction, its full metabolic effects remain unclear. Some research have indicated that KD may also lead to contrary effects, including dyslipidemia, increased inflammation, elevated risk of metabolic syndrome, as well as cardiomyocyte apoptosis and cardiac fibrosis [12].

To explore the potential effects of KD on hepatic tissue in obese and non-obese rats, obesity was induced by using high-fat diet for 8 weeks in male albino rats. HFD induced obesity is more or less similar to human obesity.

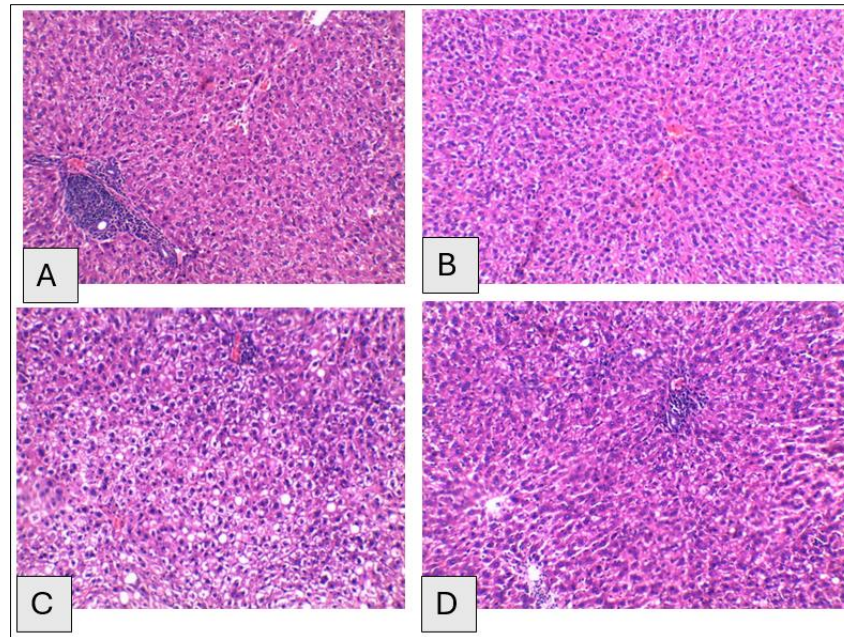


Fig 3: Histopathological results of liver tissue

Our study successfully induced NASH, as evidenced by elevated liver enzymes (ALT and AST) and significant increases in triglycerides (TG), total cholesterol (TC), and LDL-C levels, along with a marked reduction in HDL-C levels. Histopathological analysis of the liver further revealed characteristic features of NASH, including prominent macrovesicular steatosis, thickened portal tracts, and lobular inflammation. These results collectively confirm the successful establishment of NASH.

Interestingly, consumption of KD revealed marked reduction in serum glucose, liver enzymes (ALT, AST) activities and improved lipid profile as demonstrated by Yang *et al.* (2021) [13]. In our study, Group IV showed significant amelioration of histopathological changes relative to Group III, characterized by a fewer number of fat vacuoles, reduced necrosis, and fewer inflammatory foci.

In line with the above-mentioned data, Liu *et al.* (2022) [14]; Rinaldi *et al.* (2023) [15] revealed that, consumption of KD markedly reduces HFD-induced hepatic steatosis by decreasing overall adiposity and the ectopic deposition of fat. Additionally, KD ameliorates NAFLD by suppressing appetite, enhancing lipolysis, reducing lipogenesis, increasing satiety, and producing a thermogenic effect through protein intake, all of which contribute to weight loss. However, and in contrast, Garbow *et al.* (2011) [16] showed that long-term consumption of the KD can support weight loss and improve glycemic control, it may also contribute to hyperlipidemia, elevated hepatic enzymes, and NAFLD. This effect is likely attributable to prolonged high-fat intake, which can induce inflammation and endoplasmic reticulum stress, resulting in change membrane permeability of periportal hepatocytes. These hepatocytes may become overloaded when the amount of fat they receive exceeds their capacity for oxidation or VLDL-mediated export. Moreover, the buildup of ceramides in macrophages or hepatocytes, driven by excessive saturated fatty acids, may activate the inflammasome, resulting in macrophage recruitment and activation. Our study didn't show these results, this may be because the period of consumption of KD wasn't sufficient.

FGF21 action is mediated through binding to FGF receptor in presence of an essential co-receptor β Klotho [17]. Our study demonstrated a marked increase in serum FGF21 levels alongside a significant reduction in hepatic KLB gene expression in the HFD-induced obesity group (Group III) than other groups. Matching with these findings, Fisher, *et al.* (2010) [18]; Martínez-Garza *et al.* (2019) [19] revealed that obesity is associated with resistance to FGF21. This resistance has been attributed to a reduced capacity of FGF21 to trigger signaling through the ras-raf-MAPK pathway, evidenced by notably diminished ERK1/2 phosphorylation in both hepatic and adipose tissue. Furthermore, other studies reported by So *et al.* (2013) [20]; Díaz-Delfín, *et al.* (2012) [21] have also shown that both high serum TNF- α and blood glucose level which are elevated in NAFLD can impair the FGF21 action by repressing KLB gene expression in both adipose tissue and pancreatic islets. The present work verified that consumption of KD can greatly lower FGF21 resistance as evidenced by significant decrease in FGF21 levels and significant upregulation of the hepatic KLB gene in the HFD followed by the KD group (group IV) when compared to group III. This was already documented in some studies which proved that the consumption of the KD can lower FGF21 resistance that was developed in obesity [22]. Also, this is likely due to the ketogenic diet's potential to decrease elevated TNF- α and blood glucose levels, both of which are known to suppress KLB gene expression. In addition, ketogenic diets may enhance KLB expression through epigenetic mechanisms: β -hydroxybutyrate functions as a natural inhibitor of histone deacetylases (HDACs), greater transcription of specific genes. Ketone bodies can also activate the AMP kinase signaling pathway, which supports the expression of various metabolic genes, including KLB Kawakami *et al.* (2022) [23]; Guo *et al.* (2024) [24].

TNF- α exerts multiple effects across various cell types. It has role in the pathogenesis of chronic inflammatory conditions [25]. Our results showed that group III exhibited marked elevation in serum TNF- α levels than the other groups. serum TNF- α levels were markedly increased in group III compared with the other groups. This rise could be

attributed to obesity, which is recognized as a state of low-grade chronic inflammation that contributes to various metabolic disorders. Enlarged adipocytes release inflammatory cytokines which recruit and activate immune cells while inhibiting the release of adiponectin and anti-inflammatory cytokines [26].

A marked decrease in TNF- α levels was observed in the group receiving HFD followed by a ketogenic diet (Group IV) compared with the HFD-induced obesity group (Group III). These results align with Jani *et al.* (2022) [27], demonstrating that the ketogenic diet reduces inflammation by suppressing TNF- α expression.

Oxidative stress (OS) plays a key role in the development of NAFLD. The mitochondria, endoplasmic reticulum, and peroxisomes all contribute to the generation of oxidative stress in this disease. Our research highlighted the interlink between oxidative stress biomarkers and NAFLD as it revealed a marked increase in liver tissue MDA levels in group III relative to the other groups. This increase is likely attributed to enhanced lipid peroxidation resulting from oxidative stress, a key contributor to NAFLD progression. Previous research has also reported higher MDA concentrations in liver tissue as an indicator of oxidative stress in NAFLD [28].

Interestingly, we explored that KD has antioxidant effect, evident by significantly decreased liver MDA levels. These findings matched those reported by Rojas-Morales *et al.* (2022) [29] that explained that based on the potent antioxidant activity of the KD via inhibiting lipid peroxidation and restoring antioxidant enzymes. In contrast Arsyad *et al.* (2020), found that prolonged use of the KD can induce oxidative stress. This can be attributed to ketone bodies promote increased ATP production in the mitochondria compared to glucose. Fat metabolism involves multiple biochemical reactions including reduction, oxidation, hydroxylation, and conjugation that can generate excessive reactive oxygen species, potentially exceeding the liver's antioxidant defense capacity [30].

In this study, liver tissue from the HFD-induced obesity group (Group III) showed a significant elevation in hydroxyproline levels, a recognized marker of hepatic fibrosis, compared with the other groups. These findings align with existing research, which evidenced that NASH can progress to fibrosis and lead to elevated hepatic collagen fibers expression [31]. Liver tissue from the HFD followed by KD group (Group IV) exhibited marked decrease in hydroxyproline levels relative to the HFD-induced obesity (Group III). Charlot *et al.* (2022) Clarified this anti-fibrotic effect based on that the KD can decrease the hepatic steatosis and swelling which promote hepatic stress, that contributes to fibrosis. This also can be explained by the repressive effect of the KD on the expression of collagen fibers [32]., Zhang *et al.* (2020)

On the other hand, a study by, Zhang *et al.* (2020) demonstrated that KD induces hepatic fibrosis in diabetic mice through transforming growth factor-beta 1-mediated activation of hepatic stellate cells. These results differ from those of the current study, likely due to variations in the duration of ketogenic diet administration in the diabetic mice [33].

Based on our findings, the KD appears to improve both obesity and NAFLD through its anti-lipogenic, lipolytic, anti-inflammatory, and glucose-regulating effects. These benefits are associated with the upregulation of KLB gene

expression and improved FGF-21 resistance. Additionally, this evidence suggests that the KD may aid in weight loss, reduce visceral adiposity, and support appetite control, positioning it as a promising adjunctive therapy.

Conclusion

Assessed data stated that the ketogenic diet has therapeutic potential by reducing FGF21 resistance in the treatment of NASH and represents a promising co-therapeutic strategy in its management. It may promote regulation of lipid and carbohydrate metabolism besides retarding the propagation of fibrogenesis and inflammatory cascade leading to cirrhosis and hepatocellular carcinoma.

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