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Proceeding Paper

Beneficial Effects of Ketogenic Diet on Nonalcoholic Steatohepatitis in Obese Mice Model †

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Abstract: Obesity is associated with a low-grade inflammation, characterized by the secretion of inflammatory mediators, that contribute to non-alcoholic fatty liver disease (NAFLD) development. Steatosis may be complicated by hepatocellular injury and liver inflammation (steatohepatitis or NASH). Ketogenic diet (KD), a high-fat and low-carbohydrate diet, seems to present anti-inflammatory properties which could reduce NAFLD development. However, the mechanisms involved in its beneficial effects remain unclear. Obesity was induced in C57/Bl6 mice (n = 20) by using a high-fat, high-sugar diet (HFD). After 16 weeks of HFD, mice were split into two groups for six weeks: KD mice (n = 10) and HFD mice (n = 10). At the end of the 22-week protocol, we measured liver weight, hepatic lipid accumulation and inflammatory infiltrates with histological staining, and hepatic gene expression by RT-qPCR. Both HFD and KD were isocaloric and compared with a control diet (Ctrl) group of mice (n = 10). After 22 weeks of HFD, mice developed obesity (+82% of weight gain, $p < 0.001$) associated with an increase in liver weight (+113%, $p < 0.001$) and hepatic lipid accumulation (+158%, $p < 0.001$), compared with Ctrl. RT-qPCR revealed an increase in TNFa ($p < 0.05$), IL-1 ($p < 0.05$) and collagen 1 ($p < 0.01$) gene expression, but no changes of IL-10, TGFb and IFNg, compared to Ctrl. Histological staining showed an important steatosis and inflammatory infiltrates. Compared to HFD, six weeks of KD allow to reduce the liver weight (−31%, $p < 0.01$), reduce steatosis and ballooning hepatocytes, and decreased IL-6 ($p < 0.05$) and collagen 1 ($p < 0.05$) gene expression. However, KD had no effect on IL-1, TNFa, IFNg, IL-10 gene expression, compared to HFD. These results suggest that in a context of hypercaloric diet and NAFLD development, replacing sugar by lipids is efficient to prevent the onset of NASH at least partly due to a reduction in lipid accumulation and hepatocellular injuries.

Keywords: obesity; NASH; ketogenic diet

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1. Introduction

Obesity, defined by the World Health Organization as "an abnormal or excessive fat accumulation that may impair health", is a major societal concern of the 21st century [1]. In 2018, the obesity prevalence reached 42.4% in the United States [2]. Obesity is associated with a high risk of metabolic comorbidities, particularly the development of non-alcoholic fatty liver disease (NAFLD) [3]. The increase in weight gain induces the accumulation of subcutaneous, abdominal, and hepatic fat. The excessive expansion of visceral fat alters adipose tissue histology and function, inducing the secretion of inflammatory cytokines as IL6, IL1b or TNFa [4]. In the liver, fat disposition and inflammatory cytokines is responsible for the development of NAFLD and its evolution into non-alcoholic steatohepatitis (NASH),

characterized by the presence of steatosis and fibrosis. Finally, it can evolve to cirrhosis or liver cancer [5]. To reduce obesity and prevent the complication development, the general health-care guidelines recommend lifestyle modifications, with changes in food behavior and an increase in physical activity [6]. The principal goal of these recommendations is inducing weight loss to reduce the risk of complication development. Indeed, Koutoukidis and al. demonstrated that weight loss interventions were statistically significantly associated with improvements in NAFLD biomarkers [7]. Dietary approaches have become increasingly widespread to lose weight. One of them, the Ketogenic Diet (KD), a high-fat, adequate-protein, and low-carbohydrate diet, seems to be a promising strategy to prevent obesity complication development. Rodent studies showed that KD decreased weight gain and improved glucose tolerance in mouse model [8,9]. In clinical trials, the use of a KD in obese patients decreased weight gain and blood glucose levels [10,11]. KD also decreased liver enzyme levels which are liver injury indicators and are used as a biomarker of NAFLD prediction [12]. However, the mechanisms involved in its beneficial effects remain unclear, particularly for NASH development. This study aims to evaluate the effect of six-week Ketogenic but isocaloric Diet on NASH development in obese mice. Our study shows that a high fat diet induces obesity and NASH, while a high caloric diet but without sugar (i.e., Ketogenic diet) reduces weight gain and prevents the transition from NAFLD to NASH, by a decrease in hepatic swelling and fibrosis.

2. Materials and Methods

2.1. Animals

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animal experiments were approved by our local ethics committee (CREMEAS, agreement numbers: 2018042013495170 and 2020111316012887). The study was performed on 30 eight-week-old male C57BL/6J mice that were obtained from ENVIGO (Gannat, France) and maintained in a thermo-neutral environment of 22 ± 2 °C on a 12 h day/night cycle. Mice had ad libitum access to tap water and chow diet (consisting of 7.7% fat, 15.7% protein, and 61.7% carbohydrate, Safe® Diets), high-fat, high-sugar diet (consisting of 35.9% fat, 19.9% protein, and 37.3% carbohydrate, Safe® Diets) or ketogenic diet (consisting of 64.4% fat, 20.9% protein, and 3.8% carbohydrate, Safe® Diets), depending on their group assignment. Animals were divided into three groups: (1) Control (Ctrl) group, fed with a chow diet for 22 weeks (n = 10); (2) High fat high sugar diet (HF) group, fed with high fat high sugar diet for 22 weeks (n = 9); and (3) Ketogenic diet (KD) group, fed with high fat high sugar diet for 16 weeks then fed with KD for 6 weeks (n = 9). Body weight and food intake were measured once a week throughout the intervention until the end of experiment. HF diet and KD diet were isocaloric.

2.2. Anatomical Measurements and Histological Stanning

Mice were anesthetized in a hermetic cage, ventilated with gas mixture of 4% isoflurane (Aerrane, CSP, Cournon, France) and oxygen. Liver was weighed and harvested for extemporaneous studies, snap-frozen for biochemistry or fixed in isopentane for histological analysis. Mice were euthanized by cervical dislocation and exsanguinated. Serum was separated and frozen for further analysis.

The liver tissue was frozen-fixed in OCT and sectioned at −20 °C on a cryostat microtome (10-µm thick, Cryostar NX70). Cryosections were rehydrated in acetone for 3 s and dry at 37 °C for 1 h. Then, they were stained in Harris' hematoxylin solution for 2 min, washed in tap water for 3 min, in 1% acid alcohol for 2 s then again in tap water for 3 min. The slides were counterstained by dipping them in Eosin solution for 1 min, washed in tap water for 3 s, in 80% ethanol, in 100% ethanol and finally mounted in aqueous medium with Permount medium.

2.3. RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR

Gene expression was measured by real-time quantitative PCR. Total liver RNA was isolated using MagMAX™ mirVana™ Total RNA Isolation Kit (Applied Biosystems™, Foster City, CA, USA) with the Kingfisher Duo Prime (Fisher Scientific, Waltham, MA, USA) and was stored at −80 °C. Quantity and purity were assessed with Qubit™ RNA Broad Range (BR) and Integrity Quality (IQ) assay kits (Invitrogen™, Carlsbad, CA, USA) using the Invitrogen Qubit 4 Fluorometer (Invitrogen™, Carlsbad, CA, USA). cDNA was synthesized from 1 µg of total liver RNA with Maxima™ H Minus cDNA Synthesis Master Mix (Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed in duplicate in a total reaction volume of 15 µL using either PowerTrack™ SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) with primers described in Table 1 and measured in automated QuantStudio 3 Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA). Linear ranges and optimal RNA concentrations for each primer set were previously determined. Each primer sets were designed to span an exon/exon junction to minimize amplification of genomic DNA and obtained from Applied Biosystems. HPRT gene was used as housekeeping gene.

Table 1. Primers for liver qPCR.

Forward Primer	Reverse Primer
GTTGGATACAGGCCAGACTTTGTTG	GATTCAACTTGCGCTCATCTTAGGC
TGGTACTCCAGAAGACCAGAG	AACGATGATGCACTTGCAGA
AGTTGACGGACCCCAAAAG	AGCTGGATGCTCTCATCAGG
ATGGCCCAGACCCTCACA	TTGCTACGACGTGGGCTACA
GGCCCAGAAATCAAGGAGCA	AGACACCTTGGTCTTGGAGCTTAT
ACGTGGAAATCAACGGGATCA	GTTGGTATCCAGGGCTCTCC
TCATCGTGGCTTCTCTGGTC	GACCGTTGAGTCCGTCTTTG

2.4. Statistical Analysis

Data shown are means ± SEM. Normal distribution of data was verified with a Shapiro–Wilk test and parametric statistical tests were used throughout. Comparisons were made with a one-way ANOVA and uncorrected Fisher's LSD test for post hoc testing was used to explore significant differences between groups, using GraphPad 8® (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance is shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. Ketogenic Diet Decreases Weight Gain

The mice placed with HF diet presented an important weight gain, compared to the mice placed with chow died (Figure 1). After 16 weeks of HF diet, mice had a significant weight gain, compared to Ctrl group. This result showed, as expected, the development of obesity induced by HF diet.

The HF diet group continued to gain weight, reaching final body weights of 45.75 ± 1.77 g after 22 weeks (+82% of weight gain ($p < 0.001$, compared to Ctrl)). The cohort of HF diet fed animals transitioned to the KD, stopped their weight gain. At 16 weeks, these mice had a body weight of 43.04 ± 1.42 g while at 22 weeks mice reached a final body weight of 41.04 ± 1.88 g. KD mice final body weigh was lower compared to HF mice ($p < 0.05$).

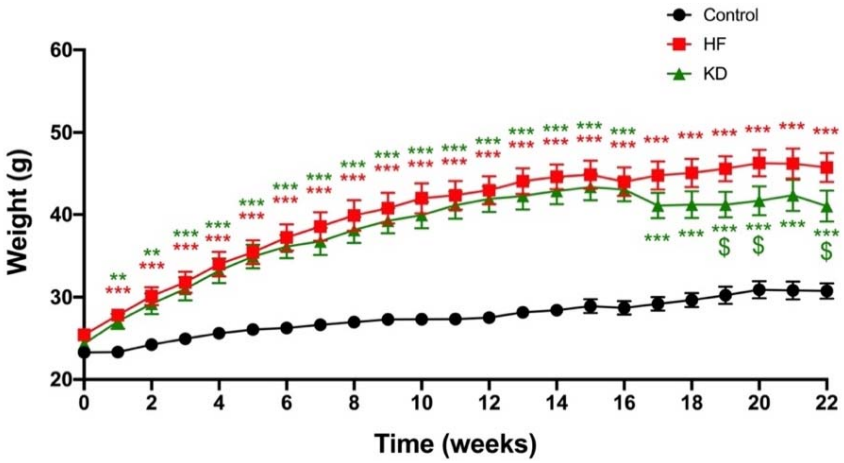

Figure 1. Effect of KD on dietary-induced obesity. Animals were switched to KD after 16 weeks of HF diet. Graph shows mean ± SEM. ** $p < 0.01$ and *** $p < 0.001$ compared to Control mice by One-way ANOVA; $ $p < 0.05$, compared to HF diet mice by one-way ANOVA.

3.2. Ketogenic Diet Decreases Liver Weight Gain

After 22 weeks of HF diet, mice doubled their liver weight, compared to control (2.496 g ± 0.3138 g in HF group versus 1.169 ± 0.08 g in Ctrl group, $p < 0.001$) (Figure 2A). During the dissection, HF livers presented a yellow color and a hepatomegaly, suggesting the presence of NASH based on liver appearance [13] (Figure 2B). The histological staining with Hematoxylin and Eosin coloration (Figure 2C) showed a major steatosis, a ballooning degeneration, and the presence of lobular inflammation. KD mice livers appeared tan and smaller than the HF diet livers. The weight confirmed this observation, showing a significant difference of −0.791 g between the two groups ($p < 0.05$) (Figure 2B). KD weight livers were not significatively different from Ctrl (Figure 2A).

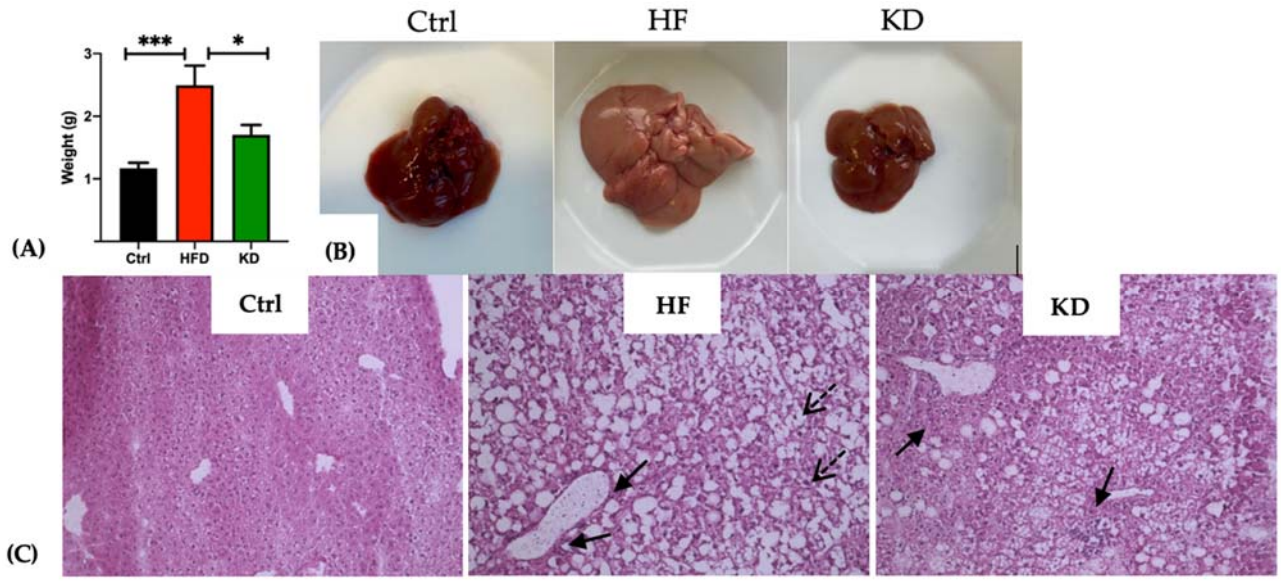

Figure 2. Effects of KD on liver. KD decreases liver final weight (**A**), prevents from hepatomegaly (**B**), and reduces steatohepatitis (**C**). Black arrows point lobular inflammation; dotted-line arrows point hepatocellular ballooning. Graph shows mean ± SEM. * $p < 0.05$, and *** $p < 0.001$ by one-way ANOVA.

Histological findings revealed an important reduction in steatosis, with less ballooning degeneration but still the presence of inflammatory infiltrates.

3.3. Ketogenic Diet Decreases Collagen-1 and IL6 Genes Expression but Had No Effect on the Other Cytokines

The analyses of gene expression revealed that HF mice presented a significant increase in several inflammatory genes (Figure 3), particularly IL1b and TNFa ($p < 0.05$), compared to Ctrl. No changes were observed for IL6, TGFb and IL10 RNAm levels. HF mice also presented a significant increase in Collagen 1 (Col1a1) gene expression ($p < 0.01$) compared to Ctrl. KD mice showed a significant lower IL6 and Col1a1 mRNA levels compared to HF. However, KD had no effect on IL1b, TNFa, TGFb, and IL10 gene expression.

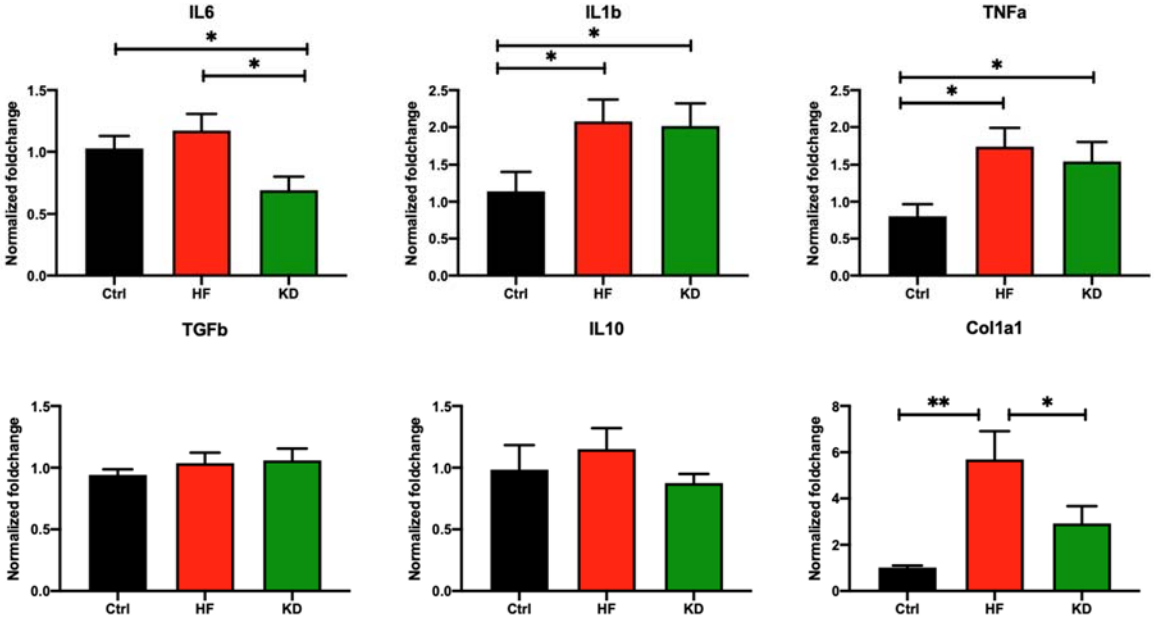

Figure 3. Effects of KD on inflammatory (IL6, IL1b, TNFa, TGFb), anti-inflammatory (IL10), and collagen (Col1a1) genes expression in control (full black-coloured bars), HF (full red-coloured bars) or KD (full green-coloured bars). Real-time PCR was performed in duplicate. Graph shows mean ± SEM. * $p < 0.05$ and ** $p < 0.01$ by one-way ANOVA.

4. Discussion

NAFLD is the most common cause of liver disease and its prevalence is increasing worldwide [14]. This pathology is associated with the presence of steatohepatitis, liver inflammation, and can progress to fibrosis [14]. Visual liver observation can reveal changes in color, size, and surface component [13]. These observations were found in the HF group: livers were larger, had a higher total weight than control mice, and presented yellow spots distributed on all the tissue. The histological staining with Hematoxylin and eosin revealed a severe steatosis associated with hepatocellular ballooning and lobular inflammation These results are according to the literature in NAFLD mice models, and confirmed the NASH diagnosis [15]. Moreover, HF mice presented a significant increase in TNFa, IL1b and collagen-1 gene expression, confirming the hepatic inflammation. IL1b and TNFa play a major role in the development of NAFLD and progression to advanced stages, by promoting inflammation and fibrosis [16], especially by inducing an increase in Col1a1 expression [17].

The main results of our study show that isocaloric KD decreases weight gain, and prevent the onset of NASH, by a decrease in liver weight, a prevention of hepatomegaly and a reduction in steatosis. First of all, this study showed that a hypercaloric diet, such as KD, without sugar, is efficient to reduce the weight gain, which is beneficial for the mice, according to guideline recommendation for obesity care [6]. Indeed, weight loss is associated with a decrease in complication risk development. For NAFLD management, recent guideline updates recommend a lifestyle intervention based on weight loss and dietary approaches rather than drugs [18]. Our results show in a very interesting way that

KD diet reduces steatosis and ballooning hepatocytes. This positive effect can be explained in different ways. The first is that the decrease in body weight can directly be the cause of the decrease in steatosis [19]. Secondly, it is known that the KD diet leads to a reprogramming of hepatic metabolism by orienting it towards the oxidation of lipids and a reduction in its storage (catabolic effect). Finally, KD mice presented a decrease in Col1a1, suggesting an anti-fibrotic effect of the diet, mediated by the decrease in steatosis and hepatic swelling. Indeed, the hepatic injuries, as ballooning and steatosis, promote hepatic stress, that contributes to fibrosis [20]. The results of KD on inflammation are mixed. Hepatic IL-6 production also play an important role in NAFLD development, so its decrease could be benefic to reduce NAFLD progression [21]. Nevertheless, KD did not exhibit protective effects on the other inflammatory genes and presented significant increase in IL1b and TNFa gene expression. Moreover, histological analysis revealed the presence of inflammatory infiltrates also in this group, suggesting a low effect of KD on inflammation. In the knowledge that IL1b and TNFa are critical cytokines involved in NAFLD development, it will be essential to perform more analysis to understand the KD effect in liver and NAFLD pathogenesis, that could go through hepatic protein quantification by Western blot, and plasmatic analysis of liver enzyme and circulating cytokines.

In conclusion, these results show that the concomitant intake of sugars and fat leads to the development of obesity and its complications. Carbohydrate restriction, with isocaloric KD, induces beneficial effects to prevent NAFLD development toward severe stages, by a reduction in weight gain, decrease in liver weight, steatohepatitis, and Col1a1 gene expression.

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