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Effects of ketogenic and western diets on proliferation, vasculogenesis and oxidative stress in the liver

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Abstract

In this study, it was aimed to investigate the effects of different diets on lipid peroxidation, antioxidants, proliferation and vasculogenesis in liver tissue. BALBC female rats (21) were divided as the group fed with standard chow (SC), the group fed with a ketogenic diet (KD) and the group fed with western diet (WD). The rats were fed with tap water and a rat diet specially prepared according to the determined diets for four weeks. Liver tissue oxidative damage, proliferation and vasculogenesis were evaluated using spectrophotometric and immunohistopathological methods. At the end of the experiment, it was found that the highest weight gain was in the WD group and the least weight gain was in the KD group. The mean body weight of the WD group was statistically significantly higher compared to the SC and KD groups ($p<0.05$). MDA levels were found to be lower in the KD group compared to the SC and WD groups. GSH and CAT levels were higher in the KD group compared to the SC and WD groups. In IHC evaluation results, it was seen that Ki-67 percentage in the KD group increased compared to the WD and SC groups ($p = 0.000$), VEGF was close to each other in all three groups and there was no significant difference in the comparisons between groups ($p>0.05$). These results revealed that ketogenic diet prevents tissue damage by decreasing lipid peroxidation in liver tissue as well as inducing cellular proliferation and vasculogenesis.

Keywords: ketogenic diet, oxidative stress, proliferation, vasculogenesis

1. Introduction

Reactive oxygen derivatives (ROS) are consistently produced in low and measurable concentrations in cells and tissues under normal physiological conditions. Their concentrations are determined by the balance between the production and destruction rate of various antioxidant compounds and enzymes (Apel and Hirt, 2004). On the other hand, cells defend themselves against potential damages of ROS through their own antioxidant mechanisms, including multiple enzyme systems, some antioxidant molecules, vitamins and trace elements. There is a strong balance between ROS production and destruction. If this balance is lost, ROS is produced in excess and oxidative damage begins to occur in all tissues (Parihar et al., 2008). In other words, oxidative damage in the cell depends on two factors. The first is the increase in ROS production as a result of chronic diseases or exogenous sources; and the second is the reduction of antioxidant and enzymatic cofactors in the diet. In addition, the dietary composition can affect both conditions (Vetrani et al., 2013).

Ketogenic diet is a diet consisting of high fat, sufficient protein and low carbohydrate that mimics the metabolic changes of hunger in the body (Freeman and Kossoff, 2010). Various studies showed that the ketogenic diet has some effects on epilepsy, obesity, liver, cancer and diabetes. It has been reported to have an effect on various diseases and systems (Wright and Simone, 2016; Newman et al., 2017). Diet improves mitochondrial function in the brain, reduces epileptic seizures, decreases weight, waist circumference, Body Mass Index (BMI), body fat mass, low-density lipoprotein (LDL), triglyceride, HbA1c, fasting insulin; and also increases high-density lipoprotein (HDL) cholesterol, increases insulin sensitivity, inhibits tumor growth in various types of cancer, reduces circulating glucose that causes rapid tumor growth, creates a therapeutic effect in cancer, strengthens memory, affects social behavior. (Boison, 2017; Wright and Simone, 2016). Western diets (WD) are rich in terms of animal fats and food additives; but are poor in other plant-derived molecules such as fiber, vitamins, minerals and antioxidants, and are often consumed in the west and in other countries under the influence of the west (Hariharan et al., 2015). That frequently consumed diet affects processes that have an impact on health such as

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exacerbating the symptoms of kidney failure, obesity, hypertension, colitis; shortening the colon, increasing tumor formation and insulin resistance, causing fatty liver development, increasing liver triglycerides, causing liver damage, increasing Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) levels and changing microbiota composition. (Baena et al., 2017; Wu et al., 2015).

Besides both the ketogenic diet and the western diet have various health consequences indicated above, it is reported especially in recent years that they also affect oxidative stress. While some studies have reported that the ketogenic diet increases oxidative stress (Allen et al., 2013), in others it has been observed that ketones in the ketogenic diet prevent oxidative stress-mediated by mitochondrial dysfunction by providing alternative substrates and antioxidant properties (Greco et al., 2016). Western diet has been reported to increase oxidative stress by the studies examining the effects of Western diet on oxidative stress (Heinonen et al., 2014; Jenkins et al., 2016). Therefore, in this study, the effects of different diets on oxidative stress changes in liver tissue, cellular proliferation and vasculogenesis were examined pathologically and biochemically.

2. Materials and methods

2.1. Ethical approval and drugs

Approval was obtained from Sakarya University Animal Care and Use Ethics Committee for the study (Approval date: 01/07/2020; no: 33). All applications were carried out in Sakarya University Animal Laboratory in accordance with international guidelines. Animals were kept in appropriate wire cages and under standard laboratory conditions (12/12-hour light / dark-light cycle, temperature 22 ° C, humidity 50-60%). All rats were given tap water and specially prepared ad libitum diets for four weeks. Ketamine HCL (Ketalar®, Pfizer, Istanbul) and Xylazine HCL (Rompun®, Bayer, Istanbul) were preferred for anesthesia.

2.2. Study design and creating groups

Twenty-one BALB C female rats (weight 15-19 g and 10-12 weeks) were randomly divided into three groups. *Standard Chow Group (SC);* The rats were fed regular standard rat chow consisting of 77.3% carbohydrates, 2.7% fat and 20% protein of calories. *Western Diet Group (WD);* The rats in this group were fed a western diet consisting of 39.70% of calories from carbohydrates, 39.51% from fat, 19.53% from proteins and 1.26% of other ingredients for 4 weeks. *Ketogenic Diet Group (KD);* This group of rats was fed a ketogenic diet consisting of 4.95% carbohydrates, 74.24% fat, 19.53% proteins and 1.28% other components of calories.

At the end of the experiment, the animals were killed by cervical dislocation while under general anesthesia with 65 mg / kg (i.p) ketamine and 7 mg / kg xylazine (i.p) injection. Samples taken for biochemical analysis were kept at -20 C. Tissue samples reserved for histological evaluations were taken into 10% formaldehyde.

2.3. Biochemical assay

Tissues were washed with 0.9% NaCl solution after weighing. After washing, they were centrifuged (+4 ºC, 3000 RPM, 10 min). They were then homogenized in a cold solution of 1.15% KCl, 0.01M sodium potassium phosphate (pH=7.4). 10% tissue homogenates were prepared. Homogenates were centrifuged at 10.000g for 20 minutes, at + 4°C. Supernatant was taken and used to determine the MDA, GSH and CAT parameters in the tissues. Protein measurement of the samples was performed using the Lowry method (Lowry et al., 1951). The determination of MDA, one of the lipid peroxidation products, in tissue homogenates was carried out using Buege and Aust's (Buege and Aust, 1978) method, based on the principle that MDA reacts with thiobarbituric acid (TBA) to give a colored compound that can be measured at 532 nm wavelength. Results are given as nM / mg protein. For GSH determination, the reaction between 5'5'-Dithiobis 2-nitrobenzoic acid (DTNB) and GSH was used to generate TNB showing maximum absorbance at 412 nm. Results are given as µM / mg protein (Beutler, 1963) CAT activity was determined by a spectrophotometric method based on the degradation of H_2O_2 by CAT. The calculation was made using the absorbance difference that decreases over time as a result of the CAT-peroxide reaction at 240 nm wavelength. Results are given as U / mg protein (Beers and Sizer, 1952).

2.4. Histopathological examination

For histopathological analysis, liver tissues were washed in 10% neutral buffered formaldehyde for 48 hours and in tap water for one day. Then, samples were passed through alcohol series and dehydration process was applied. They were then passed through xylol series to make the tissues transparent and embedded in paraffin blocks. Sections taken by microtome (4µm) were stained with hematoxylin and eosin (H&E). Photographs were taken by examining under a light microscope (Olympus CX31-Japan). Histopathological evaluations were made using Suzuki's quasi-numerical modified scoring system (Suzuki et al., 1993).

2.5. Immunohistochemistry staining

The 4µm paraffin blocks were deparaffinized and were boiled in a microwave oven for 20 minutes. They were kept in 3% hydrogen peroxide for 10 minutes and, UV block was performed for 15 minutes. The primary antibody diluted as 1/300 was dropped and incubated overnight at +4 degrees in a humid environment. Then, they were secondary antibody (Thermo scientific, HRP TP-125-HL-UK) staining following the procedure recommended by the producer company. Finally, they were rehydrated by counter staining with the H&E and the procedure was completed. In our study, the proliferation index of Ki-67 (MS-106-B, Thermo LabVision), which was previously reported in a study, was used to evaluate the groups in terms of proliferation (Hazan et al., 2002). VEGF (Gene TEX-102643) immunohistochemistry application, the staining degree was scored in five randomly selected areas and the area with the highest score was determined. Within both

groups, at least 100 cells were marked in each x40 magnification field. Percentage of stained cells and the degree of staining in sections were taken as criteria. Scoring was done with a semi-quantitative method (Ulloa-Padilla et al., 2020).

2.6. Statistical evaluations

Statistical analyzes were performed using SPSS 22.0 package program (SPSS Inc. and Lead Tech. Inc. Chicago. USA). Shapiro Wilk test was used for the normal distribution of data. One-way ANOVA test was used to compare more than two normally distributed variables. TUKEY HSC test was used for in-group significance. All results are presented as mean ± SD. Results with P <0.05 were considered significant.

3. Results

3.1. Body weight analysis

The change of average body weights of the groups at the beginning and end of the experiment is presented in Fig. 1. Before starting the study, there was no statistically significant difference between all groups in terms of body weight (p> 0.05). Average body weights in body weight analysis performed at the end of the experiment were found to be 17.042 ± 0.237 in the SC group, 17.714 ± 0.397 in the WD group and 16.800 ± 0.509 in the KD group. In the comparisons between the groups, it was observed that the average body weight of the WD group was statistically significantly higher than the SC and KD groups (p = 0.014, p = 0.001, respectively). Although the average body weight of the KD group was lower than the SC group, this difference was not statistically significant (p> 0.05).

3.2. Biochemical Results

As a result of the biochemical measurements, MDA, GSH and CAT levels were found to be significantly different between the groups (Fig. 2). In the comparisons between the groups, MDA levels were found to be statistically significantly lower in the KD group compared to the SC group and the WD group (respectively; p = 0.000, p = 0.000). The mean GSH levels in the KD group were statistically significantly higher compared to the SC and WD groups (p = 0.002, p = 0.019, respectively). When CAT levels were examined, it was found that there was a significant increase in the KD group compared to the SC group (p = 0.020). The average CAT values increased in the KD group compared to the WD group, but this increase was not statistically significant (p> 0.05). When compared with the WD group, there was no statistically significant difference in MDA, GSH and CAT levels of SC group (p> 0.05).

3.3. Histopathological examination results

The preparations were evaluated with sinusoidal occlusion, necrosis, and vascularization evaluation criteria. Vascularization was observed in the SC and WD groups (Fig. 3A-B). When the sinusoidal occlusion and necrosis were examined, it was seen that the SC and WD groups (Fig. 3A-B) had a higher rate compared to the KD group. Portal vein liver sinusoids in the KD group were observed to have more normal cell contours compared to the SC and WD groups (Fig. 3C).

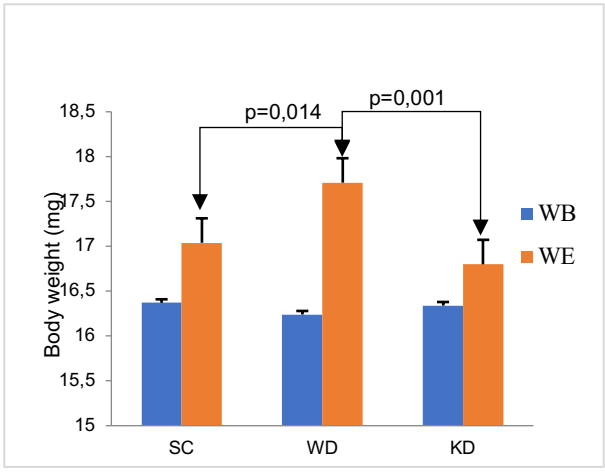

Fig. 1. Evaluation of body weight changes in experimental groups. SC: Standard chow; WD: Western diet; KD: Ketogenic diet; WB: Average body weight at the beginning of the experiment; WE: Average body weight at the end of the experiment; The mean difference is significant at the p <0.05 level

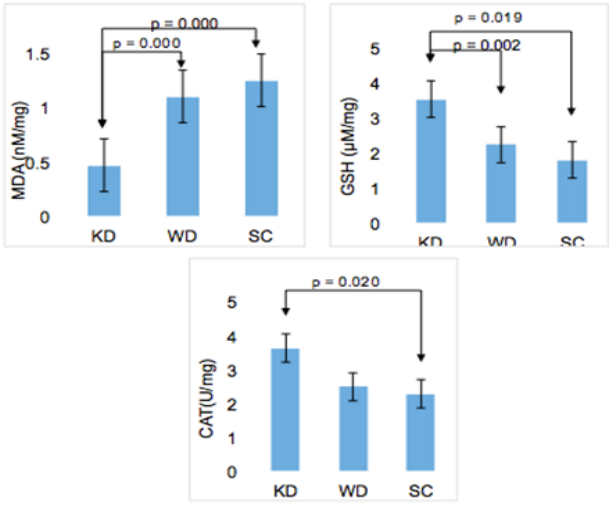

Fig. 2. Evaluation of liver tissue biochemical parameters in experimental groups SC: Standard cow; KD: Ketogenic diet; WD: Western Diet. MDA: Malondialdehyde; GSH: Glutathione; CAT: Catalase; Significance level p <0.05. Results are presented as mean ± SE

3.4. Immunohistochemical staining results

Evaluation results of liver proliferation and vasculogenesis are presented in Fig. 3. It was observed that proliferation (Ki-67) in the KD group increased compared to the WD and SC groups, and vasculogenesis (VEGF) was close to each other in all three groups. Among the groups, the lowest percentage of Ki-67 was detected in the WD group, and this low percentage was significantly lower than the KD group (p = 0.000) (Fig. 3). The highest Ki-67 percentage was found in the KD group. This increase was significant compared to the percentages in all other groups (p = 0.000 for both) (Fig. 3). When VEGF concentrations in the experimental groups were examined, all three groups gave similar results. Among the groups, VEGF was found to be the lowest in SC group and the highest in the KD group. However, there was no significant difference in

comparisons between groups (p> 0.05) (Fig. 4).

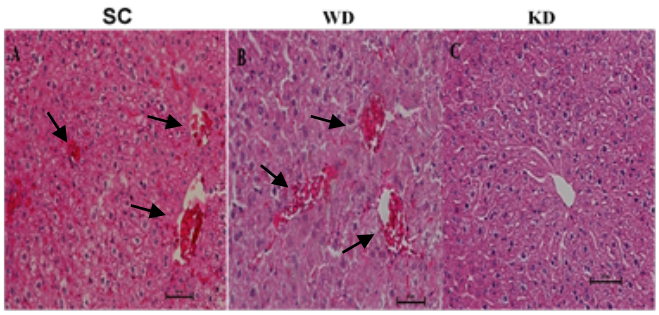

Fig. 3. SC: Standard chow; WD: Western diet; KD: Ketogenic diet. SC Group; Intense congestion of ciosides in the central vein (arrows); WD group; Intense congestion of ciosides in the central vein (arrows); KD group; normal central vein and synosid areas are seen. HE, X200

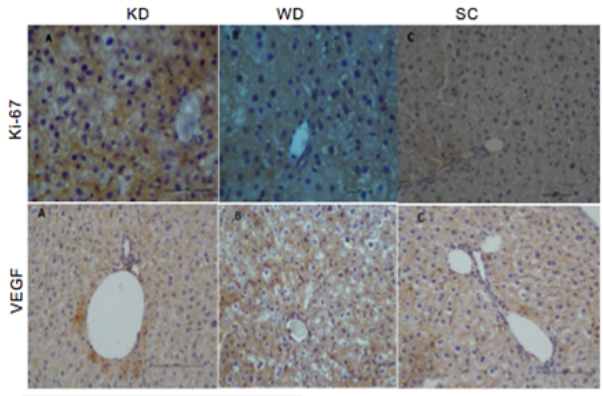

Fig. 4. SC: Standard chow; WD: Western diet; KD: Ketogenic diet. Representative photographs of IHC positivity and statistical evaluation of the effect of diets on liver proliferation and vasculogenesis in rats (400X (50 scale bar)). It is observed that Ki-67 immunopositivity is increased in the KD group compared to WD and SC groups, and VEGF immunopositivity is close to each other in all three groups

4. Discussion

The change in eating habits with modern life causes many metabolic diseases. As a result of the lack of macro or specific micronutrients in the tissues, structural deficiencies in the tissues and dysfunction in the organs occur. It has been suggested that nutritional dietary components may prevent the development of many chronic diseases, as they increase sensitivity to or protect against free radicals (Krenkel and Tacke, 2017). Affecting liver metabolism at the cellular level causes acute and chronic liver diseases. Differences in the diversity and concentrations of nutrients in the content of different diet types are thought to affect human health in different ways (Fleet, 2014). Therefore, in our study, we investigated the effects of different diets (KD, WD and SC) on oxidative stress in the liver tissue of rats, cell proliferation and vasculogenesis with some antioxidant enzymes.

In our study, MDA levels were statistically significantly lower in the KD group compared to the WD group. MDA is the end product of lipid peroxidation and is an essential biochemical parameter used as an oxidative stress marker. Both the ketogenic diet and the western diet were reported to affect oxidative stress (Norton et al., 2020). In the present study, it was observed that feeding with short-term KD caused increases in antioxidant parameters in the liver. Liver GSH levels were significantly higher in the KD group compared to the SC and WD groups. The KD group had the highest average CAT levels. However, there was no significant difference compared to the SC and WD groups. Conflicting results have been reported in dietary studies in the literature. As in some studies, in our study, KD clearly reduced lipid peroxidation, thus oxidative damage. In a study, it was found that short and long-term feeding with KD improved liver oxidative stress markers and feeding with KD significantly increased liver antioxidant capacity and glutathione peroxidase levels compared to those fed with standard rat feed, and decreased liver protein carbonyls (Kephart et al., 2017). In another study, it was shown that long-term feeding with KD (75% kcal fat) regulates GSH biosynthesis in the brain tissue of rats and increases mitochondrial antioxidant capacity (Jarrett et al., 2008). Lu et al. (Lu et al., 2018). found that KD reduces oxidative stress by suppressing some signal pathways after spinal cord injury (Pinto et al., 2018). It has been shown that KD improves mitochondrial function and reduces oxidative stress, and improves mitochondrial respiration by reducing the production of reactive oxygen species of β-hydroxybutyrate. Parry et al. reported that liver SOD and CAT levels were higher in the KD group compared to the SC group, although it was not significant. They noted that the ketogenic diet increased the volume of mitochondria in the liver and the average lifespan in rats, and they reported that the increase in mitochondrial volume occurred without oxidative damage or change in antioxidant protein levels in the liver or brain (Parry et al., 2018).

In studies examining the effects of WDs, it has been shown that their long-term consumption causes weight gain by creating physiopathological changes in lipid and energy metabolism (Wilson et al., 2007). Unlike WDs, short-term (4-6 weeks) and long-term (up to 12 months) studies have shown that KDs cause more fat loss in obese individuals (Harmancey et al., 2010). In our study, it was seen that the highest increase in body weight at the end of the experiment was in the WD group. There are studies in the literature that support our results. It has been shown that consuming a meal of WD causes a decrease in plasma SOD activity in morbidly obese individuals (Mazzoli et al., 2019). Although the physiological mechanisms of these diets on organs are not well understood, both experimental and human studies have shown that obesity is associated with increased oxidative stress markers and lipid peroxidation (Wilson et al., 2007). In the studies examining the effects of feeding with WD; unlike KD, WD has been reported to increase oxidative stress (Jenkins et al., 2016; Norton et al., 2020). Irregularity of hepatic lipogenesis and prolonged exposure of the heart to a high fuel supply have been shown to be important causes of heart failure in rats fed with high-fat WD (Harmancey et al., 2010). In a different study, it

was stated that even though WD increases oxidative stress, vascular cells adapt to this stress by resisting (Norton et al., 2020). In rats fed a diet like a WD diet which is rich in saturated fatty acids and fructose, oxidative stress markers (lipid peroxidation and Nitro-Tyrosine content) were significantly increased, but the antioxidant enzyme CAT decreased (Mazzoli et al., 2019).

In the literature, many markers such as DNA synthesis, mitosis number, cell proliferation and mitochondrial activity have been used to define liver regeneration criteria. Ki-67 antigen in the cell nucleus found by Gerdes et al. and the monoclonal antibody formed against it were described (Garcia-Fuentes et al., 2010). The increase in Ki-67 level is directly proportional to liver recovery and cell renewal. All stages of the cell cycle can be classified since all stages of the Ki-67 cell cycle except the resting stage (G0) can be shown. In our study, we used Ki-67 to show the effects of diets on cellular proliferation in the liver, and the effects on vascularization as IHC using VEGF. In our study, we observed that feeding with KD caused an increase in the percentage of Ki-67 compared to feeding with WD and SC. Thus, in our study, we showed that KD increases cellular regeneration due to cellular proliferation in the liver. VEGF positivity, which is a marker of vasculogenesis, was higher in the KD group, although it did not differ between the groups. In a study, WD was found to cause vascular oxidative stress and a decrease in endothelial function (Jenkins et al., 2016).

The results of our study showed that a low-carbohydrate high-fat diet prevents oxidative damage by decreasing lipid peroxidation in the liver and increasing antioxidant enzymes; also increases cellular proliferation and vasculogenesis. For this reason, we think that feeding with ketogenic diets can prevent the occurrence of diseases and should be preferred as a potential diet (which can be considered as additional treatment) in the treatment of diseases. Besides, changes in DNA and gene transcription levels can be investigated in future studies and their effects on other tissues can be strengthened.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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