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Investigation of the Effects of Ghrelin and Kisspeptin Levels in Liver Tissue of Rats Fed with High Fructose Diet -A Histological Study

Yüksek Fruktozlu Diyetle Beslenen Sıçanların Karaciğer Dokusunda Ghrelin ve Kisspeptin Düzeylerinin Etkilerinin Histolojik Olarak Araştırılması

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ÖZ

Amaç: Yüksek fruktozlu mısır şurubu(YFMŞ), gıda endüstrisinde yaygın olarak kullanılmakta olup; obezite, diyabet ve yağlı karaciğer hastalığı gibi birçok hastalıkla ilişkilidir. Ghrelin, merkezi ve periferik etkilere sahip birçok dokuda yaygın reseptörlere sahip olmasının yanı sıra oreksijenik bir hormondur. Gıda alınımını azaltabilmektedir. Kisspeptin, üreme hormonlarında önemli bir rol oynamakta ve karaciğer dâhil olmak üzere yüksek metabolik aktiviteye sahip birçok dokuda reseptörü bulunmaktadır. Bu çalışmada YFMŞ tüketiminin karaciğer dokusu üzerindeki etkilerinin ve bunun Kisspeptin ve Ghrelin seviyeleri ile ilişkisinin incelenmesi amaçlanmaktadır.
Araçlar ve Yöntem: Çalışmada 8-10 haftalık, 14 adet erişkin Wistar albino erkek sıçan kullanıldı ve 2 gruba ayrıldı (Kontrol, YFMŞ n=7). Sıçanların karaciğer dokularındaki Kaspaz 3, TNF-α, Ghrelin ve Kisspeptin seviyeleri immünohistokimyasal yöntemle ölçüldü ve ardından histoskorlama ile analiz edildi.
Bulgular: Verilerimize göre YFMŞ grubunda kontrol grubuna göre Kisspeptin, Kaspase 3, TNF-α düzeylerinde anlamlı artış ve Ghrelin düzeylerinde azalma gözlemlendi.
Sonuç: Sonuç olarak, çalışmamızda yüksek fruktozlu diyetin karaciğer Kisspeptin düzeylerinde neden olduğu değişiklikler ilk kez gösterilmiştir. Ayrıca, gıdada YFMŞ kullanımı inflamatuar aktivasyona, doku hasarına ve Ghrelin düzeylerinin düşmesine neden olmuştur.

Anahtar Kelimeler: apoptoz; ghrelin; kisspeptin; proinflamatuvar sitokinler

ABSTRACT

Purpose: High fructose corn syrup (HFCS) is used commonly in the food industry and has been associated with various diseases including obesity, diabetes and fatty liver. Ghrelin, an orexigenic hormone with widespread receptors in many tissues, exerts various central and peripheral effects. Food intake may reduce its synthesis. Kisspeptin plays a major role in reproductive hormones and its receptors are expressed in tissues with high metabolic activity, such as the liver. This study aims to investigate the effects of HFCS consumption on liver tissue and its relationship with Kisspeptin and Ghrelin levels.
Materials and Methods: Fourteen adult male Wistar albino rats, aged eight to ten weeks, were used in this study and divided into two groups; Control and HFCS (n=7). Tumor Necrosis Factor-alpha (TNF-α), Ghrelin, and Kisspeptin levels in the liver tissues of the rats were measured using an immunohistochemical method and subsequently analyzed through histoscoring.
Results: Our data revealed a significant increase in Kisspeptin, Caspase 3, and TNF-α levels and a decrease in Ghrelin levels in the HFCS group compared to the Control group.
Conclusion: In conclusion, our study demonstrates, for the first time, changes in liver Kisspeptin levels induced by a high fructose diet. Furthermore, the consumption of HFCS in food led to inflammatory activation, tissue damage, and reduced Ghrelin levels.

Keywords: apoptosis; ghrelin; kisspeptin; proinflammatory cytokines

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INTRODUCTION

High fructose corn syrup (HFCS) that is a viscous mixture of 42 or 55 percent fructose with sucrose, has been increasingly utilized in the food industry, especially in beverous soft drinks, due to its cost-effectiveness, enhanced taste and provided freshness. Fructose metabolism has been found to be less energetically productive and more inclined towards lipid synthesis compared to glucose metabolism. Additionally, it can be stored in the liver as glycogen to only about one-fifth of the extent of glucose.[1] Previous studies have established a connection between HFCS consumption and various diseases, including obesity, insulin resistance, diabetes, cardiovascular disorders, fatty liver, altered gut flora, and metabolic syndrome (MS).[2]

Ghrelin is primarily produced in the gastric mucosa and functions as an orexigenic neurohormone. Its receptors are widely distributed in many tissues, leading to various central and peripheral effects.Its synthesis is notably reduced by food intake, especially glucose and amino acids, as well as hormones like insulin. Moreover, it is modulated by numerous factors, including peptide hormones, neurotransmitters, and long-chain fatty acids.[3] Ghrelin exerts diverse effects, such as increasing gut motility, promoting acid secretion and cardiac output, displaying anti-inflammatory properties, providing tissue and neuronal protection, facilitating lipogenesis, influencing taste sensation, modulating the learning process, reducing thermogenesis, and regulating sleep patterns.[4]

Kisspeptin exists in four isoforms and plays a fundamental role in regulating reproductive hormones, particularly gonadotropin-releasing hormone (GnRH). Furthermore, it is predominantly located in the preoptic and infundibular nucleus within the nervous system. Kisspeptin and its receptor are expressed in tissue with high metabolic activities including liver, suggesting a potentially crucial role in metabolic processes.[5] A study has shown that inhibiting Kisspeptin can lead to glucose intolerance in pregnant mice.[6] Plasma Kisspeptin levels in humans are typically very low, although they increase significantly during pregnancy due to placental secretion. In another study, elevated levels of fat were observed in knockout mice, leading to the hypothesis that Kisspeptin might play a significant role in weight gain.[8]

In this study, the aim is to examine the effects of HFCS consumption on liver tissue and its relationship with Kisspeptin and Ghrelin levels.

MATERIALS and METHODS

Ethics Committee Permission

Approval for this study was obtained from the Munzur University Animal Experiments Local Ethics Committee (dated 09.12.2022 and numbered 2022-18/02).

Animals

Fourteen adult Wistar albino male rats, aged 8-10 weeks, were used and divided into 2 groups (7 rats in each group) with ad libitum access to food and water. The experimental period was designed to last for 6 weeks.

Groups

Control Group: No action was taken during the experimental period.

HFCS Group: Commercial corn syrup containing 42% fructose, 53% glucose, and 5% other saccharides was added to the rats' drinking water at a concentration of 30% for 6 weeks. The concentration of HFCS was determined based on the sugar content of many soft drinks, which typically range from 7% to 15% sugar content.

Termination of Experimental Applications

Twenty-four hours after the last experimental application, experimental animals were anesthetized by i.p administration of 10% ketamine (Alfamine; Alfasan IBV, Woerden, The Netherlands) and 2% xylazine (Alfazine; Alfasan IBV, Woerden, The Netherlands).[10] After the liver tissues were fixed in 10% formalin solution for 24 hours, they were passed through routine histological follow-up series and embedded in paraffin blocks. Sections of 4–6 μm thickness from these blocks were deparaffinized for immunohistochemical examinations and stored under appropriate conditions.

Immunohistochemical Measurements

Caspase-3, TNF-Alpha, Ghrelin and Kisspeptin, expression were evaluated within liver tissue sections. For this purpose, sections transferred to Poly-L-Lysine slides were rehydrated, then boiled in a microwave oven (750 W) seven times for 5 min each in citrate buffer solution, pH 6 for retrieving antigen. Sections were allowed to cool at room temperature for 20 min, washed three times for 5 min each with phosphate-buffered saline (PBS) (P4417; Sigma Chemical Co.), then treated for 5 min with hydrogen peroxide block solution (TA-125-HP; Lab Vision Corp. USA) to block endogenous peroxidase activity. Sections then were washed three times for 5 min each with PBS and were treated with Ultra V Block (TA-125-UB; Lab Vision Corp.) for 5 min to prevent background staining. Next, sections were incubated with primary antibodies [(Rabbit monoclonal Caspase-3, ERP18297-ab184787 abcam, London, UK), TNF-α (Rabbit monoclonal IgG, ab220210, abcam, London, UK), Ghrelin (Anti-Ghrelin Monoclonal IgG antibody EPR20502 ab209790 abcam, London, UK), Kisspeptin (Anti-Kisspeptin Rabbit monoclonal antibody EPR23770-189 ab275874 abcam, London, UK)] for primary antibodies immunostaining (60 min). Following this process, sections were incubated with secondary antibody (biotinylated goat anti-mouse/rabbit Ig G, TP-125-BN; Lab Vision Corp.) for 30 min, streptavidin peroxidase (TS-125-HR; LabVision Corp.) for 30 min. 3-Amino-9-ethylcarbazole (AEC) substrate + AEC chromogen (AEC substrate, TA-015 and HAS, AEC Chromogen, TA-002-HAC; Lab Vision Corp.) solution was dripped on the sections and washed with PBS. Next, tissue sections were counterstained with Mayer's haematoxylin and mounted with Large Volume Vision Mount (TA-125-UG; Lab Vision Corp). Stained sections were inspected and photographed using a Leica DM500 microscope (Leica DFC295).

It was was established histoscore based on prevalence (0.1: <25%, 0.4:26-50%, 0.6:51-75%, 0.9:76-100%) and severity (0:no, +0.5: very little, +1: little), +2: moderate, +3: severe) of the staining. Histoscore=prevalence x severity.[11,12]

Statistical Analysis

All data were reported as mean ± standard deviation. Statistical analysis was conducted using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com). Groups were compared using Student's t-test, with $p < 0.05$ values considered statistically significant.

RESULTS

Caspase 3 Immunoreactivity

The images acquired by staining liver tissues for the determination of Caspase 3 immunoreactivity are presented in Figure 1. As a result of the statistical analysis of histoscoring, significantly higher Caspase 3 levels were observed in the HFCS group compared to the Control group ($p < 0.0001$) (Figure 2). The mean and standard deviation values were 0.3014 and 0.1148 in the control group, and 1.695 and 0.1066 in the HFCS group, respectively.

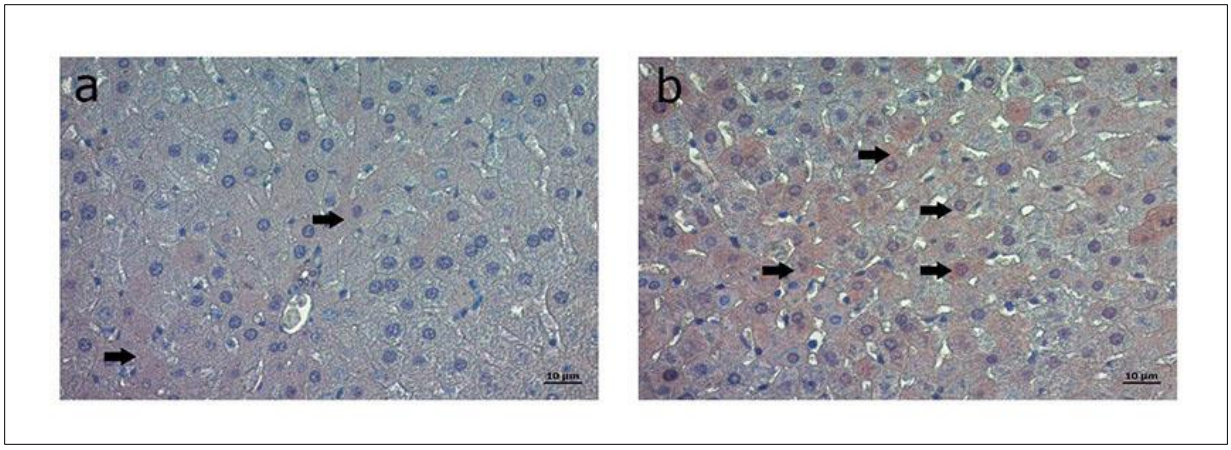

Figure 1. Caspase 3 immunoreactivity was observed in hepatocytes (black arrow) in liver tissue. Control (a), HFCS (b). Accordingly, Caspase 3 levels were increased in the HFCS group compared to the Control group. Streptavidin-biotin-peroxidase method, AEC chromogen, Mayer hematoxylin, Scala bar: 10 µm.

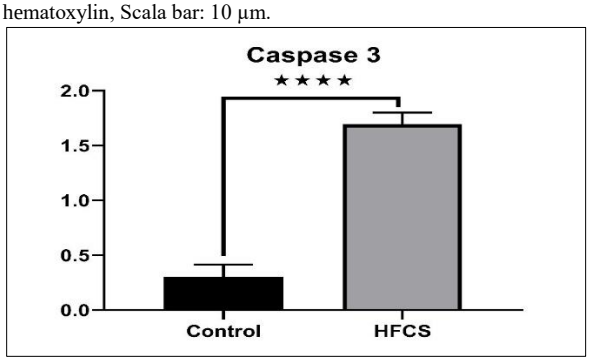

Figure 2. Analysis of Caspase 3 immunoreactivity**.** The meaning of the asterisks on the graph is as follows: *: P ≤ 0.05 **: P ≤ 0.01 ***: P ≤ 0.001 ****: P ≤ 0.0001.

Images taken under the light microscope with immunostaining are provided in Figure 3. It was observed significantly increased immunoreactivity of TNF-α in the HFCS group compared to the Control group ($p < 0.0001$) (Figure 4). The mean and standard deviation values were 0.7511 and 0.1130 in the control group and 1.175 and 0.1530 in the HFCS group, respectively.

TNF-α Immunoreactivity

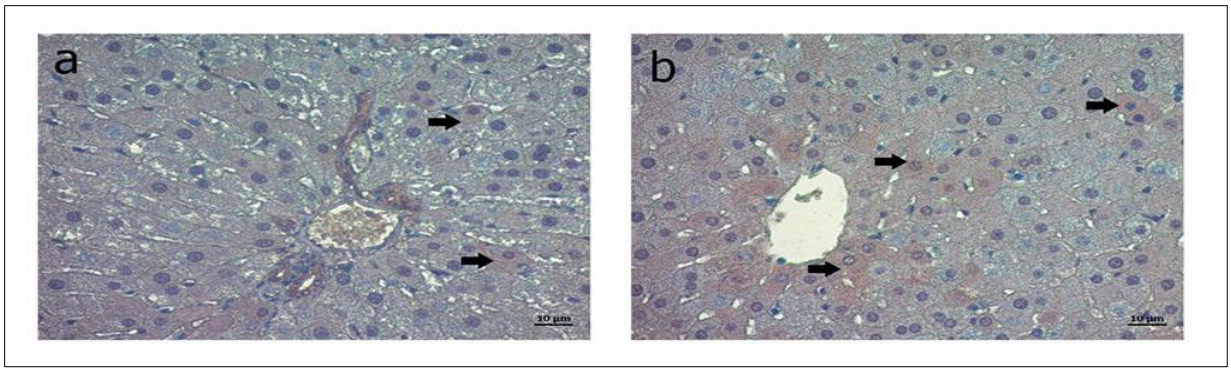

Figure 3. TNF-α immunoreactivity was observed in hepatocytes and sinüzoidal cells (black arrow) in liver tissue. Control (a), HFCS (b). Accordingly, TNF-α levels were increased in the HFCS group compared to the Control group. Streptavidin-biotin-peroxidase method, AEC chromogen, Mayer hematoxylin, Scala bar: 10 µm**.**

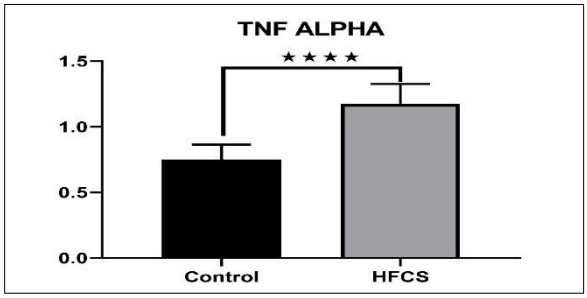

Figure 4. Determination of TNF-α levels in liver tissues. The meaning of the asterisks on the graph is as follows: *: P ≤ 0.05 **: P ≤ 0.01 ***: P ≤ 0.001 ****: P ≤ 0.0001.

Ghrelin Immunoreactivity

The images obtained through immunostaining of liver tissues to determine Ghrelin immunoreactivity are displayed in Figure 5. Statistically significant increased immunoreactivity of Ghrelin was observed in the Control group compared to the HFCS group ($p < 0.0001$) (Figure 6). The mean and standard deviation values

were 0.9167 and 0.06126 in the Control group and 0.3856 and 0.1602 in the HFCS group, respectively.

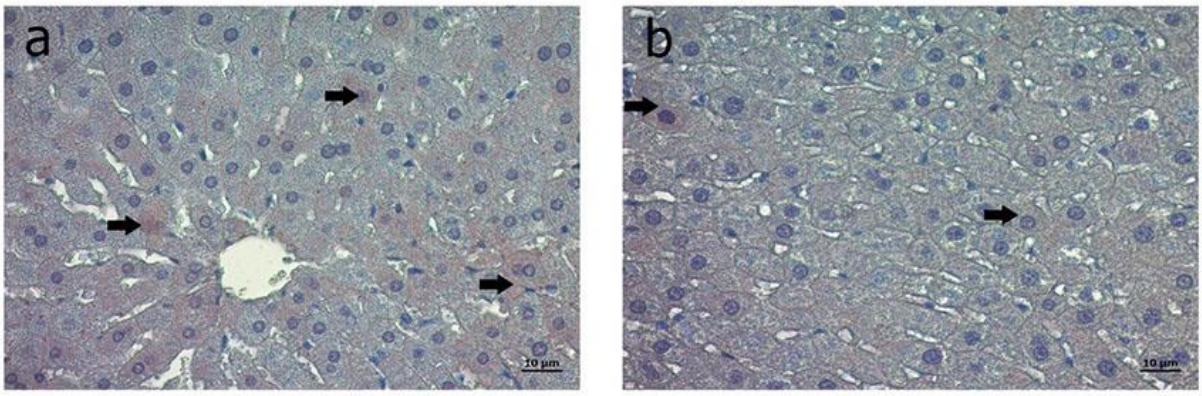

Figure 5. Ghrelin immunoreactivity was observed in hepatocytes and sinüzoidal cells (black arrow) in liver tissue. Control (a), HFCS (b). Accordingly, Ghrelin levels were decreased in the HFCS group compared to the Control group. Streptavidin-biotin-peroxidase method, AEC chromogen, Mayer hematoxylin, Scala bar: 10 µm.

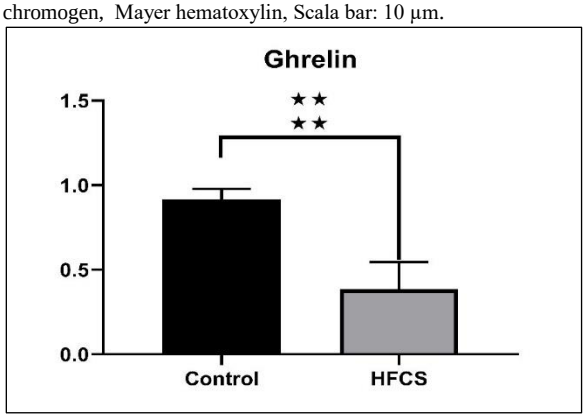

Figure 6. Demonstration of decreased Ghrelin levels in the HFCS group. The meaning of the asterisks on the graph is as follows: *: P ≤ 0.05 **: P ≤ 0.01 ***: P ≤ 0.001 ****: P ≤ 0.0001.

As depicted in Figure 7, images of Kisspeptin immunoreactivity were presented. The Kisspeptin immunoreactivity of the HFCS group was significantly higher than that of the Control group ($p < 0.0001$) (Figure 8). It was determined that the mean and standard deviation values were 0.9363 and 0.08871 in the control group and 2.096 and 0.2673 in the HFCS group, respectively.

Kisspeptin Immunoractivity

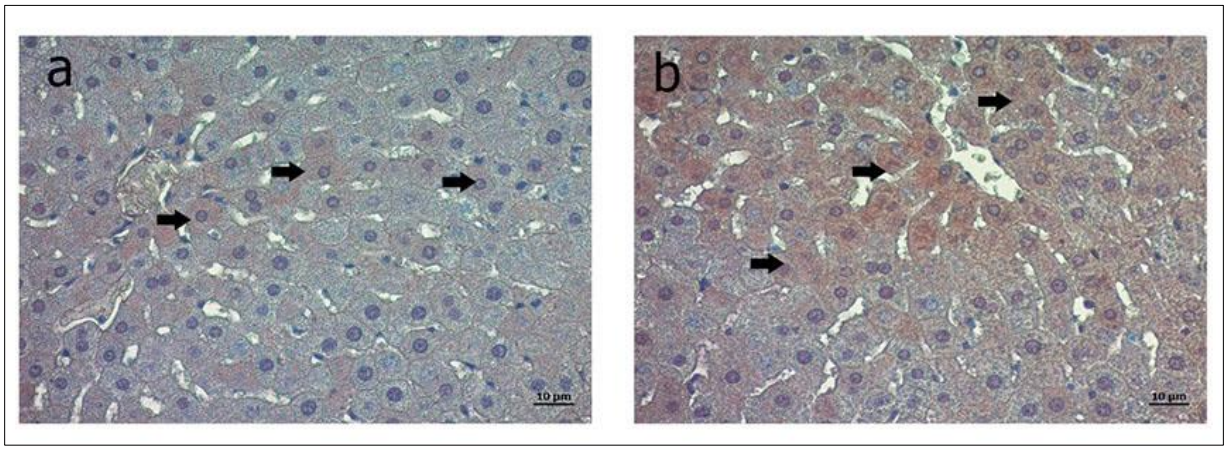

Figure 7. Kisspeptin immunoreactivity was observed in hepatocytes (black arrow) in liver tissue. Control (a), HFCS (b). Accordingly, Kisspeptin level was increased in the HFCS group compared to the Control group. Streptavidin-biotin-peroxidase method, AEC chromogen, Mayer hematoxylin, Scala bar: 10 µm.

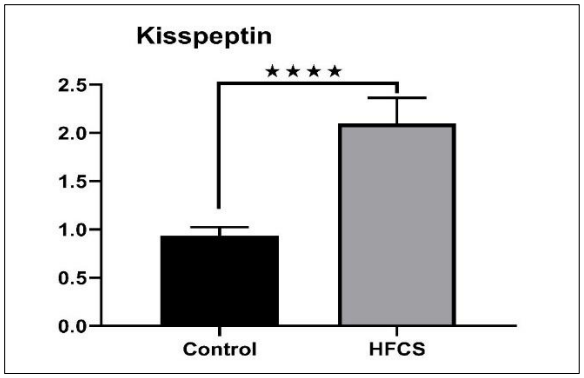

Figure 8. Comparison of Kisspeptin levels between groups**.** The meaning of the asterisks on the graph is as follows: *: $P \leq 0.05$ **: $P \leq 0.01$ ***: $P \leq 0.001$ ****: $P \leq 0.0001$.

DISCUSSION

In the 21st century, dietary habits are gradually changing. With the development of the food industry, canned, fried, baked or processed foods are increasingly becoming a part of our daily diet. The primary reason for the food industry's preference for HFCS is its lower price compared to glucose. Additionally, HFCS can enhance the taste, color, shelf life, and freshness of foods, particularly soft beverages.[13] Previous studies have consistently shown a strong association between HFCS consumption and various health issues such as obesity, metabolic disorders, cardiovascular diseases, diabetes, inflammation, and even cancer. [14] In our study, the primary objective was to investigate the effects of HFCS on Ghrelin and Kisspeptin hormones, which play crucial roles in the regulation of metabolic activity and food intake, along with assessing potential damage to liver tissue.

We measured levels of Caspase 3, the protein responsible for cell death during apoptosis, to determine whether HFCS intake induces liver damage. It has been suggested that a high dietary fructose intake can lead to increased proliferation and permeability of intestinal bacteria, resulting in the entry of endotoxins into the bloodstream and the liver, ultimately causing damage.[15] We discovered that the consumption of HFCS led to increased cell death in hepatocytes. Similar results were also reported in the liver tissue of female rats after 10 weeks of HFCS exposure by Topsakal et al.[16] In another study, it was suggested that increased HFCS consumption led to liver damage by elevating the expression of Caspase 3 and TNF-α in the liver tissue. Interestingly, this damage could be mitigated or prevented through swimming exercise.[17]

Similarly, in our study, TNF-α levels were significantly increased in the HFCS group, and this difference was statistically significant. Noha et al. reported that researchers were able to reduce the significant complications of metabolic syndrome (MS) induced by feeding rats a high-fat high-fructose diet for 8 weeks using etanercept, which is an anti-TNF-α monoclonal antibody.[18] Korkmaz et al. reported that dietary fructose intake increases TNF-α and sodium-glucose cotransporter-2 levels in renal tissue. However, they also found that these effects could be improved or ameliorated with the use of probiotics.[19] In a study conducted on primary human fetal hypothalamic cells expressing Kisspeptin receptors and capable of releasing GnRH, it was discovered that the application of TNF-α reduced the secretion of GnRH and the expression of KISS1R (Kisspeptin receptor).[20]

It was observed that Ghrelin hormone, which typically decreases with food intake, exhibited significantly lower levels in the liver tissues of rats fed with HFCS. Similar results were obtained by Catak et al. In their study, which showed decreased Ghrelin levels in both serum and urogenital tissues in rats fed with fructose.[21] In another study involving ghrelin receptor null mice fed with HFCS, it was observed that they exhibited decreased adiposity, a reduction in proinflammatory macrophages, and improved insulin resistance. [22] It was observed that a 30-day treatment with enalapril brought Ghrelin levels closer to normal in rats with metabolic syndrome (MS) induced by a 60-day fructose diet. [23] In their study on Ghrelin knockout mice, Ma et al. discovered that while HFCS consumption resulted in less weight and adiposity compared to sucrose intake, it led to greater inflammation and insulin resistance. Additionally, it was observed that Ghrelin has a protective effect against complications associated with metabolic syndrome (MS).[24] In a study using a heart transplantation model in mice, it was reported that Caspase-3 activity decreased and the Bcl-2/Bax ratio improved after Ghrelin injection, without adverse effects. Therefore, the decreased Ghrelin levels observed in our study could be considered as one of the potential reasons for liver damage.[25]

In our study, it was observed that HFCS intake increased Kisspeptin levels. However, there is a lack of studies in the

literature that investigate the effect of HFCS intake on Kisspeptin levels. In a study by Ramzan et al., it was reported that chronic application of Kisspeptin reduced seminal fructose levels.[26] In a study conducted on high-fat-fed rats, Kisspeptin levels were found to increase in the liver but decrease in adipose tissue and the pancreas. In another study, it was demonstrated that the deletion of the Kisspeptin receptor worsened fatty liver conditions, whereas its stimulation was protective against fibrosis.[28] The observation that the administration of Kisspeptin in rats reduced both blood and hypothalamic mRNA Ghrelin levels aligns with the findings from our study.[29]

In conclusion, our study holds significance as it marks the first demonstration of immunohistochemical changes in the level of Kisspeptin in liver tissue following a high fructose diet. Furthermore, our findings highlight that the incorporation of HFCS into food products elicits a proinflammatory response and diminishes Ghrelin immunoreactivity in liver tissue. As a result, we conclude that further research is warranted to explore the appropriate HFCS concentrations for use in food and beverages, especially in larger study populations.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Ethics Committee Permission

Approval for this study was obtained from Munzur University Animal Experiments Local Ethics Committee (dated 09.12.2022 and numbered 2022-18/02).

Authors' Contributions

Concept/Design: AT, AK, BZ, YO, OG. Data Collection and/or Processing: AT, BZ, OG, YO. Data analysis and interpretation: AT, AK, BZ. Literature Search: AT, AK, BZ, YO, OG. Drafting manuscript: AT, AK. Critical revision of manuscript: AT, AK, BZ, YO, OG.

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