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RESEARCH ARTICLE

The Effects of High Calcium and Vitamin D on the Fructose- Induced Lipogenesis Pathway in Rats

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ABSTRACT

In this study, the protective effects of high amounts of dietary calcium and vitamin D on fructose-induced lipogenesis were investigated at molecular and biochemical levels. Control group (Con, n=8), High fructose diet group (F, n=8), High fructose diet+75 mg $CaCO_3$ and 26.4 IU vitamin D_3 group (FCaD1, n=8), High fructose diet+150 mg $CaCO_3$ and 52.8 IU vitamin D_3 group (FCaD2, n=8) including experimental groups were fed for 28 days. The significant difference between the living weights was determined firstly as 278.64 ± 5.61^{a}; 268.94 ± 2.80^{ab}; 257.93 ± 4.86^{b} and 257.38 ± 3.42^{b} g in the Con, F, FCaD1, FCaD2 groups, respectively, at the end of the first week ($P<0.05$). This difference was seen to persist during the 3rd week of the trial. It was observed that this difference continued until the end of the 3rd week of the study. In the study, plasma Triglyceride (TG) amount was found to be in the form of 36.25 ± 2.76^{b}; 99.13 ± 15.63^{a}; 98.50 ± 18.00^{a} and 79.88 ± 9.33^{ab} $mg.dl^{-1}$ in Con, F, FCaD1 and FCaD2 groups, respectively ($P<0.05$). The SCD-1 gene's liver expression levels were assessed as 11.83 ± 2.08 ($P<0.001$); 5.31 ± 1.40 ($P<0.05$) and 5.18 ± 1.43 ($P<0.05$), respectively, compared to the Con group; *SREBP-1c* was determined as 2.11 ± 0.37 ($P<0.05$); 3.41 ± 1.20 ($P<0.05$) and 1.79 ± 0.30 ($P<0.05$), respectively. In this study, it has been shown through the *SREBP-1c* and *SCD-1* genes that calcium-vitamin D supplementation can have positive effects in a short time against lipogenesis induced by high fructose diet.
Keywords: Calcium, Fructose, Lipogenesis, Liver, Vitamin D

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Ratlarda Yüksek Kalsiyum ve Vitamin D'nin Fruktozla İndüklenmiş Lipogenez Yolağı Üzerine Etkileri

ÖZ

Bu çalışmada diyetle alınan yüksek miktarlardaki kalsiyum ve vitamin D'nin fruktozla indüklenmiş lipogenez üzerine olabilecek koruyucu etkileri moleküler ve biyokimyasal düzeyde araştırılmıştır. Kontrol grubu (Kon, n=8), Yüksek fruktozlu diyet grubu (F, n=8), Yüksek fruktozlu diyet+75 mg $CaCO_3$ ve 26,4 IU vitamin D_3 alan grup (FCaD1, n=8), Yüksek fruktozlu diyet+150 mg $CaCO_3$ ve 52,8 IU vitamin D_3 alan grup (FCaD2, n=8) olmak üzere 4 grup rat 28 gün boyunca beslenmiştir. Canlı ağırlıklar arasındaki anlamlı farklılık ilk olarak 1. hafta sonunda Kon, F, FCaD1, FCaD2 gruplarında sırasıyla $278,64\pm5,61^{a}$; $268,94\pm2,80^{ab}$; $257,93\pm4,86^{b}$ ve $257,38\pm3,42^{b}$ g olarak tespit edilmiştir ($P<0,05$). Bu farklılığın çalışmanın 3. haftasının sonuna kadar devam ettiği görülmüştür. Çalışmada plazma TG miktarının Kon, F, FCaD1 ve FCaD2 gruplarında sırasıyla $36,25\pm2,76^{b}$; $99,13\pm15,63^{a}$; $98,50\pm18,00^{a}$ ve $79,88\pm9,33^{ab}$ $mg.dl^{-1}$ şeklinde olduğu görülmüştür ($P<0,05$). Karaciğerde *SCD-1* geninin ekspresyon seviyeleri Kon grubuna göre sırasıyla $11,83\pm2,08$ ($P<0,001$); $5,31\pm1,40$ ($P<0,05$) ve $5,18\pm1,43$ ($P<0,05$) olarak, *SREBP-1c* geninin ekspresyon seviyeleri ise sırasıyla $2,11\pm0,37$ ($P<0,05$); $3,41\pm1,20$ ($P<0,05$) ve $1,79\pm0,30$ ($P<0,05$) olarak tespit edilmiştir. Yapılan bu çalışmada, yüksek fruktozlu diyet ile indüklenmiş lipogeneze karşı kalsiyum-vitamin D takviyesinin kısa sürede olumlu etkilerinin olabileceği *SREBP-1c* ve *SCD-1* genleri üzerinden gösterilmiştir.
Anahtar Kelimeler: Fruktoz, Kalsiyum, Karaciğer, Lipogenez, Vitamin D

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INTRODUCTION

There are many homeostasis systems in the body. Energy metabolism is one of these systems. The liver is a metabolic organ that controls the body's energy metabolism and plays a central role in metabolically binding to various tissues, including skeletal muscle and fat tissue, and energy metabolism is strictly controlled in this organ. Due to imbalances in diet and exercise conditions, disturbances in energy metabolism can occur and homeostasis can be disrupted. Disorders in energy metabolism can lead to chronic diseases associated with obesity and even cause the development of these diseases (Rui 2014). Today, approximately 2 billion adults are reported to be overweight, and about 650 million of these people are expected to be obese (Marziou et al. 2020). Due to the rising prevalence of obesity, the metabolic disease known as the metabolic syndrome may manifest (Eckel et al. 2005). Obesity can lead to non-alcoholic fatty liver disease (NAFLD), one of the characteristics of the metabolic syndrome (Marziou et al. 2020).

The signs of NAFLD include by more than 5% accumulation of triglycerides (TG) in hepatocytes. This is a result of the increased intake of fatty acids by the liver. TGs can also be formed as a result of de novo lipogenesis (DNL) (Agame-Lagunes et al. 2021). DNL is one of the metabolic pathways that synthesize fatty acids from excess carbohydrates (Gathercole et al. 2013). Approximately 26% of liver lipids in patients with NAFLD have been found to be caused by DNL (Agame-Lagunes et al. 2021). In researching metabolic disorders such as NAFLD and developing treatment strategies, experimental animals are often used as model organisms (Kleinert et al. 2018). The use of simple carbohydrates such as fructose in animal models can induce DNL, and changes in the body can be studied at molecular and biochemical levels (Chiu et al. 2018; Özkan and Yakan, 2019). However, the consumption of fructose can be a risk factor for the development of NAFLD and may trigger fatty liver for an average of 1 week when added to drinks that are consumed extensively (Agame-Lagunes et al. 2021).

Despite various dietary strategies, it is difficult for obese people to lose weight and then maintain their weight. Obese people struggle to lose weight and then keep it off despite trying a variety of dietary approaches. When the distribution and composition of dietary macronutrients (carbohydrates, fats, and proteins) are altered, variable amounts of weight loss and improvements in metabolic syndrome characteristics can be achieved (Abete et al. 2010). On the one hand, while attention is paid to the effects of dietary macronutrient surplus rates on body weight, studies have shown that calcium in the diet can play a role in regulating body fat and body weight (Melanson et al. 2003). In addition to calcium, vitamin D, one of the fat-soluble vitamins, is also a micronutrient extract with significant effects on human health (Ning et al. 2015). In addition to its well-known effects on calcium metabolism and skeletal formation, vitamin D has been shown to be active in the expression levels of more than 200 genes in various metabolic pathways (Yin et al. 2012). Epidemiological studies have also shown that vitamin D deficiency is associated with obesity (Mansouri et al. 2019).

Calcium is the 5th most abundant element found in the skeleton in more than 99% of the body of the vertebrate (Li et al. 2020). Calcium plays an important role in many physiological processes. It is also known that calcium plays a role in lipid and glycogen metabolism and may be responsible for balancing body weight (Li et al. 2018). Calcium may

have an anti-obesity effect through the regulation of adipogenesis. This adipogenesis is regulated by the stimulation of mesenchymal stem cells and the inhibition of preadipocytes in the differentiation phase. Calcium can also have an effect by reducing lipogenesis and increasing lipolysis. It can also have an anti-obesity effect in such ways as promoting the proliferation of preadipocytes and apoptosis, increased activation of brown fat tissue and increased thermogenesis by browning of white fatty tissue, suppression of fat absorption and promotion of fecal fat discharge, and modification of the composition and diversity of the intestinal microbiome (Zhang et al. 2019).

Studies examining the effects of both calcium and vitamin D on body weight and/or abdominal fat have been insufficient (Zarghani et al. 2016). In general, however, the recommended anti-obesity mechanisms of calcium and vitamin D include regulation of adipocyte death, adipogenesis, and lipid metabolism (Song and Sergeev 2012).

The active form of vitamin D increases the absorption of calcium in the duodenum (Thacher and Clarke 2011). Considering this effect of vitamin D on calcium absorption, rats were given calcium and vitamin D supplements together in this study. Although the relationship between vitamin D and body weight gain is still a matter of debate, a lack of vitamin D is linked to both overall and abdominal obesity according to a report by Mansouri et al. (2019). Maia-Ceciliano et al. (2019) in a study feeding adult male mice of the C57BL/6 breed with or without a control diet containing vitamin D (10.000 $IU.kg^{-1}$ vitamin D_3) or a high-fructose diet (474.3 $g.kg^{-1}$ fructose), there was no significant difference in body weight between the groups. In another study, rats found that vitamin D_3 supplementation or exposure to sunlight during pregnancy helped to control weight and thus prevent pregnancy complications associated with obesity (Kang et al. 2015). There are many studies on the anti-obesity and anti-lipogenic effects of calcium or vitamin D supplements alone. This study examined the protective effects of high amounts of calcium and vitamin D in the diet on fructose-induced lipogenesis. From the transcription factors that play an important role in the formation of hepatic lipogenesis in this scope, the expression levels of *SREBP-1c*, *ACACA*, *FASN* and *SCD-1* genes have been determined.

MATERIALS and METHODS

Experimental groups and applications

The study used male rats of Wistar albino race aged 8–10 weeks, weighing about 200–250 g and the rats were subjected to environmental conditions for a week before the experimental protocol began. At the beginning of the study, four groups were formed so that there was no statistically significant difference in the body weight of the rats ($P>0.05$). The groups were formed as the control group (Con), the high fructose diet group (F), the high fructose diet+75 mg $CaCO_3$ and the 26.4 IU vitamin D_3 group (FCaD1), the high fructose diet+150mg CaCO3, and the 52.8 IU Vitamin D_3 Group (FCaD2). The animals were housed in transparent polycarbonate cages with 4 rats in each cage. Rats were also given food and water as ad libitum, and their daily consumption was recorded. During operation, the light in the environment is set to be 12 hours light, 12 hours dark (07:00–19:00 light, 19:00-07:00 dark), moisture ratio 55%, and ambient temperature 21±2 °C. However, the rats were checked at least twice a day during the working period.

Rats in the formed groups were given different diets for 4 weeks. Rats in the groups F, FCaD1 and FcaD2 were given 2600 $kcal.kg^{-1}$ of metabolically energized pellets. Rats in group F were given ad libitum water/fructose solution with 1 kcal of metabolic energy per ml in addition to pellets. Rats

in the FCaD1 and FCaD2 groups were given different doses of calcium and vitamin D through oral gavage in addition to the diet in the F group. Oral gavage was administered to rats in the FCaD1 and FCaD2 groups daily at 8:00 a.m. until the end of the feeding period. Fructose solutions were prepared daily and offered for fresh consumption.

The daily amounts of energy that rats in the experimental groups would receive were planned similarly to the requirements (National Research Council, 1995) (Table 1).

Weekly changes in body weight and amounts of water/fructose solution consumed with feed were recorded during the feeding period. At the end of the feeding period, the rats were left hungry for 12 hours but were able to reach the water ad libitum. Rats were anesthetized (80 $mg.kg^{-1}$ ketamine and 12 $mg.kg^{-1}$ xylazine, IP) and euthanized by taking blood from the heart. In order to inhibit the nuclease activity and maintain the RNA quality by washing it with nuclease-free water, the rat liver tissue was rapidly removed and frozen in liquid nitrogen. After being frozen, the tissues were kept at -86 °C for molecular analysis.

Laboratory Analyses

Blood samples taken during euthanasia were centrifugated at +4 °C at 3000xg for 10 minutes and plasma was obtained. These plasmas were preserved at −86 °C until biochemical parameters were determined. Plasma High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), total cholesterol, triglycerides, and hunger blood glucose parameters were determined using an autoanalyzer (SIEMENS Advia 1800, Japan).

Total RNA isolation was done according to the TRIzol method (Rio et al. 2010) and the purity and concentration of the resulting RNAs were measured in nucleic acid meter (Merinton, SMA-1000). RNA quality was assessed with 100 V in electrophoresis and 1% agarose gel in 25 minutes and later, RNA integrity was checked rRNA subunits. Examples suitable for purity (A260/280: 1.8–2.2), concentration (>125 $ng.\mu l^{-1}$) and agarose gel electrophoresis were used. To prevent possible DNA contamination in these examples, DNA digestion was carried out using the DNase kit (DNase I, RNase free, Thermo Scientific, Catalog No: EN0521, USA) protocol, then RNA samples were converted to cDNA with the Revertaid First Strand cDNA Synthesis Kit (Thermo Science, Catalyst No: K1622, USA). The final volume of the post-reaction samples was completed at 200 µl with non-nuclease water and stored at -20 °C until analysis.

Amplification of *SREBP-1c*, *ACACA*, *FASN*, and *SCD-1* genes from cDNA samples was performed using real-time PCR (Rotor-Gene Q, Qiagen, USA). For amplification, the SYBR Green kit (Power SYBR Green PCR Master Mix, ThermoFisher Scientific, Catalog No: 4367659, USA) was used. The forward and reverse primary sequences of the genes to be amplified are given in Table 2. Real-time PCR is regulated to 15 seconds at 95 °C, 60 seconds at 60 °C and 40 cycles after 10 minutes of denaturation. Peptidyl prolyl isomerase A (*PPIA*) is used as a housekeeping gene. The Ct values of the genes are normalized according to the housekeeping gene and are calculated as layer changes. The RT-qPCR application is done as a duplicate. Post-reaction RT-qPCR products were controlled to be amplified by the correct gene by running at 100 V for 45 min in 1.5% agarose gel electrophoresis.

Statistical analysis

Variance analysis in repeated measurements was used to evaluate the weekly changes in the body weight of the rats. In cases where differences were detected, Bonferroni correctional simple effect analysis was used as a post-hoc test. The differences in biochemical parameters between groups were

assessed by Variance analysis. The Tukey test was used as a post-hoc test for variables with differences. All statistical calculations are performed using the IBM SPSS version 23.0 program. For the statistical calculation of gene expression results, the $2\text{-}\Delta\Delta^{ct}$ method was used and the results were given as fold change (Livak and Schmitgen 2001). In all analyses, $P<0.05$ was accepted as meaningful.

Table 1. Rations and applications in experimental groups
Tablo 1. Deneme gruplarında rasyon ve uygulamalar

Contents	Experiment groups			
	Con	**F**	**FCaD1**	**FCaD2**
Feed	2600 kcal.kg^{-1}	2600 kcal.kg^{-1}	2600 kcal.kg^{-1}	2600 kcal.kg^{-1}
Water	0 kcal.ml^{-1}	1 kcal.ml^{-1}	1 kcal.ml^{-1}	1 kcal.ml^{-1}
Calcium	-	-	75 mg $CaCO_3$ (30 mg Ca)	150 mg $CaCO_3$ (60 mg Ca)
Vitamin D_3	-	-	26,4 IU	52,8 IU

Table 2. Forward and reverse primer series of amplified genes
Tablo 2. Genlere ait forward ve reverse primer dizileri

Genes	Accession no*	Forward and Reverse Primer Sequence	Bp**	Reference
PPIA***	NM_017101.1	F:5'-CAGACAAAGTTCCAAAGACAGCA-3' R: 5'-CACCCTGGCACATGAATCCT-3'	117	Santos et al. 2016
ACACA	NM_022193.1	F: 5'-CAATCCTCGGCACATGGAGA-3' R:5'-GCTCAGCCAAGCGGATGTAGA-3'	149	Gou et al. 2016
FASN	NM_017332.1	F: 5'-GCTGCTACAAACAGGACCATC-3' R: 5'-TCCACTGACTCTTCACAGACCA-3'	98	Mock et al. 2017
SCD-1	NM_139192.2	F: 5'-CCTTAACCCTGAGATCCCGTAGA-3' R: 5'-AGCCCATAAAAGATTTCTGCAAA-3'	95	Yasari et al. 2010
SREBP-1c	NM_001276707.1	F: 5'-GCAACACTGGCAGAGATCTACGT-3' R: 5'-TGGCGGGCACTACTTAGGAA-3'	104	He et al. 2004

*: NCBI GenBank accession number, **: Base pair, ***: Housekeeping gene

RESULTS

Energy Consumption and Living Weight Change

The feed, water and energy consumption of the animals in the groups was measured over 4 weeks, with live weights and daily. Significant differences in living weight between the groups were first observed at the end of week 1 ($P<0.05$), whereas the group's live weight averages were initially approximately 220 g ($P>0.05$). This difference was shown to persist at different levels of significance until the end of the third week of the study. At the end of the first week, fewer body weight gains were observed in the FCaD1 and FCaD2 groups compared to the Con group ($P<0.05$). At the end of week 2 of the study, a lower body weight gain was observed in both the FCaD1 and FcaD2 groups compared to both the Con and F groups ($P<0.001$). By the end of week 3 of the study, a lower body weight gain was observed in the FCaD1 and FCaD2 groups compared to the Con group ($P<0.01$). At week 4 of the study, there was no significant difference in living weight between the groups (Table 3).

Biochemical parameters

Values for certain biochemical parameters in plasma are shown in Table 4. There were no statistically significant results for plasma glucose, total cholesterol, HDL and LDL among the groups. The biochemical parameters studied in the study showed that the plasma TG levels were 36.25±2.76[b]; 99.13±15.63[a]; 98.50±18.00[a]; 79.88±9.33[ab] in the Con, F, FCaD1 and FCaD2 groups, respectively, and that the increases in plasma TG levels in the Con group were statistically significant (P<0.05). Plasma TG levels in the FCaD2 group are similar to those in the Con groups (Table 4).

Gene Expression

RNA isolation samples with sufficient purity (A260/280:1.87±0.01) and concentration (675.30±31.12 ng.µl^{-1}) were used after integrity and quality checks in agarose gel electrophoresis. Results from the expression levels of *ACACA*, *FASN*, *SCD-1*, *SREBP-1c* genes are shown in Table 5. According to the control group, *ACACA* gene expression was approximately twice as expressed in the F group, about seven times in the FCaD1 group, and about two and a half times (P<0.05). *FASN* gene expression increased approximately twice in all trial groups. However, a statistically significant increase was observed only in the FCaD2 group (P<0.05). *SCD-1* gene expression was expressed approximately 12 times in group F (P<0.001) and in groups FCaD1 and FCA2 (P <0.05) about 5 times. The levels of gene expression of *SREBP-1c* were expressed approximately twice in group F; approx. 3.5 times in group FCaD1; and more than 1.5 times (P<0.05) in group FCaD2 (Figure 1).

Table 3. Living weight in groups, total energy intake, feed and water intake during the feeding period (X̄±Sx̄)
Tablo 3. Gruplarda besleme süresince canlı ağırlık, toplam enerji alımı, yem ve su tüketimi (X̄±Sx̄)

Feeding period	Traits	Con	F	FCaD1	FCaD2	P
Beginning	**Live weight (g)**	212.93±4.06	217.78±2.62	218.79±2.56	222.11±4.36	0.334
	Live weight (g)	278.64±5.61[a]	268.94±2.80[ab]	257.93±4.86[b]	257.38 ±3.42[b]	**<0.05**
	Total consumed energy (kcal/rat/week)	563.65	632.65	586.24	558.64	
	Energy taken from food (kcal/rat/week)	563.65	371.77	331.68	319.64	
First week	**Energy taken from water (kcal/rat/week)**	-	260.88	254.56	239.00	
	Weekly consumed feed (g/rat)	216.79	142.99	127.57	122.94	
	Weekly consumed water (ml/rat)	398.50	260.88	254.56	239.00	
	Live weight (g)	319.78±5.25[a]	311.90±4.36[a]	292.06±5.16[b]	275.19±4.01[b]	**<0.001**
	Total consumed energy (kcal/rat/week)	637.26	646.28	587.25	552.11	
	Energy taken from food (kcal/rat/week)	637.26	356.15	333.81	308.00	
Second week	**Energy taken from water (kcal/rat/week)**	-	290.13	253.44	244.11	
	Weekly consumed feed (g/rat)	245.10	136.98	128.39	118.46	
	Weekly consumed water (ml/rat)	431.13	290.13	253.44	244.11	
	Live weight (g)	352.79±6.27[a]	344.35±4.29[ab]	323.15±7.57[b]	322.93±7.53[b]	**<0.01**
	Total consumed energy (kcal/rat/week)	657.80	687.02	590.03	629.21	
	Energy taken from food (kcal/rat/week)	657.80	399.39	314.70	319.88	
Third week	**Energy taken from water (kcal/rat/week)**	-	287.63	275.33	309.33	
	Weekly consumed feed (g/rat)	253.00	153.61	121.04	123.03	
	Weekly consumed water (ml/rat)	432.50	287.63	275.33	309.33	
	Live weight (g)	368.89±7.56	376.43±6,44	345.93±10.84	356.93±8.13	0.076
	Total consumed energy (kcal/rat/week)	727.35	616.91	656.34	705.42	
	Energy taken from food (kcal/rat/week)	727.35	319.41	336.15	355.03	
Fourth week	**Energy taken from water (kcal/rat/week)**	-	297.50	320.19	350.39	
	Weekly consumed feed (g/rat)	279.75	122.85	129.29	136.55	
	Weekly consumed water (ml/rat)	496.27	297.50	320.19	350.39	

[a, b]: Different letters in the same rows indicate the difference between groups.

Table 4. Biochemical parameters
Tablo 4. Biyokimyasal parametreler

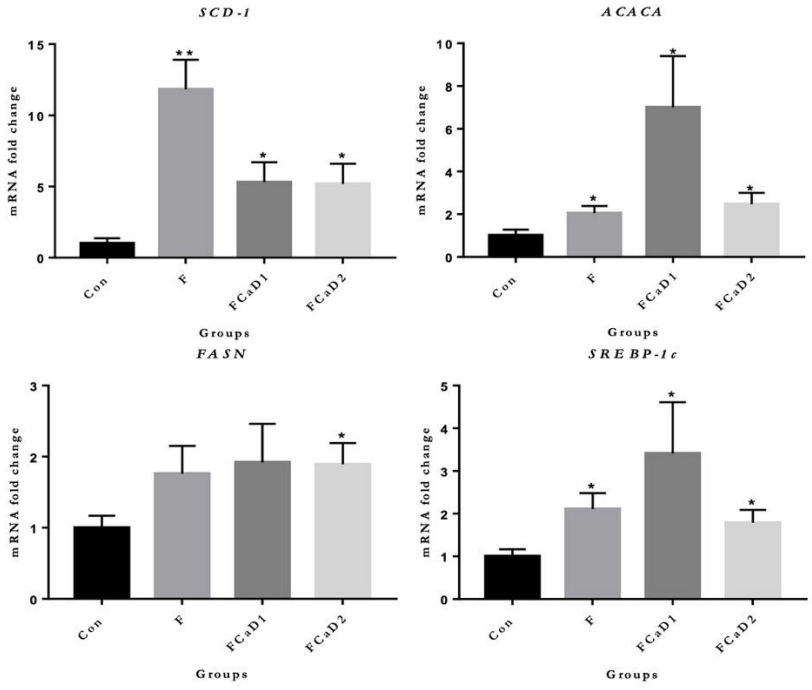
Biochemical parameters	Groups				P
	Con	F	FCaD1	FCaD2	
Glycose (mg/dL)	160.63±9.59	141.88±11.00	136.13±11.47	145.38±11.94	0.453
T. Col (mg/dL)	47.88±2.60	46.25±1.76	50.00±3.07	49.38±1.79	0.687
HDL (mg/dL)	34.69±2.57	32.22±1.22	33.80±1.48	33.29±1.31	0.787
LDL (mg/dL)	9.53±0.81	7.58±0.56	8.96±1.37	9.69±0.78	0.381
TG (mg/dL)	36.25±2.76[b]	99.13±15.63[a]	98.50±18.00[a]	79.88±9.33[ab]	**<0.05**

T. Col: Total Cholesterol
[a, b]: Different letters in the same rows indicate the difference between groups.

Table 5. Fold changes in liver tissue of ACACA, FASN, SCD-1, SREBP-1c genes according to control group (X̄±Sx̄)
Tablo 5. Karaciğer dokusunda kontrol grubuna göre ACACA, FASN, SCD-1, SREBP-1c genlerinin kat değişimi (X̄±Sx̄)

*Genes	F		FCaD1		FCaD2	
	Fold change	P	Fold change	P	Fold change	P
ACACA	2.05±0.34	**<0.05**	7.00±2.40	**<0.05**	2.47±0.53	**<0.05**
FASN	1.76±0.39	0.094	1.92±0.54	0.091	1.89±0.30	**<0.05**
SCD-1	11.83±2.08	**<0.001**	5.31±1.40	**<0.05**	5.18±1.43	**<0.05**
SREBP-1c	2.11±0.37	**<0.05**	3.41±1.20	**<0.05**	1.79±0.30	**<0.05**

* Gene expression levels are given as fold change according to control group.



*: P<0,05; **:P<0,001.

Figure 1. Levels of *ACACA*, *FASN*, *SCD-1*, *SREBP-1c* gene expression in liver tissue

Figür 1. Karaciğer dokusunda *ACACA*, *FASN*, *SCD-1*, *SREBP-1c* genlerinin ekspresyon seviyeleri

DISCUSSION

It is that high fructose corn syrup (HFCS) can lead to excessive energy consumption, weight gain and an increased prevalence of obesity (Melanson et al. 2008). This finding can be attributed to the rapid absorption of fructose by the liver and its entry into the glycolysis pathway after skipping the phosphofructokinase regulatory step (Kawasaki et al. 2009). Bocarsly et al. (2010) in a study in which male Sprague-Dawley rats were offered diets containing 8% HFCS or 10% sucrose for 8 weeks, even though they consumed the same total calories, rats fed a diet containing HFCS gained significantly more body weight compared to rats fed the sugar diet. These results suggest that excessive consumption of HFCS may contribute to the incidence of obesity. During this study, however, it was not observed that there was any difference in living weight between the Con and F groups. Depending on the duration of the rat's exposure to fructose (4 weeks), there is no significant change in body weight in rats in group F. According to calcium and vitamin D dosages, there were no appreciable variations in body weight changes between the FCaD1 and FCaD2 groups of rats in the current investigation. According to Zarghani et al. (2016), there were no discernible differences in body weight and Body Mass Index (BMI) parameters between the groups in a study in which male rats with a starting weight of 216 g, fed a high-fat and high-fructose diet for 60 days, were given 4 different doses of calcium and vitamin D3 to supplement their diets (suboptimal, normal, high, and very high calcium and vitamin D3). When it comes to the lack of discernible changes in the body weight of rats that are dose-dependent (FCaD1 and FCaD2), Zarghani et al.'s (2016) study and the current study are moving in the same way. The rats in the FCaD1 and FCaD2 groups gained less live body weight than the Con group during the study's first week (P0.05), and this difference persisted at various levels of significance (P0.001 and P0.01) during weeks 2 and 3. Nevertheless, calcium and vitamin D supplementation had no significant dose-dependent effects on the rats' body weight. Accordingly, the current study discovered that groups taking calcium and vitamin D supplements without regard to dose saw lower increases in live body weight than the Con group.

During the study period, the highest energy intake was found in group F in weeks 1, 2, and 3. The feeling of saturation occurs as a result of the insulin hormone triggering the release of leptin. Fructose does not trigger the release of insulin and therefore does not produce a feeling of satiety. In the F-group fed fructose, it is believed that a feeling of satiety may arise due to feed consumption (Özkan 2018). Therefore, in the light of literature scans and the findings of this study, it is believed that the decrease in insulin and leptin levels in the circulation after fructose intake may result in less appetite suppression and consequently increased energy intake (Bantle 2009; Özkan 2018). The lowest energy intake in the current study was observed in the FCaD2 group at weeks 1 and 2 of the feeding period and in the FCaD1 group on week 3. Calcium has been shown to reduce the absorption of fatty acids by creating intestinal calcium and fatty acid soaps and increase the excretion of fecal fat, as well as decrease the

energy taken due to these mechanisms that increase the feeling of satiety (Teegarden 2005; Abete et al. 2010). Papakonstantinou et al. (2003) in a study in male Wistar albino rats who were given a high calcium diet (2.4% calcium) for 85 days that there was no significant difference from the control group in terms of energy consumption at the end of the feeding period.

For 10 weeks, in the study in female Sprague-Dawley rats fed normal or high energy density diets with different rates of calcium (0.2% or 0.6% calcium or 1.8% calcium), rats fed high-energy diets (4.6 $kcal.g^{-1}$) consumed significantly more energy than rats fed a normal energy diet (3.8 $kcal.g^{-1}$). However, the researchers found that there were no significant differences in energy consumption between groups on diets that contained different amounts of calcium (Zhang and Tordoff 2004). Siddiqui et al. (2008) in a study in male Wistar albino rats, who were given a high-fat or high-sugar diet containing calcium-vitamin D (low-calcium (0.25%) and vitamin D (400 $IU.kg^{-1}$) or high calcium (1.5% and vitamin D (10 000 $IU.kg^{-1}$ diet) in different doses for 13 weeks, that the high-fat diet group consumed more calories at the end of the feeding period than the high glucose diet group, but there were no significant differences in energy intake between the groups on a calcium vitamin D diet in various doses. The results of studies on the effect of dietary calcium-vitamin D content on energy intake may vary. In this study, Wistar albino rats were given high-dose calcium and vitamin D supplements in addition to a high-fructose diet for 28 days. When examining the amount of energy consumed by the rats, it was found that the rats who took calcium and vitamin D supplements for the first 3 weeks consumed less energy than the control group. The scans of the literature and the results of this study are thought to vary depending on the type of animal, the duration of feeding the animal, and the differences in the calcium-vitamin D doses supplemented with the diet (Papakonstantinou et al. 2003; Zhang and Tordoff 2004; Siddiqui et al. 2008).

High fructose intake has been shown to have adverse effects on insulin sensitivity/glucose homeostasis (Tappy and Le 2010). As a result of chronic exposure to fructose, glucose regulation is impaired and plasma glucose concentrations increase (Özkan 2018). In this study, rats on high-fructose diets found no significant difference in plasma glucose concentrations. Depending on the duration of the rat's exposure to fructose (4 weeks), it is believed that glucose regulation in rats on a high-fructose diet may not be sufficiently impaired. A study found that the levels of plasma glucose increased significantly when rats were given fructose with their diet for a long period (3 months), which was explained by the metabolic syndrome resulting from chronic consumption of fructose (Özkan 2018). Sergeev and Song (2014) that in a study in which four-week-old male C57BL/6J mice were given a 10-week control diet (10% fatty), a high-fat diet (60% fat), a high-calcium diet, a high-vitamin D diet, or high-calcium+vitamin D diet with a high-fat diet, fasting plasma glucose concentration was significantly lower in mice fed a high-calcium and high-vitamin D diet than in the high-fat diet group. In addition, these researchers found that the concentration of fasting plasma glucose in mice fed a high calcium+vitamin D diet was at the level of the control group concentration. In the current study, rats who consumed different doses of calcium+vitamin D in addition to a high fructose diet found no significant changes in plasma glucose concentrations. This finding is believed to be related to the time rats take calcium+vitamin D supplements (4 weeks). It also confirms that the metabolism of the energy taken from fat and carbohydrates works through different mechanisms of glucose regulation.

In a study conducted in male Wistar Albino rats on a high-fat-fructose diet for 15 weeks, plasma

levels of TG, total cholesterol and LDL increased significantly compared to the control group, while HDL decreased markedly (Aragno et al. 2009). In this study, it is similar to the findings of Aragno et al. (2009) that the TG levels of rats in groups F and FCaD1 increased significantly compared to rats in the Con group. This increase can be attributed to rats in groups F and FCaD1 on high fructose diets (Aragno et al. 2009, Özkan 2018). Some studies in rats have shown that fructose is hyperlipidemic (Tappy and Le 2010; Özkan 2018). However, in the current study, no significant changes were observed in total cholesterol, LDL and HDL levels. As with plasma glucose concentrations, the findings of these biochemical parameters are thought to be affected by the duration of rats' exposure to fructose. In a study in which male rats fed a high-fat and high-fructose diet for 60 days were supplemented with 4 different doses of calcium + vitamin D_3, they did not observe any significant difference in serum TG, cholesterol and non-HDL cholesterol levels between the groups. However, in rats receiving high and very high doses of calcium-vitamin D_3 supplementation, a significant increase in serum HDL cholesterol was observed compared to the group receiving a normal dose of calcium-vitamin D_3 (Zarghani et al. 2016). In this study, there were no significant differences in the levels of TG, cholesterol and LDL cholesterol in the rats in the FCaD1 and FCaD2 groups compared to rats in group F who were on a high fructose diet. Similar to the study carried out by Zarghani et al. (2016) this study found that calcium-vitamin D supplementation in fructose diet had no effect on plasma TG, cholesterol and LDL levels. In the current study, there was also no significant difference in plasma HDL levels between the groups.

Fructose supplementation to diet is a method often used in animal models to experimentally induce DNL (Chiu et al. 2018; Özkan and Yakan 2019). *SREBP-1c*, which regulates hepatic lipid homeostasis, has been shown to be a transcription regulator that stimulates the expression of certain genes in DNL such as *ACC* and *FASN* (Yin et al 2012). Dietary calcium, a nutrient that does not provide energy, has been shown to play an important role in regulating energy and lipid metabolism (Jacqumain et al. 2003). Also, higher vitamin D intake has been associated with lower body fat and metabolic health (Zhu et al. 2013). In this study, expression levels were also studied to study the effects of calcium and vitamin D on fructose-induced metabolism due to the possible lipogenic effects of *ACACA*, *FASN*, *SCD-1* and *SREBP-1c* genes.

High fructose intake has been shown to increase hepatic expression of the *ACACA* gene compared to high glucose intakes (Shimada et al. 2019). In this study, *ACACA* gene expression levels were significantly increased in rats in groups F, FCaD1 and FCaD2, who were fed high fructose and calcium and vitamin D in different doses during the 4-week feeding period, compared with the Con group. Studies in rats on high fructose diets have shown increased levels of *ACACA* gene expression (Softhic et al. 2016; Distefano 2020).

In a study of rats fed a high-fat diet, a stronger increase in the expression levels of the *FASN* gene was observed as a result of the addition of fructose to the drinking water of rats compared to a high fat diet (Softhic et al. 2016). Similarly, a study in female Sprague-Dawley rats found that rats on high-fructose diets significantly increased hepatic expression of the *FASN* gene compared to rats fed a high corn starch diet (DiStefano 2020). In this study, a significant increase in the level of expression of the *FASN* gene was observed only in the FCaD2 group.

Depending on the fructose diet, up-regulation of *ACACA*, *FASN* and *SREBP-1c* has been observed in many studies despite the use of different experimental designs, animal models and treatment protocols (Softhic et al. 2016; Maia-

Ceciliano et al. 2019, DiStefano 2020). Despite these findings, the molecular mechanisms affecting the gene expression of fructose are not clearly known. It has been shown that fructose can regulate a number of genes involved in de novo lipogenesis through transcriptomic and epigenetic mechanisms. Fructose intake has also been shown to alter the levels of specific microRNAs (miRNAs) that target molecules involved in lipid metabolism and other biological processes related to hepatic function (DiStefano 2020). It has been suggested that more work is needed to illuminate the mechanisms by which miRNAs play a role in the etiology of NAFLD caused by fructose (Pan et al. 2021). It is believed that the expression levels of the *ACACA* and *FASN* genes in the FCaD1 and FCaD2 groups may have increased more than the F group in comparison with the Con group because of the inadequate activity of the factors suppressing *SREBP-1c*, *ACACA*, and *FASN* such as miR-33, and the unsufficient suppression of the levels of expression of factors such as miR-122, miR-370. It is believed that may have triggered lipogenesis of fructose by affecting the mechanisms that control, *SREBP-1c*, *ACACA* and *FASN* genes in F, FCaD1, FCaD2 groups. Thus, it is believed that changes in the levels of expression of *ACACA* and *FASN* genes in the FCaD1 and FCaD2 groups may be influenced by epigenetic mechanisms.

In the current study, the level of *SCD-1* gene expression in the liver of rats in group F increased significantly. In addition, the increased level of *ACACA* gene expression in the liver of rats in group F suggests that the path through Adenosine Monophosphate- Activated Protein Kinase (AMPK) described in the literature may affect hepatic lipogenesis. Das and Choudhuri (2020), in their study on male Wistar albino rats, found that the lipogenic pathway was suppressed by reactivation of AMPK as a result of calcium supplementation given to rats on a high-fat diet. Another study found that the expression levels of the *SCD-1* gene were suppressed by vitamin D in both the liver and fat tissue of pregnant rats. Depending on these findings, researchers report that a decrease in body weight and fat accumulation following treatment with vitamin D may be linked to the modulation of genes associated with lipogenesis (Kang et al. 2015). In this study, the FCaD1 and FCaD2 groups that received calcium and vitamin D supplementation also observed fewer increases in the levels of *SCD-1* gene expression in the liver than in the F group compared to the Con group. It is believed that calcium and vitamin D supplementation may have suppressed the level of *SCD-1* gene expression, in line with some reports (Kang et al. 2015; Das and Choudhuri 2020). The level of *SCD-1* gene expression in the FCaD2 group was observed to be less elevated compared to the Con group than in the FCaD1 group. Therefore, the current study found that the level of *SCD-1* gene expression was influenced by the dose of calcium and vitamin D.

It has been shown that the effect of dietary carbohydrates such as glucose, fructose and sucrose on significantly increasing hepatic *SCD-1* is dependent on *SREBP-1c* as well as due to independent mechanisms. (Flowers and Ntambi 2008). The activation of *SREBP-1c* plays an important role in the progression of NAFLD caused by a high-fat and fructose diet (Aragno et al. 2009). Fructose has been shown to induce *SREBP-1c* and other lipogenic genes more potently than glucose. Increasing levels of expression of the *SREP-1c* gene in the liver of rats on a high-fructose diet containing 67% carbohydrates (98% fructose) for 8 weeks have been reported (DiStefano 2020). In this study, the levels of *SREBP-1c* gene expression in rats from trial groups F, FCaD1 and FCaD2 increased significantly compared to the Con group. Inadequate calcium intake during maternal pregnancy and lactation has been shown to increase the expression of *SREBP-1c* in the liver or fat tissue of mouse offspring (Li et al.

2018; Zhang et al. 2019). In one study, vitamin D supplementation in mice on a high fructose diet caused a decrease in the levels of expression of lipogenic genes such as *SREBP-1c* in the liver (Maia-Ceciliano et al. 2019). Vitamin D deficiency in rats has been shown to increase hepatic lipogenesis with fructose intake, while vitamin D supplementation can weaken hepatic steatosis by regulating lipid metabolism through negative regulation of *SREBP-1c* and target genes (Maia-Ceciliano et al. 2019). Yin et al. (2012) showed that vitamin D_3 regulates lipid metabolism by reducing hepatic steatosis in the liver of male adult rats. The protective effect of vitamin D_3 against hepatic steatosis has been achieved by reducing the expression levels of *SREBP-1c* and the target genes *ACC* and *FASN*. Although researchers generally reported that both vitamin D deficiency and fructose intake increased the expression of lipogenesis-related genes in the liver, they reported that the expression levels of these genes were mainly affected by fructose intake (Maia-Ceciliano et al. 2019). In this study, *SREBP-1c* gene expression levels increased less in the FCaD2 group than in the Con group. As with the *SCD-1* gene expression level, the *SREBP-1c* gene expression level was also affected by calcium and vitamin D doses. However, increases in the level of *SREBP-1c* expression in the liver in rats in the FCaD1 group were observed to be greater than in rats in the F group. This outcome observed in the FCaD1 group is thought to have been shaped by epigenetic mechanisms, as in the *ACACA* and *FASN* genes.

CONCLUSION

The results showed that when the rats were exposed to fructose for 28 days, there were no significant changes in body weight. Regardless of calcium and vitamin D doses, it was found that during the first week of the study there was less live body weight gain in rats in the FCaD1 and FCaD2 groups than in the Con group, and this difference persisted during the second and third weeks. Effects of short-term calcium-vitamin D intake on energy intake with the diet have shown that they can vary depending on the type of animal, the duration of feeding, and the differences in calcium and vitamin D doses supplemented by the diet. While the hyperlipidemic effect of fructose has been observed to disappear due to dietary doses of calcium and vitamin D, it has not been found to affect plasma glucose, total cholesterol, HDL and LDL levels. It is believed that changes in the expression levels of *ACACA* and *FASN* genes due to calcium-vitamin D supplementation in high-fructose diets may be affected by epigenetic mechanisms. The level of *SREBP-1c* and *SCD-1* gene expression varied depending on the dose of calcium and vitamin D. It has been determined by the changes in the expression levels of SREBP-1c and SCD-1 genes that dietary treatment with calcium and vitamin D supplementation may be a good option against lipogenesis triggered by high fructose diet. Some genes have shown that calcium and vitamin D supplementation against lipogenesis induced by a high fructose diet can have positive effects in a short time. But it is believed that more and longer-term studies are needed to clarify the mechanisms underlying adequate intake of calcium and vitamin D for their anti-obesity and anti-lipogenic effects.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: AY and SÖK contributed to the project idea, design and execution of the study. AY and SÖK contributed to the acquisition of data. AY and SÖK analysed the data. AY drafted and wrote the manuscript. AY and SÖK reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Hatay Mustafa Kemal University Experimental Research Practice and Research Center. This research was approved by the Hatay Mustafa Kemal University Local Ethics Board for Animal Experiments (HMKÜ-HADYEK) at the meeting of January 28, 2021 (Decision number: 2021/01-07).

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Explanation: This article is summarized by the first author's master thesis

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