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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Fructose Consumption Induces Cell Death Through Endoplasmic Reticulum Stress in Pancreas and Changes Biochemical Parameters in Blood

Fruktoz Tüketimi Pankreasta Endoplazmik Retikulum Stresi Yoluyla Hücre Ölümüne Neden Olur ve Kandaki Biyokimyasal Parametreleri Değiştirir

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Abstract
Fructose is the natural sugar found in fruits. This sugar is widely used in all ready-to-eat foods, especially soft drinks. The present study aims to investigate how fructose consumption affects biochemical parameters in the blood and whether it leads to cell death in pancreatic tissue, triggered by endoplasmic reticulum (ER) stress. Sprague-Dawley rats were divided into a control and a fructose group. The control group (n=7) had free access to tap water and standard pellets, whereas the fructose group (n=7) received 20% fructose in drinking water for eight weeks. Food and fluid intake of the rats were measured daily during the experiment. Lipid levels and total oxidant/antioxidant statuses in blood were analyzed. The expression of glucose-regulated protein-78 (Grp-78), inositol-requiring enzyme-1α (IRE1-α), protein kinase R-like ER kinase (PERK), activating transcription factor (ATF)-4 and -6, CCAAT/enhancer-binding homologous protein (CHOP), and caspase (Cas)-3/-8/-9/-12 mRNA were detected in the pancreas. Consumption of fructose resulted in an increase in serum triglycerides and very low-density lipoprotein levels and in mRNA expression levels of Grp-78, IRE-1α, PERK, ATF-4, -6 and Cas-3/-8/-9/-12 in the pancreas compared with control group. Fructose consumption can lead to disruption of the lipid profile and the balance between oxidants and antioxidants, as well as trigger ER stress, which causes programmed cell death. These alterations can lead to the development of many diseases.
Keywords: Apoptosis, cell death, ER stress, fructose, oxidative stress

Öz
Fruktoz meyvelerde bulunan doğal bir şekerdir. Bu şeker, başta meşrubatlar olmak üzere tüm hazır gıdalarda yaygın olarak kullanılmaktadır. Bu çalışma, fruktoz tüketiminin kandaki biyokimyasal parametreleri nasıl etkilediğini ve pankreas dokusunda endoplazmik retikulum (ER) stres kaynaklı hücre ölümüne neden olup olmadığını araştırmayı amaçlamaktadır. Sprague-Dawley sıçanlar kontrol ve furktoz gruplarına ayrıldı. Kontrol grubu (n=7) standart pellet ve çeşme suyu ile beslendi, fruktoz grubunun (n=7) içme suyuna %20 fruktoz 8 hafta boyunca eklendi. Deney süresince sıçanların günlük yem ve sıvı tüketimleri ölçüldü. Kandaki lipid seviyeleri ve toplam oksidan/antioksidan durumları analiz edildi. Pankreas dokusunda glukozla düzenlenen protein 78 (Grp-78), inozitol gerektiren enzim1α (IRE1-α), protein kinaz R benzeri ER kinaz (PERK), aktive edici transkripsiyon faktörü (ATF)-4 ve -6, C/EBP homolog protein (CHOP), Kaspaz (Cas)-3/-8/-9/-12 mRNA ekspresyon seviyeleri tespit edildi. Kontrol grubu ile karşılaştırıldığında fruktoz tüketimi, serumda trigliserid ve çok düşük yoğunluklu lipoprotein düzeylerini ve pankreasta Grp-78, IRE-1α, PERK, ATF-4, -6 ve Cas-3/-8/-9/-12 mRNA ekspresyon düzeylerini artırdı. Fruktoz tüketimi lipid profilinin ve oksidan/antioksidan dengesinin bozulmasına neden olabileceği gibi ER stresini tetikleyerek programlı hücre ölümüne neden olabilir. Bu değişiklikler birçok hastalığın gelişmesine yol açabilir.
Anahtar Kelimeler: Apoptoz, hücre ölümü, ER stresi, fruktoz, oksidatif stres

I. INTRODUCTION

Lately, the use of high-fructose corn syrups has become widespread due to increase in industrialization and the effects of increase in fructose consumption on human health have begun to be questioned. The hypothesis that fructose-rich foods and beverages delay the feeling of fullness and, cause them to be consumed more, increase the lipogenesis by influencing some regulatory mechanisms such as insulin and leptin hormones and thus induce insulin resistance and obesity-related diseases is discussed [1, 2].

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In healthy fed individuals, small adipocytes in the adipose tissue protect the main metabolic organs such as the liver, pancreas and muscles from excessive lipid burden. On the other hand, the hypertrophy in adipose tissues develops due to excessive triglyceride accumulation from high carbohydrate consumption. As a result, triglyceride and/or other lipid metabolites are distributed to non-adipose tissue such as liver, muscle, pancreas, and interact with insulin signaling pathways [3, 4].

Pancreatic beta cells have primary role in glucose homeostasis associated with insulin secretion. In cases of increased insulin secretion, there is a discrepancy between protein synthesis and protein folding capacity of endoplasmic reticulum (ER) due to impaired insulin secretion in overloaded pancreatic beta cells. Accordingly, incorrectly folded or unfolded proteins accumulate in ER lumen, resulting in ER stress [5-7]. Initially ER-associated degradation (ERAD), an integral part of the ER stress response, removes excess or misfolded intracellular proteins. The ERAD mechanism tries to change environment and reestablish normal ER function. When ERAD fails to maintain ER homeostasis, excessive and prolonged ER stress activates unfolded protein response (UPR). UPR consists of three pathways; Protein kinase R-like ER kinase (PERK), inositol requiring enzyme-1 (IRE-1) and activating transcription factor-6 (ATF-6) signal pathways. When UPR is activated, the capacity of ER and the translation of genes encoding folding enzymes are stimulated to fold unfolded proteins in the ER lumen. If unfolded protein-induced ER stress continues, the cell is directed to programmed cell death [8, 9].

It has been reported that oxidative stress induced by the production of excessive reactive oxygen species (ROS) is one of the most important factors causing the increase of UPR and ER stress by changing the functions of the enzymes that fold proteins, and is a potent inducer of apoptosis called programmed cell death [10, 11]. Studies suggest that fructose causes oxidation of lipid, protein and DNA damage by increasing ROS production and disturbing antioxidant defense mechanisms [12, 13].

This study aims to investigate the effects of fructose consumption on blood biochemical parameters and apoptosis induced by ER stress in rat pancreas. For this purpose, the mRNA transcription levels of ER stress and of ER stress-induced cell death markers in pancreatic tissues, lipid profile in serum, the total amount of oxidant/antioxidant in plasma and the lipid peroxidation levels were determined.

II. MATERIAL AND METHODS

2.1. Experimental Design

This study was performed according to the approval of the Local Ethics Committee of the Animal Experiments of Bezmialem Vakif University. The 8-10 week old male Sprague-Dawley rats were fed ad libitum with a standard pellet diet and tap water. The rats maintained under standard laboratory conditions of 12 h light/12 h dark period at 21 ℃ ± 2 ℃ room temperature. Animals were randomly divided into two groups as control and fructose groups. Control group (n = 7) was given tap water and standard feed for 8 weeks. Fructose group (n = 7) received 20% fructose dissolved in tap water, prepared freshly every day during the 8-week experiment (fructose was consumed orally as daily drinking water). The animals were sacrificed at the end of 8 weeks. The blood and pancreas tissues were removed from the animals. Pancreas tissue samples were fixed in liquid nitrogen and stored at -85 ° C.

2.2. Body Weight Gain (BWG), Food and Water Consumptions

BWG was calculated by taking the weight of body weights measured at the end of the 8th week and the body weights before starting the experiment. The amount of feed and water consumed daily was measured during the experiment.

2.3. Serum Lipid Profile

Total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and triglycerides (TG) were measured with used colorimetric assays using the Roche Cobas c501 (Roche Diagnostics Ltd. Switzerland).

2.4. The Measurement of Total Oxidant/Antioxidant Status (TAS/TOS) in Plasma

The plasma TOS and TAS levels were determined using the Total Oxidant / Antioxidant Status Kit (Rel Assay Diagnostics, Gaziantep, Turkey). The absorbance values of the samples for TOS and TAS were read using UV-1280 UV-VIS spectrophotometer (Shimadzu, Tokyo, Japan) at wavelengths of 530 and 660 nm, respectively.

2.5. Analysis of Lipid Peroxidation

The level of lipid peroxidation was analyzed by the procedure of Ledwozyw et al. [14]. Plasma specimens were mixed with a solution of 30% trichloroacetic acid (T6399, Sigma-Aldrich, USA), 0.75% thiobarbituric acid (TBA, 108180, Merck, Germany) and 5 M HCI. Malondialdehyde (MDA) reacts with TBA to develop a colored product. The color produced in plasma samples was measured at 532 nm by using UV-1280 UV-VIS spectrophotometer (Shimadzu, Tokyo, Japan).

2.6. Quantitative Real-Time PCR (qRT-PCR) Assay

Expressions of mRNAs for the ER stress and apoptosis, GRP-78, PERK, ATF-4, IRE-1α, ATF-6, CHOP, caspase (Cas)-12 and Cas-3/-8/-9 were quantified by qRT-PCR. Total RNA was extracted

from pancreas tissues using the RNA Extraction Kit (Hybrigen, R1051, Turkey) according to the manufacturer protocol. The purity and concentration of RNA were determined by Qubit® 2.0 Fluorometer (Invitrogen, USA). Reverse transcription was conducted by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814, CA, USA) using 1 µg of total RNA per sample. Transcriptional levels of targeted genes were analyzed by qRT-PCR using SYBR Green qPCR Master Mix reagent system. Each run consisted of 95℃ for 5 min followed by 45 cycles of 95℃ for 30 s, 59℃ for 25 s, and 72℃ for 30 s in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Beta actin (β-actin) was used as a housekeeping gene for normalizing the transcription data. The primer designs are presented in table 1. The relative mRNA levels were normalized to β-actin mRNA levels using the Pfafll method [15].

2.7. Statistics

All results were presented as mean ± SEM and median (interquartile, IQR). Data were calculated with the GraphPad Prism 5 computer program. The Shapiro - Wilk test was used to detect whether the data were normally distributed. The comparisons between groups were statistically determined using independent samples t-test and the Mann-Whitney U test. $p < 0.05$ was considered statistically significant.

Table 1. Primers used for the mRNA expression studies.

Genes		Primer Sequence
GRP-78	Glucose-Regulated Protein-78	F 5'-CCG TAA CAA TCA AGG TCT ACG A-3' R 5'-AAG GTG ACT TCA ATC TGG GGT A-3'
PERK	Protein kinase-like Endoplasmic Reticulum Kinase	F 5'-GAT CCG TCT CCC AAA CAG G-3' R 5'-TAG CCA AGG CTT TGA CTT CC-3'
IRE-1α	Inositol-Requiring Enzyme-1α	F 5' CCT GAG GAA TTA CTG GCT TCT C-3' R 5' TCC AGC ATC TTG GTG GAT G-3'
ATF-4	Activating Transcription Factor-4	F 5'-GTT GGT CAG TGC CTC AGA CA-3' R 5'-CAT TCG AAA CAG AGC ATC GA-3'
ATF-6	Activating Transcription Factor-6	F 5'-GGG AGT GAG CTG CAG GTG TA-3' R 5'-TTA TGG GTG GTA GCT GGT AA-3'
CHOP	CCAAT/enhancer-binding Homologous Protein	F 5'-AGC TGG AAG CCT GGT ATG AGG A-3' R 5'-GCT AGG GAT GCA GGG TCA A-3'
Cas-3	Caspase-3	F 5'- ACT GGA AAG CCG AAA CTC TTC-3' R 5'-AGT TCC ACT GTC TGT CTC AAT A-3'
Cas-8	Caspase-8	F 5'- TCT GCT GGG GAT GGC TAC T -3' R 5'- CAT GTT CCT CGG GTT GTC TT -3'
Cas-9	Caspase-9	F 5'- CTC CTG GAG AGA CAA GAA GAG C -3' R 5'- AAA ACA GCC AGG AAT CTG CT -3'
Cas-12	Caspase-12	F 5'-TAA CAA AGG CCC ATG TGG AG-3' R 5'-TCG GAA AAT TTC TTC CAA ATG AT-3'
β-actin	Beta actin	F 5'- CTA AGG CCA ACC GTG AAA AG -3' R 5'- TCT CCG GAG TCC ATC ACA AT -3'

III. RESULTS

3.1. BWG and Food & Fluid Intake

BWG showed a slightly increase in fructose group compared to control group rats. We observed that the consumption of fluid was high in rat with fructose group compared to control group ($p < 0.01$). However, the food consumption in fructose group was low compared to control rats ($p < 0.001$, Table 2).

3.2. The Measurement of Serum Lipids

Serum total cholesterol, VLDL and TG levels significantly increased in fructose group compared to control group ($p < 0.01$ for all). On the contrary, serum HDL and LDL levels were the same between two groups (Table 2)

3.3. Plasma TOS, TAS and MDA Levels

The levels of TOS, TAS and MDA in the blood plasma of control and fructose groups are shown in table 3. TOS levels did not differ between the two groups but the level of TAS was lower in the fructose group than the control group ($p < 0.05$). Also, plasma MDA levels of fructose group increased compared to control rats ($p < 0.05$).

3.4. Quantitative Real-Time PCR Findings

According to our results, transcriptional level of GRP78 mRNA, one of the ER chaperones, increased in the fructose group compared with the control rats ($p < 0.05$). Similarly, the ER stress markers, PERK, ATF-4, IRE-1α and ATF-6 were also expressed at a higher level in the fructose group than in the control group ($p < 0.05$). The increase in IRE-1α mRNA transcription level was higher than the others. CHOP and Cas-12 mRNA levels were high in the fructose group compared to the control group ($p < 0.05$). Cas-3/-8/-9 mRNA levels were significantly higher in the fructose group than the control group ($p < 0.05$), (Figs.1 and 2).

Table 2. Food and liquid consumptions, body weight gain and biochemical parameters of the groups.

		Control	Fructose Group	P value
BWG (g)*	mean ± SEM	123.71 ± 6.35	143.57 ± 7.63	> 0.05
	median (IQR)	125 (120- 134)	145 (125 – 168)	
Food intake (g/day)*	mean ± SEM	22.82 ± 0.60	12.35 ± 0.50	< 0.001
	median (IQR)	22.91 (21.18- 23.17)	11,98 (11.29 – 13.80)	
Water intake (mL/day)*	mean ± SEM	36.80 ± 0.89	44.01 ± 2.00	< 0.01
	median (IQR)	36.41 (35.15 – 38.71)	43.66 (38.69 – 47.01)	
Cholesterol (mg/dL)*	mean ± SEM	70.57 ± 3.12	79.71 ± 2.56	< 0.05
	median (IQR)	73 (61 – 77)	81 (76 – 86)	
HDL (mg/dL)*	mean ± SEM	58.71 ± 2.95	63.85 ± 2.93	> 0.05
	median (IQR)	57 (54 - 66)	60 (60- 72)	
LDL (mg/dL)*	mean ± SEM	18.42 ± 1.58	17.14 ± 0.85	> 0.05
	median (IQR)	18 (16 - 23)	16 (15 -19)	
VLDL (mg/dL)*	mean ± SEM	7.42 ± 0.57	11.29 ± 0.77	< 0.01
	median (IQR)	7 (6 - 9)	12 (9 – 13)	
TG (mg/dL)*	mean ± SEM	36.28 ± 2.79	56.57 ± 3.87	< 0.01
	median (IQR)	35 (30- 43)	61 (43- 65)	

*Data are shown as the mean ± SEM and median (interquartile, IQR); BWG: Body weight gain; HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TG: Trigliserid.

Table 3. The measurements of oxidative stress parameters in plasma of fructose and control groups.

	TAS (mmol/L)*	TOS (µmol/L)*	MDA (µmol/L)*
Control	4.36 ± 1.10	4.21 ± 0.61	5.38 ± 0.23
Fructose Group	2.07 ± 0.86	6.01 ± 1.39	6.36 ± 0.34
P value	< 0.05	> 0.05	< 0.05

*Data are shown as the mean ± SEM; TAS: total antioxidant status; TOS: total oxidant status; MDA: malondialdehyde.

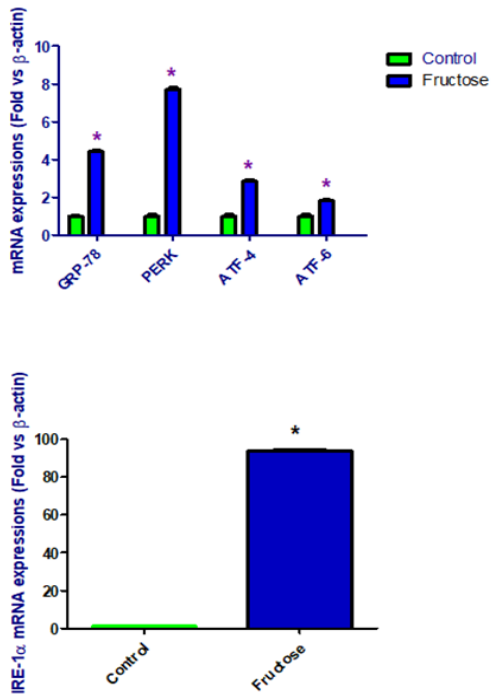

Figure 1. Relative gene expression (Mean ± SEM) data of ER stress markers: Glucose-regulated protein-78 (GRP-78), protein kinase R-like ER kinase (PERK), activating transcription factor (ATF)-4/-6 and, inositol-requiring enzyme-1α (IRE-1α) in pancreas tissues from high- fructose diet and control groups. *$p < 0.05$ compared to the control.

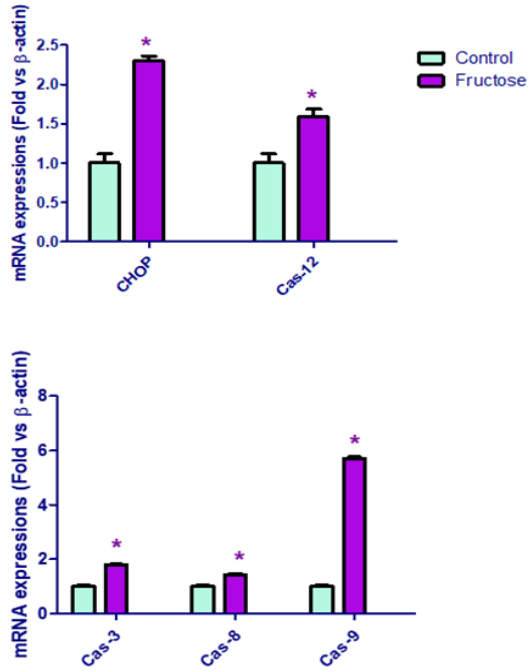

Figure 2. Relative gene expression (Mean ± SEM) data of proapoptotic/apoptotic markers: CCAAT/ enhancer-binding homologous protein (CHOP) and caspase (Cas)-3/-8/-9/-12 in pancreas tissues from high-fructose diet and control groups. *$p < 0.05$ compared to the control.

IV. DISCUSSION

It is reported that chronic fructose consumption induced the increase in body weight due to expanded, hypertrophic visceral white adipose tissue in rats [16]. On the contrary, Ramos et al. [17] showed that fructose consumption in different periods increased liquid intake and reduced food intake but the body weight did not change. Moreover, Oashi et al. [18] found that there was no significant difference in body weight of rats fed high-fructose diet (60% fructose) for 3 or 4 weeks compared to rats fed control diet. Similar to the above studies, food and liquid intake in the fructose-fed rats were inversely altered, while body weights were the same in the present study. The reason for unchanging body weight may be that the amount of calories consumed in the control and fructose groups is almost similar.

When lipid profile changes were examined, serum total cholesterol, TG and VLDL levels were different between the fructose and control groups. Recent studies have shown that high-fructose diet in different times increases serum TG and VLDL levels [19, 20]. Similarly, in our study, 20% fructose application for 8 weeks elevated the TG and VLDL levels. Studies have shown evidences that fructose increases ROS production and disrupts antioxidant defense mechanisms. It is reported that oxidative damage occurred in both lipid and protein components in rats fed a fructose-rich diet for 8 weeks, SOD activity which is one of the enzymatic components of antioxidant defense system decreased and, inflammation and cellular necrosis findings were present [13, 21, 22]. Our findings also showed an increase in TOS levels of fructose group rats but this increase did not reach statistically significant levels. There was a statistically significant increase in TAS levels of this group. These results are consistent with the findings that showing unfolded proteins in the ER lumen increased ROS production and activated UPR as the 2nd messenger and stimulated apoptosis [23]. Thus it was thought us that high-fructose diet may have an important contribution to the development and progression of oxidative stress. In studies, high-fructose diet has been associated with various oxidative stress biomarkers, especially MDA, which is a by-product of lipid peroxidation [24, 25]. Also, Kelany et al. [26] reported that high-fructose diet increased serum MDA levels, and decreased serum catalase levels. Similar to these studies, the increased plasma MDA level in the group given 20% fructose was evaluated as an indicator that fructose induced oxidative stress.

In the literature, there are few studies on the relationship between high-fructose diet and ER stress pathways in pancreas tissue. Studies with mice carrying mutations in the eIF2α phosphorylation site showed a significant relationship between ER stress pathway and pancreatic cell functions. It has been

shown that diabetic phenotype develops shortly after birth due to beta cell death resulting from ER stress in the mutant PERK-/- mice [27, 28]. Akiyamae et al. [29] indicated that XBP1 deficiency in pancreatic α-cells induces altered insulin signaling and dysfunctional glucagon secretion in α-cells created complementary in vivo (α-cell–specific XBP1 knockout mice) and in vitro (stable XBP1 knockdown α-cell line) models. Sundar Rajan et al. [30] showed that ER stress in pancreatic β cells is effective in the varying levels of insulin synthesis and development of diabetes.

Balakumar et al. [31] reported that in the liver and pancreatic tissues of rats fed with combination of high fat diet and high-fructose diet increased the Grp 78, PERK, IRE-1α, CHOP mRNA levels along with other biological markers involved in ER stress. The study demonstrated that high-fructose diet is as detrimental as high-fat diet in triggering of insulin resistance and diabetes. There are studies showing that ATF-6 is also associated with beta cell function. Especially, studies in humans have reported a relationship between ATF-6 variants and type 2 diabetes [32, 33]. It is known that ATF-6 is an important mediator in the cell's response to accumulation of misfolded proteins in ER. Therefore, it is thought that ATF-6 may be involved in the apoptosis of beta cells but there are no direct evidence indicating its exact role in pancreatic beta cell death [34]. In our study, the transcription levels of Grp-78, IRE-1α, PERK, ATF-4, ATF-6 mRNA showed statistically significant increases in fructose group compared with the control group. The increase in IRE-1α mRNA transcription level is higher than the others. It is suggested that IRE-1 was thought to be the most important pathway in UPR associated with ER stress in pancreatic tissue.

CHOP and Cas-12 act as mediators of ER stress and activate apoptotic pathway [35, 36]. In our study, similar to ER stress markers, mRNA transcription levels of CHOP and caspase-12 are important elements of the switch from pro-survival to pro-death signaling, showed statistically significant increases in fructose group compared to the control group. Likewise, Balakumar et al. [31] showed that CHOP mRNA levels increased in ER stress induced apoptosis in high-fat/fructose diet fed rats. Chen et al. [37] demonstrated that signaling switches from pro-survival to pro-apoptotic due to increasing in ER stress and the apoptotic response is initiated by caspase-12 in diabetic rats. Furthermore, Cas-9 is known as initiator, whereas Cas-3 and -8 are known as executioner in caspases cascade of apoptosis [38]. Similar to ER stress markers, the levels of Cas-3/-8 and -9 mRNA were also increased in pancreas of fructose group rats in accordance with reported data. Cheng et al. [39] showed that Cas-3/-8 and -9 protein expression levels increased in rats applied high-fructose diet. Kalra et al. [40] reported that Cas-3 expression increased in rat renal epithelial cell lineage cultured in high-fructose. Thus, it is thought that apoptosis may be induced with increased ER stress in the pancreas of rats fed with a high-fructose diet.

In conclusion, we can say that fructose consumption can decrease the level of antioxidant, which can cause the destruction of free radicals in metabolic reactions, and increase the level of MDA as a lipid peroxidation marker. In addition, fructose consumption induces ER stress by increasing the expression of PERK/ATF-4 and -6 and especially IRE-1, the major signaling pathway in ER stress-related UPR. Thus, the increase in ER stress in pancreatic tissue may also trigger increased expression levels of Cas mRNAs, which initiates programmed cell death. However, these results need to be supported by future experiments.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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