Endothelial dysfunction in high fructose containing diet fed rats: Increased nitric oxide and decreased endothelin-1 levels in liver tissue

Yüksek fruktoz içeren diyete beslenen ratlarda endotel disfonksiyonu: Karaciğer dokusunda artmış nitrik oksit ve azalmış endotelin-1 düzeyleri

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ABSTRACT

Objectives: Dietary high fructose consumption which is closely associated with endothelial dysfunction via insulin resistance has recently increased in developed countries. Insulin resistance has a promoter effect on many metabolic disorders such as syndrome X, polycystic ovary syndrome, Type 2 diabetes mellitus etc. Our aim in this study is to understand the impact of increased fructose intake on metabolisms of glucose, insulin and endothelial dysfunction by measuring nitric oxide (NO) and endothelin-1 (ET-1) levels in hepatic tissue which is crucial in fructose metabolism.

Materials and Methods: We designed an animal study to understand increased fructose intake on hepatic endothelium. Twenty adult male albino rats were divided into two groups; the study group (group 1, n=10) received isocaloric fructose enriched diet (fructose-fed rats, containing 18.3% protein, 60.3% fructose and 5.2% fat) while the control group received purified regular chow (group 2, n=10) for 2 weeks. After feeding period, blood and hepatic tissue samples were collected and glucose, insulin, NO and ET-1 levels were analysed.

Results: We found increased fasting glucose and insulin levels and impaired glucose tolerance in fructose fed rats. Higher NO and lower ET-1 levels were also detected in hepatic tissue samples of the group 1.

Conclusion: Increased fructose consumption has deleterious effects on glucose tolerance, insulin resistance and may cause to endothelial dysfunction.

Key words: Fructose consumption, endothelial dysfunction, nitric oxide, endothelin-1, liver.

ÖZET

Amaç: Gelişmiş ülkelerdeki diyetlerde fruktozun tüketim sıklığı son yıllarda giderek artmaktadır. Artan fruktoz tüketimi İnsülin rezistansı oluşturarak endotel disfonksiyonuna yol açabilir. İnsülin rezistansı, sendrom X, polistik over sendromu, tip 2 diabet gibi birçok metabolik bozukluğun patogenezinde altta yatan etkendir. Çalışmamızın amacı, artmış fruktoz tüketiminin, glukoz, insülin düzeylerine ve ayrıca metabolizmada kritik önemde sahip olan karaciğer dokusundaki endotel fonksiyonlarını olan etkilerini, nitrik oksit (NO) ve endotelin–1 (ET–1) ölçüçerek değerlendirmektir.

Gereç ve yöntem: Çalışmamızda 20 erkek sıçan iki gruba ayrıldı. Çalışma grubu (grup 1, n=10) fruktozdan zenginleştirilmiş izokalorik diyet ile (içeriği: %18.3 protein, %60.3 fruktoz ve %5.2 yağ) kontrol grubu ise (grup 2, n=10) purifiye normal besin ile 2 hafta süresince beslendi. Beslenme periyodu sonrasi kan ve hepatik doku örnekleri alınarak glukoz, insülin, NO ve ET–1 düzeyleri analiz edildi.

Bulgular: Glukoz toleransını bozuantaki glukoz tüketim sıklığını arttıran fruktoz içeren diyete beslenen sıçanlarda, normalde artmış açlık glukozu, azalmış insülin düzeyleri ve bozulmuş glukoz toleransı izlendi. Grup 1 karaciğer dokusunda ise artmış NO ve düşük ET–1 düzeyleri saptanmıştır.

Sonuç: Artmış fruktoz tüketimi bozmandaki glukoz toleransını bozmaktadır ve insülin rezistansını oluşturur. Insülin rezistansı karaciğer dokusunda endotel disfonksiyonuna yol açabilir.

Anahtar kelimeler: Fruktoz tüketimi, endotel disfonksiyonu, nitrik oksit, endotelin–1, karaciğer.
INTRODUCTION

More than 50% of energy must be provided from carbohydrates in adults on regular diet. Fructose is typically consumed as sucrose (table sugar), a disaccharide composed of equal parts of fructose and glucose, or as a component of high-fructose corn syrup (HFCS, which is used to sweeten most cola drinks). High-fructose corn syrup is a source of sucrose and the overconsumption of HFCS is related to obesity. Varying concentrations of free fructose and free glucose are used to obtain HFCS. According to US Department of Agriculture data, inclusion of fructose ratio in HFCS varies between 42-55%.

Fructose is sweeter than glucose (over than 2-fold) and it is transformed into lipids in the fastest pathway among all carbohydrates and therefore blamed for serious atherogenic effect. Fructose does not have insulin releasing effect and does not need insulin for its metabolism. An increase in fructose consumption over the past 10-20 years has been linked with a rise in the prevalence of obesity and metabolic disorders. Consumption of a high-fructose diet promotes development of three of the pathological characteristics associated with metabolic syndrome: visceral adiposity, dyslipidemia, and insulin resistance. Insulin resistance is not only an early and major feature in development of non-insulin-dependent diabetes mellitus (NIDDM), but also associated with hyperlipidemia, hypertension, obesity, enhanced oxidative stress, endothelial dysfunction and cardiovascular disease, the so-called ‘insulin-resistance syndrome (syndrome X, metabolic syndrome). Metabolic syndrome is closely associated with endothelial dysfunction. Although endothelial dysfunction occurs in many different disease processes, oxidative stress can be identified as a common denominator. Nitric oxide (NO), superoxide radical (O₂⁻), hydroxyl radical (•OH), hydrogen peroxidase (H₂O₂), and peroxynitrite (ONOO⁻) are produced in the vascular bed under both normal and stress conditions such as inflammation or injury. When NO and O₂⁻ are produced in close vicinity, they interact to form ONOO⁻. Although neither NO nor superoxide is a strong oxidant, peroxynitrite is a potent and versatile oxidant that can attack a wide range of biological targets. Reduced bioavailability of NO, an alteration in the production of prostanooids, including prostacyclin, thromboxane A₂, an impairment of endothelium-depend hyperpolarization, as well as an increased release of endothelin-1, can individually or in combination contribute to endothelial dysfunction.

Our aim in this study is to understand the impact of increased fructose intake on metabolisms of glucose, insulin and endothelial dysfunction by measuring NO and endothelin-1 levels in hepatic tissue which is crucial in fructose metabolism.

MATERIALS AND METHODS

The study was approved by the Experimental Animal Ethics Committee of the Celal Bayar University Hospital (2005/3). After 1-week acclimatization period, twenty adult male albino rats with an average weight of 205±24 grams were randomly divided into two groups. The study group (group 1, n=10) received isocaloric fructose enriched diet (fructose-fed rats, containing 18.3% protein, 60.3% fructose and 5.2% fat) while the control group received purified regular chow (group 2, n=10) for 2 weeks. After at least 10 hours fasting period, blood was withdrawn from tail veins to measure fasting glucose and insulin levels. Then the rats were given 30% glucose at 1.7 mg/kg dose, and 30 minutes later blood samples were obtained to measure glucose and insulin levels. After collecting blood samples, rats were sacrificed and hepatectomy was performed. Hepatic tissues were washed with cold saline solution, placed into clean glass tubes, labeled and stored in a deep freeze at -70°C until the day of measurement.

Plasma glucose levels were detected by photometric method using an automatic analyser (Beckman Coulter DxC 800), plasma insulin levels were measured by RIA method using a commercial kit (DSL–1600).

Hepatic tissues were homogenised at 16000 rpm in four volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) using a homogeniser (for 2 min at 5000 rpm). The homogenate was then centrifuged at 5000 g for 60 min to remove debris. Clear upper supernatant fluid was taken and NO and endothelin–1 levels were carried out in this stage. Protein assays were made by the method of Lowry.

Since plasma nitrite (NO₃⁻) and nitrate (NO₂⁻) levels can be used to estimate NO production, we measured the concentrations of these stable NO oxidative metabolites. Determination of NO₂⁻ and NO₃⁻ was made by the method of Zielke.
NO$_2^-$ was based on the Griess reaction, in which a cromophore with a strong absorbance at 545 nm is formed by reaction of NO$_2^-$ with a mixture of naphthylethlenediamine and sulfanilamide. After samples were deproteinized with Somogyi reagent, an aliquot of the sample was mixed with fresh reagent. After 40 min incubation time the absorbance was measured in a spectrophotometer (Shimadzu UV-1201, Japan) to give the NO$_2^-$ concentration. A second aliquot was treated with copper-coated cadmium granules (Cd) in glycine buffer at pH 9.7 (2.5–3 g Cd granules for a 4-ml reaction mixture) to reduce NO$_3^-$ to NO$_2^-$ as $\text{NO}_3^-$ is reduced to $\text{NO}_2^-$ in this aliquot thus gave the total NO$_3^-$ plus NO$_2^-$, finally representing total NO concentration. A standard curve was established with a set of serial dilutions (100–5 mmol/L) of NaNO$_2$. The resulting equation was then used to calculate the unknown sample concentrations.

Endothelin–1 levels were detected by enzyme-linked immunosorbent assay (ELISA) method using a commercial kit from Cayman Chemical Company (Cat. no: 583151) and were carried out in duplicate; the mean of the two measurements gave the final result.

Statistical Package for the Social Sciences (SPSS) for Windows, Version 10.0 was used to statistical analysis of data. Mean values were expressed as x ± SD. For the differences between groups, nonparametric test; Kruskal–Wallis analysis of variance (Mann–Whitney test as a post hoc test) were applied. A p value of less than 0.05 was accepted as statistically significant.

RESULTS
After 2 weeks of diet, fructose fed rats (group 1) had hyperinsulinemia and exaggerated response to glucose overload (Table 1). Plasma fasting glucose and insulin levels were significantly higher in group 1 at both 0' and 30' (p=0.001, 0.02, 0.004 and 0.008 respectively). Mean endothelin–1 and NO levels in hepatic tissue are seen in Table 1. We found significantly higher NO (p=0.04) and lower endothelin–1 levels (p=0.028) in group 1.

Table 1. Plasma glucose and insulin levels (x ± SD) at 0' and after oral glucose loading (30’) and liver tissue NO and ET-1 levels in Group 1 and Group 2.

<table>
<thead>
<tr>
<th>Time</th>
<th>Group 1 (n=10)</th>
<th>Group 2 (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0. min</td>
<td>86.87±12.33*</td>
<td>50.00±6.63</td>
<td>0.001</td>
</tr>
<tr>
<td>30. min</td>
<td>120.25±7.06*</td>
<td>109.66±9.93</td>
<td>0.020</td>
</tr>
<tr>
<td>Insulin (µIU/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0. min</td>
<td>35.10±6.92*</td>
<td>19.65±4.31</td>
<td>0.004</td>
</tr>
<tr>
<td>30. min</td>
<td>136.32±12.27*</td>
<td>87.90±9.77</td>
<td>0.008</td>
</tr>
<tr>
<td>NO (µmol/g tissue protein)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1.93 ± 1.03*</td>
<td>0.99 ± 0.31</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>ET-1 (pg/g tissue protein)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1.78 ± 0.40*</td>
<td>2.43 ± 0.55</td>
<td>0.028</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION
Insulin resistance is usually characterized by higher fasting and post-glucose loading insulin levels, and a decreased responsiveness of tissue to the insulin driven clearance of this glucose from the bloodstream. It seems to be a common feature and a possible contributing factor to several frequent health problems, including Type 2 diabetes mellitus, polycystic ovary syndrome, dyslipidemia, hypertension, cardiovascular disease, sleep apnea, certain hormone-sensitive cancers and obesity. According to results of Steinberg et al., endothelium dependent vasodilatation is reduced by 30-40% in obese humans compared to lean control group and they suggested that these patients are subject to endothelial dysfunction which may be related to insulin resistance.

Recent studies have shown that a high intake of refined carbohydrates may contribute to the risk of developing insulin resistance. In animal models, diets high in fructose have specifically been con-
Contribute to a metabolic disturbance leading to insulin resistance.\textsuperscript{12} Unfortunately, fructose consumption rate has increased year after year in humans. Until recently, humans consumed fructose amounting to 16–20 grams per day, largely from fresh fruits. Westernization of diets has resulted in significant increases in added fructose, leading to typical daily consumptions amounting to 85–100 grams of fructose per day. Indeed, one out of every four children in the United States consumes above the recommended 25% of total energy intake from sweeteners. Exposure of the liver to such large quantities of fructose leads to rapid stimulation of lipogenesis and triglyceride (TG) accumulation, which in turn contributes to reduced insulin sensitivity and hepatic insulin resistance/glucose intolerance.\textsuperscript{13}

\textbf{Figure 1.} The relationship between fructose metabolism and insulin resistance

\[ \text{FRUCTOSE} \]

\[ \text{fructokinase} \]

\[ \text{ATP} \rightarrow \text{ADP} \]

\[ \text{Fructose 1-P} \]

\[ \text{aldolase B 1-P aldolase} \]

\[ \text{Glyceraldehyde} \]

\[ \text{Dihydroxyacetone phosphate 3} \]

\[ \text{trio kinase} \]

\[ \text{ATP} \rightarrow \text{ADP} \]

\[ \text{Glyceraldehyde 3-P} \]

\[ \text{glycerol dehydrogenase} \]

\[ \text{Glycerol} \]

\[ \text{glycerol dehydrogenase} \]

\[ \text{Glycerol 3-P} \]

\[ \text{glycerokinase} \]

\[ \text{Pyruvate} \]

\[ \text{Acetyl CoA} \]

\[ \text{Acyl CoA esterification} \]

\[ \text{TRIGLYCERIDE}^{\uparrow} \]

\[ \text{LIPOGENESIS}^{\uparrow} \]

\[ \text{INSULIN RESISTANCE} \]
The relationship between fructose metabolism and insulin resistance in hepatic tissue is summarized in Figure 1 (reviewed 13,14,15).

Many studies have shown that plasma TGs increased significantly in fructose fed rats whereas plasma glucose and insulin levels were controversial from controls.16 In this study, we observed to glucose and insulin levels in fasting plasma and responses to oral glucose load were significantly greater in fructose-fed than in control rats. Increased TG levels can be harmful on liver cells. According to Porikos 17 study on healthy non-obese males who consumed a diet containing 20–35 percent of calories as sucrose, 3 of 11 participants (27%) developed markedly increased levels of alanine aminotransferase (4.33-9.22 times the upper limit of normal) and moderate increases in aspartate aminotransferase (1.04-3.64 times the upper limit of normal), changes suggestive of liver injury. Additional evidence that fructose can cause liver damage is that intravenous administration of fructose (250 mg/kg of body weight over five minutes) to healthy volunteers resulted in 75% reduction in the hepatic concentration of adenosine triphosphate (ATP) within 10 minutes. Sixty minutes after fructose administration, the ATP concentration still decreased by about 40% compared with baseline.18 The possible reason for this untoward result is overwhelming the capacity of aldolase B in fructose metabolism pathway due to ingestion of large amounts of fructose. In fact, hereditary fructose intolerance (absence of aldolase B) leads to intracellular trapping of fructose-1-P, accumulation in liver cells and eventually ATP and inorganic phosphate levels decrease. Decreased availability of hepatic ATP impairs gluconeogenesis (causing hypoglycemia), and protein synthesis (causing a decrease in blood clotting factors and other essential proteins).

Fructose overload has been shown to increase oxidative stress. Delbosc19 et al., found that high fructose feeding was also associated with an early (1-week) increase in reactive oxygen species (ROS) production and they have suggested that the production of ROS can be a key-event in the initiation and development of cardiovascular complications associated with insulin resistance. Some authors also have reported that high fructose diet (HFD) in rats led to insulin resistance and a defect in the free radical defence system.20,21 These results indicate that consumption of HFD (also sucrose) negatively affects the balance of free radical production and antioxidant defence, leading to increased lipid susceptibility to peroxidation. Fructose overload dependent insulin resistance and increased free radicals may result in unfavourable effects on endothelial cells. We found increased NO and decreased endothelin-1 levels in hepatic tissues of fructose overload rats. High fructose diet may responsible that increased NO levels in the hepatic tissue and also some other tissues, such as kidney, heart, aorta etc. Cosenzi et al.,22 have demonstrated increased urinary NO excretion in high fructose fed rats and suggested a role for NO in the pathogenesis of the early renal changes induced by HFD. In another study on female rats, decreased heart superoxide dismutase activity and 3-fold increase in plasma NO concentration were reported in fructose fed group compared with starch-fed females.23 In this study, they suggested that estrogen protected female rats against the pro-oxidant effect of high sucrose diet. Furthermore, while serum nitrite/nitrate (NO) levels did not significantly differ between the fructose-fed and control groups, NO levels in the aorta significantly increased. Our study indicates that plasma and tissue NO levels may differ. ET-1 mRNA expression in the aorta increased 195% in fructose-fed rats and the authors suggested that increased expression of vascular ET-1 might be causally related to the development of hypertension in fructose-fed rats.24 Catena et al.,25 have found increased systolic blood pressure (BP) in fructose fed rats (control, 151±4 mmHg; fructose-fed, 179±10 mmHg; p<0.05) and they suggest, fructose-enriched diets induces an increase in BP that is associated with insulin resistance. Decreased endothelin levels found in our study, may reply for increased BP in hepatic tissues. However, kept in mind that endothelin levels may differ at other tissues especially aorta.

We consider that the changes in NO and endothelin levels may contribute to amelioration of tissue endothel dysfunction by decreasing insulin resistance at the target tissue and also manage the BP. A recent study, gene therapy with pcDNA3.1-human eNOS decreased fructose-induced hypertension and insulin resistance in rats.26 According to authors, eNOS gene therapy may be important in the treatment of hypertension and insulin resistance. However, increased NO levels may also help prevent or delay the occurrence of atherogenic cardiovascular diseases associated with insulin-resistant.
states. It should be kept in mind that increased free radicals, especially superoxide anion, may reduce NO bioavailability. Superoxide radicals cannot be dismutated in the absence of Cu-Zn SOD enzyme and these increased radicals can interact with NO to form peroxynitrite radicals eventually leading to aggravation of cellular injury via membrane damage.20-21 However, no reduction of Mn-SOD, GPx and catalase levels found by Busserolles et al. 20 in fructose fed rats may protect probable mitochondrial damage.

In conclusion, increased fructose consumption has beyond doubt deleterious effects such as impaired glucose tolerance, insulin resistance, hyperlipidemia, oxidative stress and endothelial dysfunction. Frequent fructose intake may induce endothelial dysfunction leading to progressive hepatic injury. Increasing of NO levels may help to improve these deleterious effects. Keeping dietary fructose ratio in an optimum range and addition of some antioxidant agents in the western diets may be effective in prevention of cellular damage.

REFERENCES