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The effects of melatonin on brain nitrosative stress and energy balance in fructose-mediated metabolic syndrome model

Fruktoz-aracılı metabolik sendrom modelinde melatoninin beyinde nitrozatif stres ve enerji dengesi üzerine etkisi

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Abstract: Objective: Metabolic syndrome (MetS), one of the common health problems seen with increasing frequency in today’s modern societies, is also an important risk factor for neurological disorders such as stroke, depression, Alzheimer’s disease. On the other hand, melatonin is a neurohormone, has potent antioxidant and neuroprotective activities. In the present study, we aimed to investigate the possible protective effects of melatonin administration on brain tissue in fructose-mediated MetS model.

Methods: Male adult Sprague-Dawley rats were randomly divided into four groups (n=8); control, fructose, melatonin and fructose plus melatonin. MetS was induced by fructose solution 20% in tap water, and melatonin was administered at the dose of 20 mg/kg bw/day by oral gavage. Systolic blood pressures (SBP) were measured by tail-cuff method. After the experimental period of 8 weeks, serum triglyceride, glucose, insulin, and tissue ATP/ADP ratio, nitric oxide (NOx) and 3-nitrotyrosine (3-NT) levels were measured. Also tissue endothelial and inducible nitric oxide synthase (eNOS and iNOS) protein levels were determined.

Results: Fructose consumption increased SBP, serum triglyceride, insulin levels and induced insulin resistance significantly compared to control group and MetS model was successfully demonstrated. In comparison with control group, fructose administration did not cause significant changes in tissue ATP/ADP ratio and 3-NT levels. NOx levels did not change significantly among groups, and iNOS-eNOS proteins were not detected in any groups. Interestingly, tissue 3-NT levels were elevated significantly while ATP/ADP ratio was diminished in fructose plus melatonin group compare with both control and fructose groups.

Conclusion: These results indicate that high fructose diet for 8 weeks does not influence nitric oxide production, energy metabolism and protein nitration in brain. Nevertheless melatonin acted as a pro-oxidant at that dose when administered with fructose.

Keywords: Fructose, metabolic syndrome, melatonin, brain, energy

Özet: Amaç: Metabolik Sendrom (MetS), bugünün modern toplumlarında yüksek görülme şıklığına sahip, yaygın sağlık problemlerinden biri olup; felç, depresyon ve Alzheimer hastalığı gibi nörolojik bozukluklar için de önemli bir risk faktörü oluşturmaktadır. Diğer taraftan, melatoninin güçlü antioksidan ve nöron koruyucu özelliğine sahip bir nörohormon olduğu bilinmektedir. Bu çalışmada, fruktoz-aracılığıyla MetS oluşturulan sıçanlarda, melatonin uygulanmasının beyin dokusunda olması koruyucu etkilerini araştırılmıştır.

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Metod: Yetişkin erkek Sprague-Dawley sıçanlar kontrol, fruktoz, melatonin ve fruktoz+melatonin olmak üzere rastgele 4 gruba ayrıldı (n=8). MetS, içme suyu içerisinde %20 oranında fruktoz verilerek oluşturuldu ve melatonin 20 mg/kg/gün dozunda oral gavaj yoluyla uygulandı. Sistolik kan basıncı (SKB) kuyruktan kan basıncı ölçüm yöntemi ile belirlendi. Sekiz haftalık deney periyodu sonunda, serum triglisiter, glukoz ve insülin seviyeleri ile doku ATP/ADP oranı, nitrik oksit (NOx), 3-nitrotirozin (3-NT) seviyeleri ölçüldü. Ayrıca, dokulardaki endotel-yal ve indüklenebilir nitrik oksit sentaz (eNOS ve iNOS) protein düzeyleri belirlendi.

Bulgular: Kontrol grubuyla karşılaştırıldığında, fruktoz grubunda SKB, serum trigliserit, insülin düzeylerinin ve insülin direncinin belirgin şekilde arttığı belirlendi. MetS modelinin başarılı oluştuğu görüldü. Fruktoz uygulanan grupta, doku ATP/ADP oranı ve 3-NT düzeylerinde kontrol grubuna göre anlamlı bir farklılık bulunmamıştır. Doku NOx seviyeleri tüm gruplar arasında belirgin bir farklılık göstermemiştir; dokularda iNOS ve eNOS proteinleri saptanamamıştır. Fruktoz+melatonin grubunda ise kontrol ve fruktoz gruplarıyla karşılaştırıldığında, ilginc bir şekilde 3-NT düzeyleri artarken, ATP/ADP oranı belirgin şekilde azalmıştır.

Sonuç: Bu sonuçlar, 8 haftalık yüksek fruktoz diyetinin beyn dokusunda nitrik oksit üretimi, enerji metabolizması ve protein nitrasyonu üzerinde henüz bir etkisi olmadığını göstermiştir. Melatonin ise, beklenenin aksine, bu doza ve fruktozla birlikte uygulandığında pro-oksidan etki göstermediği bulunmuştur.

Anahtar Kelimeler: Fruktoz, metabolik sendrom, melatonin, beyn, enerji

Introduction

Of the several characteristics of metabolic syndrome (MetS), at least three should be present for it diagnosis, obesity, in conjunction with type 2 diabetes, hypertension, coronary artery diseases and dyslipidemia are important features of MetS which is usually associated with hyperinsulinemia and insulin resistance [1]. Nowadays, it is known that one of the main reasons of increasing incidence of MetS is high fructose consumption in diet [2]. While fructose is also a simple sugar, like glucose, there are emerging data from many animal and human studies showing fructose is highly lipogenic and plays important roles in the development of MetS and related complications [3].

MetS is a important risk factor for neurological disorders. The molecular mechanism underlying the mirror relationship between MetS and neurological disorders is not fully understood. However, it is becoming increasingly evident that all cellular and biochemical alterations observed in MetS like impairment of endothelial cell function, abnormality in essential fatty acid metabolism and alterations in lipid mediators along with abnormal insulin/leptin signaling may represent a pathological bridge between MetS and neurological disorders [4]. The results indicate that insulin-resistant rat model manifested impaired learning and memory ability which was associated with fructose-drinking. In addition, insulin resistance can result in oxidative/nitrosative stress, leading to neuronal degeneration. Reactive oxygen and nitrogen species (RONS) production were found significantly increased in the brain of fructose-fed rats. It has been indicated that fructose provokes oxidative stress in the brain which induces oxidation of lipids and proteins [5]. On the other hand, it was reported higher levels of fructose could change brain energy metabolism and fructose can induce neurodegeneration in brain, particularly hippocampus-dependent cognitive function is impaired in animal models [6,7].

Melatonin (N-acetyl-5-methoxytryptamine) is the hormone secreted mainly by the pineal gland and it is involved physiological functions in humans and animals having antiexcitatory, antioxidant, antiinflammatory, immunomodulatory and vasomotor effects [8]. Data obtained from animal models suggest that melatonin may improve glucose homeostasis by restoring the vascular action of insulin [9]. It demonstrated that the existence of an additional intracellular mechanism stimulated by melatonin in the rat hypothalamus, intracellular relationship between melatonin and insulin signaling may have a role in the intracellular mechanism controlling body weight, feeding behaviour and blood glucose levels [10].

Numerous in vivo and in vitro studies have demonstrated the capacity of melatonin to encounter oxidative stress and inflammation in brain in a dose-dependent manner [11-13]. Little is known about the precise mechanism of melatonin on fructose-induced MetS model in brain tissue depending on the nitrosative stress and ATP production. The aim of this study was to investigate possible melatonin effect on brain 3-nitrotyrosine (3-NT) and nitrate plus nitrite (NOx) levels as nitrosative stress markers, and ATP/ADP ratio for energy balance in high fructose-fed rats.
Materials and Methods

Chemicals

Melatonin (≥98%) and d-Fructose (≥99%) were purchased from Sigma-Aldrich (St. Louis MO, USA). Primary and secondary antibodies for western blotting were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals used were of the highest analytical grade, and purchased from Merck or Sigma-Aldrich.

Animals and Experimental Design

This study was carried out in accordance with the regulations of Animal Experimentation Ethics Committee of Gazi University (G.Ü.ET-12.006/17.01.2012). Thirty-two adult male Sprague-Dawley rats weighing 225±10 g were obtained from Laboratory Animal Husbandry and Experimental Research Center at Gazi University. The animals were housed at 20-24°C with a 12-h light/12-h dark cycle and they were provided with standard rat chow and tap water freely available.

Thirty-two rats were randomly divided into four groups (n=8) as follows:
1. Control Group: Rats received standard rodent diet and tap water.
2. Fructose Group: Rats received standard rodent diet and tap water supplemented with 20% fructose [14,15].
3. Melatonin Group: Rats received standard rodent diet and tap water, and melatonin administered at the dose of 20 mg/kg body weight in 0.1% ethanol solution per day by oral gavage [16]. Melatonin solution was prepared freshly every day.
4. Fructose plus Melatonin Group: Rats received standard rodent diet and tap water supplemented with 20% fructose, and melatonin administered at the dose of 20 mg/kg body weight in 0.1% ethanol solution per day by oral gavage.

Since ethanol was used as a melatonin’s vehicle, control and fructose groups received 0.1% ethanol solution proportionately with body weight. The experiment was carried out for 8 weeks and at the end of the 8th week, the animals were sacrificed under ketamine (30 mg/kg bw) and xylazine (6 mg/kg bw) anesthesia. Blood samples for laboratory assays were drawn intracardiacally and sera were separated by centrifugation. Sera and brain tissues were stored at -80°C until analysis.

Systolic Blood Pressure and Serum Analysis

Systolic blood pressures (SBP) were measured by tail-cuff method at the beginning of the study, at the end of the 4th week and at the end of the 8th week. All animals were preconditioned for blood pressure measurements 1 week before each experiment. At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the SBP value.

Serum glucose and triglyceride levels were measured by enzymatic methods using autoanalyzers. Serum insulin level was estimated by using commercially available ELISA kit (Millipore). Insulin resistance was evaluated by the Homeostasis Model Assessment index (HOMA-IR) using the formula: [insulin (mU/L) x glucose (mmol/L)]/22.5.

Measurement of Tissue NOx and Protein Levels

Tissue NOx (nitrite plus nitrate), which are known to be the end products of NO, was measured by using commercially available colorimetric kit (Cayman Chemical). Tissue total protein concentration was determined by the BCA protein assay kit (Thermo Fisher Scientific).

Measurement of Tyrosine Nitration (3-NT)

Tissue homogenates were prepared according to the method described by Kamisaki et al. [17]. Briefly, 0.25 g total brain tissue was homogenized in 50 mM potassium phosphate buffer (pH 7.2) and then centrifuged for 5 min at 1,000 g. Following the acid hydrolysis of the precipitate with 6 M HCl, it was evaporated under nitrogen gas. After 1 ml distilled water was added, 10 µl of the sample was auto-injected a Microtech Scientific C18 analytical column (particle size 5 μm, 50 x 1 mm). All samples were analysed by HPLC with electrochemical detector (ECD) using the method described by Maruyama et al. [18]. Mobil phase was 50 mM H₃PO₄, 50 mM citric acid, 40 mg/L EDTA, 100 mg/L octane sulfonic acid and 5% methanol (v/v) (pH 3.1 with KOH). The flow rate was 0.05 ml/min. ECD was set at -850/600 mV. Tissue 3-NT content was calculated from a 3-NT standard curve (3.125, 6.25, 12.5, 25 and 50 μmol/L) and expressed as nmol/mg protein.

Measurement of ATP-ADP Levels

Measurement of ATP and ADP levels was accomplished by HPLC, using the method described by Szabo et al. [19].
Briefly, total brain tissue (0.15 g) in 1.5 ml ice-cold 0.6 N perchloric acid was homogenized and placed on ice for 1 h, followed by neutralization with 1M of K$_2$HPO$_4$, then centrifugation for 15 min at 10,000 g at 4°C, and filtration through a 0.2 mm syringe filter. ATP and ADP were measured by the HPLC diode array detector at a wavelength of 254 nm. The analytical column was 4.6x250 mm (Allosphere ODS-2, C18 5 µm reverse-phase column). The mobile phase was 160 mM KH$_2$PO$_4$ with 100 mM KCl at pH 6.5. The flow rate was 1 ml/min. ATP and ADP peaks were identified according to the corresponding retention times and confirmed by 'spiking' with added exogenous ATP and ADP. Concentrations of ATP and ADP were calculated from the standard curve and expressed as µmol/g tissue. Results were also given as ATP/ADP ratio.

Western Blotting Assay for eNOS and iNOS

Total brain tissues were homogenized with ice cold RIPA buffer (containing protease inhibitors cocktail and EDTA), and placed on ice for 30 min, then centrifugation for 30 min at 10,000 g at 4°C. Supernatants were stored at -80°C until analysis. Immunochemical analyses against eNOS and iNOS proteins were performed wherein 20 µg protein of brain samples were separated with SDS-PAGE (8%). Initially, samples were placed into 2×loading buffer and boiled for 5 min. Electrophoresis was performed using a Bio-Rad Mini Protean tetra cell gel apparatus at 150 V for 1h. The separated proteins were then transferred from the gels onto a polyvinylidene difluoride (PVDF) membrane (200 mA) for 2 h, at 4°C. PVDF membranes were then blocked with 5% nonfat dry milk in TBST (Tris-buffered saline containing 0.1% Tween 20, pH 7.2) for 2 h. After washing for 25 min (five times for 5 min) with TBST, the membranes were incubated overnight at 4°C with the primary antibodies against either rat eNOS (1:1000) or rat iNOS (1:1000). The membranes were washed for 25 min (five times for 5 min) with TBST and incubated with the appropriate HRP-conjugated secondary antibody (anti-rabbit (1:5000)) for 1 h at 25°C. Blots were developed using enhanced chemiluminescence detection (Thermo Scientific). Band intensities were quantified using UVP Bioimaging System software and normalized to the quantity of β-actin (1:1000) in each sample lane. For sequential reprobing of the same blots, the membranes were stripped of the initial primary and secondary antibodies and subjected to immunoblotting with another target antibody. iNOS electrophoresis standard and rat aorta tissue were used as positive controls for iNOS and eNOS respectively.

Statistical Analysis

All data were expressed as mean ± standard deviation. The statistical analyses of the results were performed using a computerized statistical package (SPSS 16.0 for windows, Chicago, IL, USA). Each mean value was compared by one-way analysis of variance (ANOVA) and Tukey for multiple comparisons. All statistical tests were two-tailed, and p<0.05 was considered statistically significant.

Results

SBP and Serum Parameters

In comparison with control group, fructose administration caused significant increase in SBP, serum triglycerides, insulin levels and induced insulin resistance. Thus, MetS model was successfully demonstrated (Table 1).

Tissue NOx, eNOS and iNOS Protein Levels

As seen in Table 2, NOx levels did not change significantly among groups. iNOS and eNOS proteins were not detected in any groups (Figure 1). Brain tissue NO production was not affected by fructose or melatonin.
Tissue 3-Nitrotyrosine, ATP and ADP Levels

Tissue 3-NT levels are shown in Table 2. Fructose administration did not cause significantly changes in 3-NT levels compare to control group. In melatonin group, 3-NT levels decreased significantly compared to control group (p<0.05). In fructose plus melatonin group 3-NT levels significantly increased compared to the other groups (p<0.05).

As shown in Table 3, a significant difference between ATP, ADP levels, ATP/ADP ratio of control and fructose groups could not be found. ATP, ADP levels and ATP/ADP ratios were diminished in both melatonin and fructose plus melatonin group compare with other groups (p<0.05).

Discussion

MetS is a cluster of pathologies comprising insulin resistance, hyperinsulinemia, hypertriglyceridemia and hypertension. It has been suggested that fructose is an important nutritional factor in the development of MetS in humans. High fructose intake also induces insulin resistance, impaired glucose tolerance, hyperinsulinemia, hyperlipidemia and hypertension in animals with a very similar metabolic profile to humans [20]. Thus, high fructose feeding is widely used to induce the alterations which take place in MetS and to investigate the development mechanisms of related pathologies and search for possible treatment methods [15]. The metabolic alterations observed in fructose-fed rats are quite divergent among the studies probably due to study design. Differences between studies include the strain of rat used, the amount, period and route of fructose administration and the age of the animals [14]. In our study, we used Sprague-Dawley male rats and fructose administration was accomplished by giving daily prepared 20% d-fructose in tap water to drink for 8 weeks. At the end of the study, fructose intake induced hypertension, hyperinsulinemia, insulin resistance and hypertriglyceridemia as a MetS criteria were observed compared with control group. Thus, MetS model was successfully demonstrated as shown in Table 1.

Central nervous system (CNS) is abnormally activated in MetS [21]. There is an interaction between CNS activation and insulin resistance. The earliest events in the pathogenesis of neurodegenerative disease is oxidative/nitrosative stress [22]. Recent studies have supported the hypothesis that nitric oxide (NO) is a mediator of neuronal injury in pathological conditions. NO formed nitric oxide synthases (NOS) is major signaling functions, vasodilator, and key mediator of immunity effect. Since NO is a gas, it can diffuse freely out of the neurons producing it, react with oxygen to form peroxynitrite and induce cell death in the surrounding neurons of cerebral cortex, cerebellum and hypocampus. Neuronal NOS (nNOS) has been thought to account for 95% or more of all NOS activity in the brain [23]. The presence of nNOS appears correlate with regions of excitatory amino acid neurotoxicity both in vivo and in vitro [24,25]. It also suggested excess production of NO by nNOS is thought to mediate neuronal damage. Brain NOx (nitrate+nitrite) content decreased rapidly in parallel with the inhibition of brain NOS [25–27].

In the present study, brain tissue eNOS and iNOS proteins were analyzed. But, no detectable level was measured by western blotting. In addition, as a result of the fructose feeding, both NOx and 3-NT levels unchanged
significantly. Our results were in consistent with the data from the evaluation of the effect of fructose on oxidative stress of brain carried out by Lopes et al. [5]. They investigated the effect of acute fructose administration on oxidative/nitrosative stress in the brain and reported the levels of nitrate and nitrite were not altered. 8 weeks fructose diet may not be sufficient to observe the oxidative/nitrosative stress findings in brain. In the present study, melatonin supplementation had no effect on NOx levels when administrated neither alone nor together with fructose. In fructose plus melatonin group, 3-NT levels increased significantly compared to all groups. It is unclear how melatonin increased 3-NT levels in fructose plus melatonin group. In contrast to our data, Tan et al. reported melatonin exerts direct, non receptor-mediated actions, such as the scavenging of number of free radicals and the neuroprotective effect of melatonin involve the inhibition of nNOS [28]. In our study, increase in 3-NT levels in fructose plus melatonin group can be explain; melatonin is transformed enzymatically to active and inactive metabolites. The inhibitor effect of melatonin on nNOS activity mainly dependent on its endogenous conversion to active metabolites. The inhibition of this active metabolites formation by some substrates may increase nNOS activity and thus NO over production might been occured. We considered that when administrated melatonin together with fructose, melatonin transformation to active metabolite may be inhibited by fructose, and NO production and 3-NT levels may be increased by the effect of nNOS activity [29].

Previous studies have demonstrated a link between MetS and mitochondrial function which is essential to brain homeostasis. It was supported that mitochondrial damage was a key mediator of diet-induced disturbances of the brain [30]. In our experimental study, we evaluated the energy balance by determining ATP/ADP ratio in rat brain tissues. We could not find any differences on ATP/ADP ratio between fructose and control groups. Our results were supported by the evidences that persistent exposure of mitochondria to NO leads to peroxynitrite generates which in turn damage nitrosative phosphorylation enzymes which produce ATP. The mechanism responsible for inhibition may be nitration of essential tyrosine residue [31]. As seen our results, brain NOx and 3-NT levels after fructose administration did not increase and thus, ATP/ADP ratio also did not change compare with control group. In the present study, ATP/ADP ratio decreased in both melatonin and fructose plus melatonin groups. Even more decrease was observed in fructose plus melatonin group. Our results that decreased ATP/ADP ratio in melatonin group are aggreement with published data by Wu et al. [6]. They reported that there is a link between MetS and central sympathoexcitation-induced hypertension. They demonstrated that fructose increases pyruvate production via upregulation of GLUT 2 and 5 transporters expression for fructose and glucose. Pyruvate then enters to TCA cycle and produce a greater amount ATP via mitochondrial respiratory chain in rat brain. ATP-dependent increase in neurotransmitter release is involved sympathoexcitation and hypertension. As shown, there is a close relationship between ATP production and hypertension [6,32]. As seen in our results, hypertension was regulated by the effect of melatonin treatment. Low ATP levels in fructose plus melatonin group can be explain that melatonin requires more ATP to prevent hypertension.

In conclusion, these results indicate fructose consumption at that dose and duration did not influence NO production, energy metabolism and protein nitration in brain. When administered melatonin with fructose might act as a pro-oxidant in our experimental condition. Further experimental studies (e.g., different melatonin’s doses, nNOS analysis and/or investigation of distinct brain areas particularly) are needded to clarify the underlying the effect mechanism of melatonin on disturbances of these parameters in fructose-fed rats.

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Conflict of Interest: The authors have no conflict of interest.

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