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RESEARCH ARTICLE

Investigation of TNF-α and NF-κB Levels in Masseter Muscle of Rats with High Fructose Corn Syrup-induced Metabolic Syndrome

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ABSTRACT

Objective
In recent years, especially due to excessive sugar consumption, metabolic Syndrome (MetS) has become increasingly common in the world and in Turkey. The aim of the study was to investigate the inflammatory effects of high fructose corn syrup (HFCS) intake on the masseter muscle of young rats.

Materials and methods
Sixteen 3-week-old male Wistar rats were randomly split into two groups: control and HFCS. Animals were given HFCS in the form of 20% solutions in drinking water. Animals were sacrificed in the eighth week. Right masseter muscle was isolated for immunohistochemical and histopathological examination, and left masseter muscle was isolated for gene expression analysis.

Results
Both ELISA and real-time polymerase chain reaction (rt-PCR) measurements revealed that the HFCS group had significantly higher TNF-a and NF-kB levels than the control group ($p < 0.05$). Additionally, when comparing the HFCS group to the control group, a higher degree of lymphocyte infiltration was seen.

Conclusion
According to study results, young rats' masseter muscle tissue had significantly higher levels of TNF- and NF-B due to fructose-induced MetS. These findings suggest that MetS, through increased inflammation, can cause masseter muscle dysfunction, injury, fatigue, and pain.

Keywords: Metabolic syndrome, masseter muscle, inflammation, TNF-a, NF-κB.

INTRODUCTION

Metabolic Syndrome (MetS) is a medical condition defined by the presence of at least three of the following symptoms: central obesity, hypertension, hyperglycemia, high triglycerides, and low high-density lipoprotein (HDL) cholesterol.[1] MetS, a multifactorial disease, increases the risk of other diseases such as cardiovascular disease, non-alcoholic fatty liver disease, type 2 diabetes and cancer. The prevalence of MetS is increasing all over the world, especially in developed countries. In the USA, 15% of people obtain 25% of their daily energy requirement from sugar. It has been reported that the consumption of high-fructose corn syrup is 30-35 kg per person per year in the USA, more than 50% of the total sugar.[2] Many Mets animal models are created by high-carbohydrate or high-fat diets.[3] High-fructose diets, in particular, have been used successfully in animal models to develop the MetS model.[4]

Tumor Necrosis Factor α (TNF-α) is one of the main cytokines involved in inflammatory and immune processes. TNF-α also plays a key role in inflammation and cytotoxicity by interacting with cells or acting in a pleiotropic manner, increasing inflammation in different cell types and tissues. The Nuclear Factor Kappa B (NF-κB) is an important mediator of inflammatory responses and regulates many aspects of innate or acquired immune functions.NF- κB regulates inflammation by inducing the expression of several pro-inflammatory genes,

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including those encoding cytokines and chemokines. TNF-α and NF-κB activation thus play a role in the pathogenesis of many inflammatory diseases.

Masticatory muscles are crucial to human health, especially food consumption and chewing, through the stomatognathic system.[5] Masticatory muscles have been studied for structural changes in muscle type composition and/or muscle size as a result of eating habits, lifestyle, diet, and psychological or physiological stress. Masticatory muscles have been studied for structural changes in muscle type composition and/or muscle size as a result of eating habits, lifestyle, diet, and psychological or physiological stress.[6] Muscle weakness and reduced muscle mass have been linked to insulin resistance, glucose intolerance, and type 2 diabetes mellitus in some studies.[7] It has been previously reported that oxidative stress intensifies in the skeletal muscles of patients and animals with MetS.[8] Reid et al.[9] demonstrated in vitro that increased oxidative stress was linked to the onset of the weakness/fatigue process in the rat diaphragm muscle. In the literature, previous studies have been conducted on the antioxidant effects of metabolic syndrome in masseter muscle tissue.[10] However, as far as we know, there is no study in the literature examining the inflammatory effects of metS in masseter muscle tissue and investigating inflammatory cytokine expressions. This study aimed to explain the role and possible mechanisms of MetS in the formation of inflammation in masseter muscle tissue.

MATERIAL AND METHODS

Study Design

An animal experiment model was designed using 3-week-old male Wistar rats to examine the effect of MetS induced by HFCS. The research was approved by Afyon Kocatepe University Experimental Animals Local Ethics Committee (dated 11.05.2019 and number 49533702/160). This study was supported by Afyonkarahisar Health Sciences University Scientific Research Projects Commission under grant number (19.CAREER. 016).

Animals

Four-week-old male, average weight of 100 g, 16 Wistar Albino rats were taken from Afyon Kocatape University Experimental Animals Center. Wistar rats, 1-month-old weighing approximately 100 g, were substituted under 12 hours light and 12 hours dark. It was placed in temperature (20-22°C) and humidity-controlled rooms with free access to standard feed and water. After a one-week accommodation period, the animals were divided into 2 different groups. The body weights of the animals at the beginning and during the process, the amount of feed consumed and the amount of liquid they drink were recorded with weekly measurements.

A 20% (w/v) solution of HFCS (55% fructose) in drinking water was prepared twice a week and stored at +4 °C. It was added to the drinkers by shaking before giving to the animals. The animals were given standard feed (62% carbohydrates, 23% protein, 4% fat, 7% cellulose, standard vitamins and salt mixture), drinking water and drinking water containing fructose (20%). HFCS dose and duration were determined by preliminary experiments. No feed or fluid restriction was applied to the rats in the study. The feed and fluid needs of the animals were regularly monitored every day. Room ventilation and other parameters were kept under constant control.

Experimental groups

In the study, experimental animals were randomly divided into 2 groups as follows:

1. Control group: The animals were given drinking water and standard feed for eight weeks.
2. HFCS group: The animals were given drinking water, standard feed and 20% fructose corn syrup for eight weeks.

Blood glucose levels, plasma lipid, insulin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured from the weekly blood of the animals. The weights of animals were weighed. During the study period, no loss was experienced in the experimental animals and no unexpected side effects were observed. The MetS model was established in the fourth week of the experiment and animals were sacrificed with ketamine (100 mg/kg) and xylazine (10 mg/kg) in the eighth week. Masseter muscle tissue was removed from both sides (Figure 1). The right masseter was fixed in 10 % neutral formalin for histological analysis, and the left side was stored in RNA later (Ambion Inc., Austin, TX, USA) for gene expression analysis.

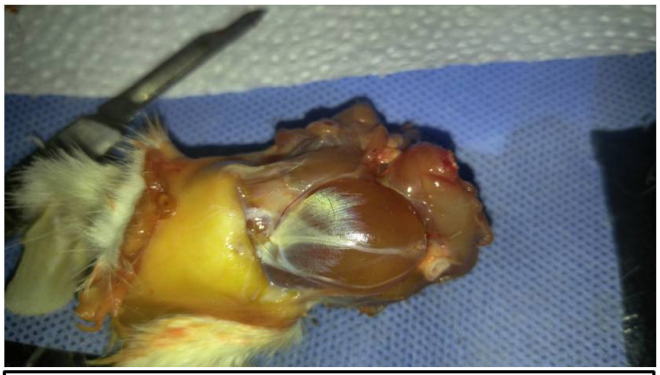

Figure 1. Example of masseter muscle tissue

Measurement of weight, feed consumption, fluid and calorie intake, and some metabolic parameters in blood samples

The blood glucose level was measured in blood samples taken from the tail at the end of the experiment using a glucose meter (Accutrend® Plus). The measurements were repeated at least 3 times and the averages were taken. 4 cc blood samples taken intracardiac were centrifuged at 2200 rpm for 30 minutes and the liquid part was separated. The separated plasma was taken with a pasteur pipette, put into Eppendorf tubes and stored frozen at -85°C. Parameters measured in plasma samples were determined using appropriate enzymatic analysis kits and/or ELISA kits.

Immunohistochemical staining

For histochemical staining, 5 µm thick sections were taken from paraffin-embedded masseter muscle tissue. After deparaffinization, samples were stained with Hematoxylin-Eosin (HE) using standard protocols and examined by light microscopy (Nikon, Eclipse E600, Tokyo, Japan).

Determination of gene expressions by a real-time polymerase chain reaction(rt-PCR)

Total RNAs were isolated from masseter muscle tissues using the RNeasy total RNA isolation kit (Qiagen, Venlo, The Netherlands) as described in the manufacturer's protocol. Gene expressions were determined by mixing 1 µl cDNA, 5 µl 2X SYBR Green Master mix (Fast Start Universal SYBR Green Master Mix, Roche, Basel, Switzerland) and primer pairs at final concentrations of 0.5 µM in a total volume. 10 mL quantitative real-time PCR (LightCycler480 II, Roche, Basel, Switzerland) reactions were performed in triplicate and the specificity of the PCR products was verified using melt analysis. Relative expression of genes relative to internal control; GAPDH was calculated with the advanced relative measurement tool with efficiency correction provided by the LightCyclerVR 480 SW 1.5.1 software.

Statistical analysis

Results were reported as mean ± standard error mean. Real Time-PCR results were given as % change compared to the control group. All data were analyzed in the GraphpadPrism 6.02 statistical software program. Differences between groups were evaluated with Student's t-test or Mann-Whitney U test. A p-value of <0.05 was considered statistically significant.

RESULTS

Evaluation of metabolic syndrome parameters

The weight, feed consumption, and fluid intake of the experimental animals were monitored weekly during the feeding period. Calorie intake was calculated from the data obtained and given in Table 1.

Table 1. Weight, feed intake and metabolic parameters measured in plasma in groups

Groups	Control	HFCS
Terminal body weight (g)	291±5	365±9 *
Omentum weight/body weight (%)	0,66±0,1	1,72±0,12 *
Foodintake (g/day)	25,5±1,2	14,4±0,9 *
Liquid intake (ml/100 g bw)	16,1±2,1	13,2±1,8
Total caloricintake (kcal)	89,4±1,2	77,5±3,4 *
Glucose (mg/dL)	73±2,9	106±4 *
Insulin (ng/mL)	0,62±0,05	1,95±0,05 *
Triglyceride (mg/dL)	109±2,8	179±1 *
VLDL (mg/dl)	21,8±0,6	36±0,2 *
Cholesterol (mg/dl)	58,5±3,1	67,1±3,1 *
Fructose (µmol/L)	144±3	159±7 *
Urea (mg/dL)	52,9±3,7	64,4±1,2 *
Creatinine (mg/dL)	0,48±0,03	0,57±0,04 *
Sodium (mmol/L)	144±1,2	145±0,8
Potassium (mmol/L)	46,6±3,9	41,4±2,6
Total Protein (g/dL)	6,51±0,24	6,64±0,11
Uricacid (mg/dL)	0,98±0,1	2,6±0,08 *
ALT(IU/L)	39,6±3,3	70,6±7,4 *
AST (IU/L)	99±7,7	140±15,9 *
Calcium (mg/dL)	0,22±0,01	0,21±0,03
Iron (µg/dL)	64,3±5,4	60,2±5,4
T4 (ng/dL)	1,86±0,17	1,65±0,14
T3 (pg/dL)	2,26±0,15	2,18±0,27
Estradiol (pg/ml)	10,7±0,9	12,9±0,9 *
Total testosterone (ng/ml)	2,82±0,29	2,81±0,19

In the study, it was observed that metabolic syndrome parameters were successfully formed in the HFCS group. When Table 1 is examined, it is seen that the body weights of the rats in the HFCS group are significantly higher than the rats in the control group. In addition, blood glucose, insulin, triglyceride, VLDL, cholesterol, fructose, urea, creatinine, uric acid, ALT, AST, and estradiol levels were significantly higher HFCS group than in the control group.

Immunohistochemical and histolopathological evaluation

NF-κB and TNF-α levels of inflammatory structures were measured using ELISA kits (Cloud-Clone Corp., USA). TNFα and NF-kB level was found to be significantly higher in the HFCS group than in the control group (Figure 2).

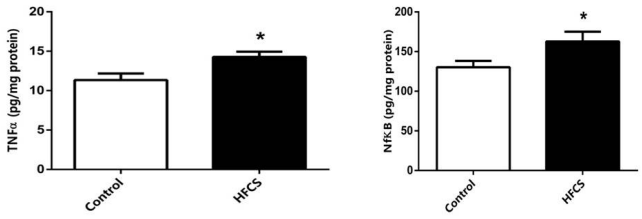

Figure 2. Comparison of the HFCS group with the control group in terms of TNF-α and NF-κB levels
* Represents significance compared to control ($p < 0.05$).

Histological examination with HE staining showed no pathological changes in the masseter muscle tissue of healthy rats, whereas the uptake of HFCS caused lymphocyte infiltration in the masseter muscle of rats (Figure 3).

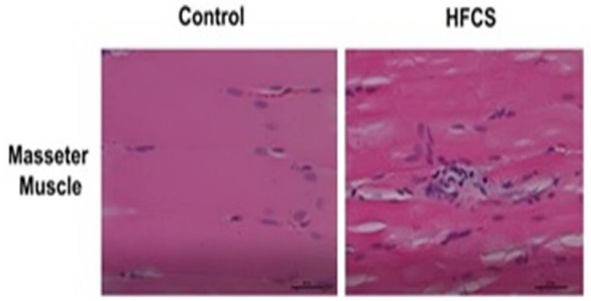

Figure 3. Histopathological features of masseter muscle tissues in the control and HFCS groups. Hematoxylin-eosin staining shows lymphocyte infiltration from both masseter muscle and gingival tissues in the HFCS groups (HE staining x 50 magnification for masseter muscle).

Evaluation of gene expressions by rt-PCR

TNF-α and NF-κB level was found to be significantly higher in the HFCS group compared to the control group (Figure 4).).

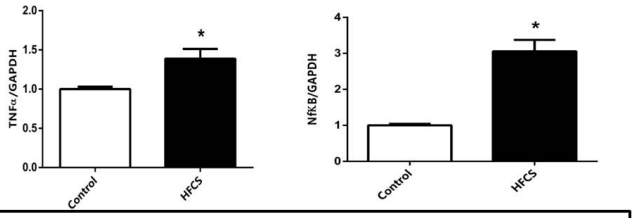

Figure 4. Comparison of the HFCS group with the control group in terms of TNF-α and NF-κB levels
* Represents significance compared to control ($p < 0.05$).

DISCUSSION

HFCS is increasingly being used to replace other caloric sweeteners, first in beverages and more recently, in thousands of other processed and packaged foods. Even though it has the same caloric value, HFCS is known to cause more harmful effects on the body than other sugars due to its high fructose content.[11] Experimental studies have shown that HFCS consumption causes many pathologies such as metabolic syndrome[12], diabetes[13], oxidative stress[14] and inflammation[15]. In addition to hyperglycemia, systemic accumulation of advanced glycation end products (AGE) was observed in rats fed a high fructose diet.[16] It has been stated that AGEs can cause oxidative stress and inflammatory reactions associated with impaired bone cell development along with metabolic disorders.[17] The findings of this study, similar to the findings of previous studies, showed an increase in parameters (plasma glucose, cholesterol, triglyceride, insulin, AST, ALT, etc.), which are the markers of metabolic syndrome, with HFCS intake. MetS criteria, including central obesity, hyperglycemia, and dyslipidemia, were successfully induced by the HFCS diet in this study. TNF-α and NF-κB levels, one of the most important proinflammatory cytokines, in the masseter muscle tissue of rats with MetS were revealed for the first time in the literature by immunohistochemical and gene expression analysis.

In this study, sections taken from the masseter muscle tissue of both sides of rats with metabolic syndrome with HFCS were examined histologically. In the histopathological examination, inflammatory effects such as increased lymphocyte infiltration were observed in the masseter muscle tissue. In addition, TNF-α and NF-κB levels were examined in masseter muscle tissue by ELISA method and a significant increase in these parameters was observed in rats with MetS. In this study, the expression of TNF-α and NF-κB genes was also examined by rt-PCR analysis, and a significant increase in the expression of these genes was observed in rats with MetS.

Previous studies in the literature have revealed that some histopathological changes occur due to oxidative increases in masseter muscle tissue. Li et al.[18] demonstrated how oxidative stress brought on by psychological stress can result in structural and functional changes in masseter muscle cells by lowering the capacity of antioxidant enzymes. On the other hand, Aghabeigi et al. [19] demonstrated that patients with chronic facial pain had higher intra-articular and systemic free radical levels. In the presence of facial pain, superoxide

dismutase (SOD) activity decreased and reactive oxygen species (ROS) formation increased, according to Viggiano et al. [20] According to Özgöçmen et al.[21] there is a significant relationship between pain and oxidative stress in fibromyalgia. In patients with myofascial pain dysfunction, Etoz et al. [22] discovered a link between increased pain and decreased total antioxidant capacity.

It has been demonstrated that MetS causes oxidative stress in the rat masseter muscle tissue. Tukel et al.[10] reported that rats with MetS had significantly lower SOD, catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities in the masseter muscles compared to the control group. These results show that the balance between oxidant formation and antioxidant defense in the masseter muscle is impaired in MetS. Again in this study, Na + / K + -ATPase activity in the masseter muscle in the MetS group was found to be significantly lower than the control group. Decreased ATPase activity has generally been considered to be one of the manifestations of cell damage in association with free radical formation, hypoxia, or acidic metabolites.[23] Similarly, it has been shown that a diabetes-induced decrease in Na + / K + -ATPase activity prevents contraction and endurance, and causes fatigue in skeletal muscle.[24] Masseter interleukin-6 levels were found to be significantly higher in rats exposed to a combination of occlusal intervention and chronic stress compared to the control group. Also, there was a positive and significant relationship between pain response and masseter interleukin-6 level in this study.[25]

In recent years, bruxism, temporomandibular joint dysfunction and myofascial pain have become increasingly common, especially in adolescents, due to increased anxiety and stress in daily life. Metabolic syndrome may contribute to myofascial pain by causing increased inflammation in the masticatory muscles as well as in other muscles. In this study, the inflammatory and destructive effects of metabolic syndrome on masseter muscle tissue due to HFCS consumption in young adult rats were experimentally demonstrated. The study findings suggest that metabolic syndrome findings that may occur as a result of excessive sugar consumption in children may be a predisposing factor in myofascial pain and temporomandibular joint dysfunction in addition to stress factors.

The most important limitation of this study is that only TNF-α and NF-κB pathway, which are among the most important proinflammatory cytokines, were examined in this study investigating the possible inflammatory effects of metabolic syndrome on masseter muscle tissue. In future studies, other inflammatory cytokines such as IL-1, IL-6 or anti-inflammatory cytokines in masseter muscle tissue can be investigated separately. This study is the first to investigate the inflammatory effects of HFCS-induced metabolic syndrome in masseter muscle tissue. In this study, the effects of metabolic syndrome on masseter muscle tissue in young rats were comprehensively demonstrated for the first time by histological, histochemical and gene expression analysis.

CONCLUSION

The results of the study demonstrated that fructose-derived MetS significantly raised the levels of TNF-a and NF-kB in masseter muscle tissue. A higher level of inflammatory cell infiltration was also seen in the HFCS group compared to the control group during the histological examination. These results show that HFCS-induced MetS causes increased inflammation in the masseter muscle tissue as well as in other tissues in rats. These results suggest that MetS may cause masseter muscle dysfunction, pain, weakness, fatigue, and injury through impaired antioxidant or anti-inflammatory defense. Further studies are needed to elucidate the increase in inflammation in the masseter muscle tissue due to MetS and its possible mechanisms.

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Conflict of Interest
The authors report no conflicts of interest related to this study.

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