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Effects of Food Restriction on PTGS2 and NRF2 Genes Expression Levels in Rat Testis

Hüseyin ÖZKAN[1,a], Sevda DALKIRAN[2,b], İrem KARAASLAN[3,c], Ufuk KAYA[4,d], İbrahim ALAKUŞ[5,e], Baran ÇAMDEVİREN[2,f], Akın YAKAN[1,3,g]

[1]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Genetics, Hatay-TURKEY
[2]Hatay Mustafa Kemal University, Institute of Health Sciences, Department of Molecular Biochemistry and Genetics, Hatay-TURKEY
[3]Hatay Mustafa Kemal University, Technology and Research & Development Center (MARGEM), Hatay-TURKEY
[4]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Biostatistics, Hatay- TURKEY
[5]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Surgery, Hatay-TURKEY
ORCID: [a]0000-0001-5753-8985; [b]0000-0002-5704-5774; [c]0000-0002-7485-192X; [d]0000-0002-4805-0993; [e]0000-0002-2031-7035; [f]0000-0003-1508-7869; [g]0000-0002-9248-828X

Corresponding author: Hüseyin ÖZKAN; E-Mail: hozkan@mku.edu.tr
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Abstract: This study aimed to investigate the effects of food restriction on oxidative status at the molecular levels of the testis in rats. A total of 16 male Wistar rats were allocated to two groups (n=8) and fed for four weeks. The first group was control (Cont) and consumed food and water ad libitum. The second one, food-restricted group (FR) was presented half of the regular consumption. But the water was presented ad libitum. The feeding period was maintained for four weeks. At the end of the feeding period, rats were euthanized by cardiac blood sampling under anesthesia. Malondialdehyde (MDA) levels in testis tissue were determined and calculated as nmol/mg protein. In addition, Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*) and Nuclear factor erythroid 2–related factor 2 (*NRF2)* genes expression levels were determined in testis tissue. While MDA and gene expression levels were found similar in groups, a positive correlation was found between *PTGS2* and *NRF2* genes (r=0.629; P<0.05). The molecular regulation of oxidative status was found strongly related with *PTGS2* and *NRF2* genes molecular activity in testis of rats. The obtained results were shown that the feeding period and restriction rate factors were mainly responsible for the oxidative status of testis tissue.
Keywords: Food restriction, *NRF2*, oxidative stress, *PTGS2*, testis

Yem Kısıtlamasının Rat Testisinde PTGS2 ve NRF2 Genlerinin Ekspresyon Seviyelerine Etkileri

Öz: Bu çalışmanın amacı, yem kısıtlamasının rat testisinde oksidatif duruma etkilerinin moleküler seviyede belirlenmesidir. Toplam 16 erkek Wistar rat iki gruba ayrılmıştır (n=8) ve dört hafta boyunca beslenmiştir. Birinci grup olan Kontrol (Cont) grubuna yem ve su *ad libitum* olarak verilmiştir. İkinci grup olan, yem kısıtlaması (FR) grubuna Cont grubunun yarısı kadar yem sunulurken su *ad libitum* olarak sunulmuştur. Beslenme periyodunun sonunda ratlar anestezi altında kardiyak kan alınarak ötenazi edilmiştir. Testis dokusunda Malondialdehit (MDA) seviyeleri belirlenmiş ve nmol/mg protein olarak hesaplanmıştır. Ayrıca testis dokusunda Prostaglandin-Endoperoxide Synthase 2 (*PTGS2)* ve Nuclear factor erythroid 2–related factor 2 (*NRF2)* genlerinin ekspresyon seviyeleri tespit edilmiştir. Gruplarda MDA ve gen ekspresyon seviyeleri benzer bulunurken, *PTGS2* ve *NRF2* genleri arasında pozitif korelasyon bulunmuştur (r=0.629; P<0.05). Rat testisinde oksidatif durumun moleküler regülasyonu, *PTGS2* ve *NRF2* genlerinin moleküler aktivitesi ile güçlü bir şekilde ilişkili bulunmuştur. Elde edilen sonuçlar, testis dokusunun oksidatif durumundan esas olarak beslenme süresi ve kısıtlama oranı faktörlerinin sorumlu olduğunu göstermiştir.
Anahtar kelimeler: *NRF2*, Oksidatif stres, *PTGS2*, testis, yem kısıtlaması

Introduction

Numerous factors are responsible for the sustainable health of organisms. Nutrition is one of the major elements that regulate the complex reproductive activity in mammals (Ajuogu et al., 2020). Male reproductive physiology is strongly associated with diet-related disorders such as obesity. Regulation of diet is considered the gold standard in treating metabolic disorders (Moszak et al., 2020). Moreover, food or calorie restrictions are frequently preferred to bring obesity-related disorders under control (Filaire et al., 2009).

Most cellular components such as lipids and proteins are vulnerable to oxidative modifications. A disturbed oxidative balance between reactive oxygen species (ROS) causes oxidative stress in the organism. It has been shown in several studies that FR has positive

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effects of diminishing the ROS in many tissues and organs (Filaire et al., 2009; Ichikawa et al., 2000; Kim et al., 1996).

As with all systems in the organism, the antioxidant mechanism is of tremendous importance in the reproductive system. In the past 20 years, researchers have focused on the molecular regulation of the biological processes in tissues and organs (Alberts et al., 2002). In addition, gene expression studies attract attention in any area of biology. On the other hand, a limited molecular study-related oxidative stress has been found about the effect of FR on testis tissue (Li et al., 2021; Mladenovic Djordjevic et al., 2021).

Oxidative balance is under the control of many genes of the oxidative pathway. *PTGS2* (Prostaglandin G/H Synthase 2) and *NRF2* (Nuclear Factor, Erythroid 2 Like 2) are responsible for constituting oxidative equilibrium (Özkan and Kutlu, 2020). *PTGS2* is triggered mainly by the activation of the inflammatory pathway and leads to ROS production (Onodera et al., 2015). *NRF2*, other vigorous responsive gene, has essential roles in response as an antioxidant regulator. MDA, a crucial indicator of lipid peroxidation, is a significant parameter that shows the oxidative status (Yang et al., 2014).

The use of laboratory animals, typically rats, as a model is substantial and advantageous in understanding molecular and biological processes of nutrition-related physiology. Although many studies have been reached about the effects of FR on metabolic organs in the literature, limited study at the molecular level has been found about the oxidative status of testis tissue. Restriction in the diet by 50% reduces the amount of calories available and provides minimum micronutrient requirements in adult rats without causing malnutrition (Smyers et al., 2015). This study aimed to investigate the oxidative effects of food restriction for four weeks to testis tissue at the molecular levels. For this aim, in addition to MDA levels, *PTGS2* and *NRF2* genes expression levels were determined. Relationships between these parameters in testis were also analyzed.

Materials and Methods

Animals and dietary experiments

The study was ethically approved by the Animal Experiments Local Ethics Committee of Hatay Mustafa Kemal University (Decision no: 2021/04-02). In the study, 16 male Wistar albino rats were divided into two groups and fed with two different diets for four weeks. The rats weighed 200-250 g and were eight weeks old. Animals were housed individually in transparent polycarbonate cages throughout the feeding period. In addition, a standard light procedure was applied (12 hours daylight/12 hours dark). Before the feeding period, the daily food consumption of the rats during the one-week acclimatization period was recorded and the average daily consumed amount of food was calculated (30.04±2.2 g/rat/day). While the first group was control (Cont) and consumed commercial food (Bil-Yem Ankara) and water ad libitum, the second was food-restricted group (FR). The Cont group was provided approximately 7 g/day of crude protein and 80 kcal/day. The FR group was provided with about half of the food daily recorded consumption of rats in Cont group, and water was presented as *ad libitum* (Gardner et al., 2010; Smyers et al., 2015). These amounts meet the daily energy and protein requirements recommended by the NRC for rats (NRC, 1995). The ingredients of standard food are presented in Table 1.

At the end of the feeding period, the rats were applied overnight fasting and euthanized by cardiac blood sampling under anesthesia (80 mg/kg Ketamine and 12 mg/kg Xylazine, IP). Following the euthanasia, the right testis tissues were quickly collected and snap-frozen in liquid nitrogen. Frozen samples were stored at -86 °C until the execution of the molecular experiments and MDA analysis.

RNA isolation and cDNA synthesis

Total RNA isolation was performed on samples according to instructions of the producer of Trizol Reagent (Thermo Fisher Scientific, Cat No: 15596026, USA). Approximately 50 mg tissue was homogenized in 1 mL Trizol Reagent. Following the isolation, pellets of RNA were dried for 10 min at room temperature and dissolved with nuclease-free water (NFW) by pipetting. While purity and concentration of samples were checked with a nucleic acid spectrophotometer (Merinton, SMA-1000 UV Spectrophotometer, China), RNA integrity was evaluated by checking 28S and 18S rRNA units on 1% agarose gel stained by Red Safe (intone Biotechnology, Cat No: 21141, Korea).

Following the RNA isolation, possible genomic DNA contamination in samples was eliminated with DNase treatment (DNase I, RNase free, Thermo Fisher Scientific, Cat no: EN0525, USA). Then, cDNA synthesis was applied to samples with a commercial reverse transcription kit (High-Capacity coda Reverse Transcription Kit, Thermo Fisher Scientific, Cat No: 4368814, USA), and one µg total RNA was used. Protocol in thermal cycler (Bio-Rad T100, USA) was as follows: Following the 60 min at 42 °C, samples were kept at 25°C for 5 min and 70 °C for 5 min, respectively. After the reaction, all samples were completed to 200 µL with NFW and stored at – 20 °C until gene expression analyzes.

RT-qPCR application

PTGS2 and *NRF2* genes expression levels were determined by RT-qPCR (Rotor-Gene Q, Qiagen,

Table 1. Ingredients and nutrient composition of food

	Ingredient (%)
Corn maize	29.00
Full-fat soybean	28.00
Sunflower seed meal	7.00
Wheat middling	13.00
Alfalfa meal	14.00
Meat bone meal	4.00
Molasses	1.75
Limestone	2.00
Dicalcium phosphate	0.50
NaCl	0.50
Vitamin-mineral premixes	0.25
Nutrient composition (calculated)	
Metabolic Energy (kcal/kg)	2600.00
Dry Matter (%)	88.40
Crude Protein (%)	22.50
Crude Cellulose (%)	8.10
Fat (%)	3.15
Carbohydrate (%)	54.72
Insoluble ash in HCl (%)	1.05
Ca (%)	1.30
P (%)	0.80

Germany) using SYBR Green Dye containing kit (Power SYBR® Green PCR Master, Thermo Fisher Scientific, Cat no: 4367659, USA). RT-qPCR protocol was as follows: Following the 10 min at 95 °C, 95 °C for 10 sec, 60 °C for 60 sec, and 40 cycles. Samples were studied as duplicates and primers of genes used for amplification were presented in Table 2. *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was used as a housekeeping gene for fold change calculations.

Determination of MDA levels

MDA analysis was applied using about 5 g of testis tissues. Samples were diluted ten times with Phosphate-buffered Saline (PBS) and homogenized under cold and sterile conditions. After that, samples were centrifuged at +4 °C for 10 min at 3000 xg. The supernatants were transferred to new sterile 1.5 mL volume tubes and levels of MDA were determined spectrophotometrically at 532 nm wavelength according to the reported method of Wasowicz et al. (1993). In addition, total protein levels of supernatants were evaluated according to the Lowry method, and the levels of MDA were calculated as nmol/mg protein (Lowry 1951). MDA and protein levels were measured by spectrophotometer (Evolution 160 UV-VIS v8.01 Spectrophotometer, Thermo Fisher Scientific, USA)

Statistical analysis

Stata 12/MP4 (License No: 50120500264) was used to analyze the data. Before performing the statistical analysis, data were examined for normality with Shapiro-Wilk test. In addition, homogeneity of variances was examined with Levene test as parametric test assumptions. Independent sample t test was used to evaluate the differences between groups for MDA. In addition, the $2^{-\Delta\Delta Ct}$ method was used to calculate expression levels of target genes. The correlation between studied parameters was determined by Spearman's Rho, and $P<0.05$ was considered as significant in all analyzes.

Results

The concentration and purity (A260/A280 ratio) of RNA samples were 156.16±40.32 ng/µL and 1.89±0.01, respectively. In addition, expression levels of *PTGS2* and *NRF2* genes were similar between Cont and FR groups. In addition, there were no significant differences between the groups in terms of MDA levels of about two nmol/mg protein in both groups (Figure 1).

According to correlation analysis, *PTGS2* and *NRF2* gene expression levels were significantly correlated ($r=0.629$; $P<0.05$). On the other hand, there were no significant relationships between MDA and *PTGS2* and *NRF2* (Table 3).

Discussion and Conclusion

In addition to genetic background, environmental factors significantly affect maintaining healthy life. Nutrition and dietary ingredients are some of the significant environmental factors for the physiological

Table 2. Forward and reverse primer sequences of genes

Genes	Forward and Reverse Sequences	Product L.	References
GAPDH	F: 5′-AGTGCCAGCCTCGTCTCATA-3′ R: 5′-TCCCGTTGATGACCAGCTTC-3′	241	Guvenc et al., 2019
PTGS2	F: 5′-TGTATGCTACCATCTGGCTTCGG-3′ R: 5′-GTTTGGAACAGTCGCTCGTCATC-3′	94	Guvenc et al., 2019
NRF2	F: 5′-TTGTAGATGACCATGAGTCGC-3′ R: 5′-TGTCCTGCTGTATGCTGCTT-3′	141	Guvenc et al., 2019

Product L: *Product Length*

processes of the organism (Özkan and Yakan, 2019). Dietary regulation is frequently used to prevent metabolic diseases such as obesity and its complications and in the treatment processes of these diseases. Also, nutrition is often taken under control in delaying aging processes. Moreover, FR is one of the most common practices for these purposes (Hamden et al., 2008; Mladenovic Djordjevic et al., 2021).

It has been reported that FR significantly affects the immune response and the activities of antioxidant parameters in the organism (Mortazavi et al., 2014). In addition, long-term food and calorie restriction have positive effects on oxidative damages in various tissues and organs (Bruss et al., 2010; Filaire et al., 2009). On the other hand, the positive effects of food restriction are strongly related to some parameters such as degree of restriction and time. A study has reported that only a two-week calorie restriction led to a decrease in hydrogen peroxide levels in skeletal muscle (Harper et al., 2004).

Responses of the tissues are different from FR. The effects of food restriction on the liver and other metabolic organs have mainly been studied (Filarie et al., 2009; Hamden et al., 2008). On the other hand, there is limited information about the oxidative status of testis in food-restricted conditions at the molecular level. In this study, the feeding period has been maintained for four weeks, and at the end of the period, the gene expression levels of *PTGS2* and *NRF2* in groups have been found similar. *PTGS2* has been reported to increase with increased oxidative stress in the aging process in testis tissue (Wang et al., 2005). On the other hand, it has been reported in another study that *PTGS2*showed similar expression patterns by food restriction for nine weeks in rat testis at different environmental temperature conditions (Bozkaya et al., 2017). The possible reason for similar *PTGS2* expression levels has been thought to be the time and restriction ratio of our study.

NRF2 is a crucial transcription factor in response to oxidative stress for maintaining homeostasis (Yang et al., 2018). This transcription factor triggers many genes expression levels, such as HO-1 and NQO1 (Ross and Siegel, 2017). In addition to function on oxidative status, it has a virtual role on cellular pathways such as autophagy, apoptosis, and cell proliferation (Özkan and Kutlu, 2020). Therefore, the determination of NRF2 activity is essential to investigate the effects of calorie restriction on testis tissue. While there have similar expression patterns in terms of NRF2 determined between the groups, a remarkable and positive correlation has been determined between PTGS2 and NRF2 gene expression levels in testis tissue. The specific correlation results have shown that the antioxidant mechanism of testis tissue is provided by effective co-regulation of these genes.

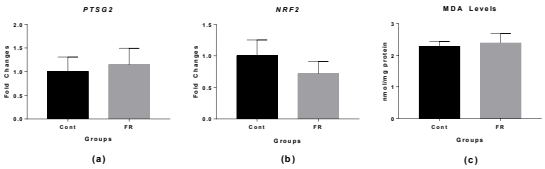

Figure 1. Gene expression results and MDA levels in groups.

The oxidative damage in testis tissue is crucial for maintaining normal physiology. The MDA levels have shown that consuming different diets for four weeks has no increasing oxidative effects on the testis. As is well known, MDA levels are measured to determine lipid peroxidation levels in various tissues and biological fluids (Özkan and Kutlu, 2020). In a study, MDA levels in plasma were reported to decrease in 8 days food-restricted rats (Filaire et al., 2009). On the other hand, it was reported that there were no significant change in terms of MDA levels in skeletal muscle and liver. In a study conducted on Sprague-Dawley rats, similar MDA levels in testis have been reported after 20% food restriction for two weeks (Jayroe et al., 2012). The possible reasons for these results are the feeding period and restriction rate.

MDA levels may increase in response to *PTGS2* activity, which is involved in pathways such as the inflammation pathway for possible oxidative stress (Özkan and Kutlu, 2020). NRF2 is the significant regulator for response to oxidative stress. In conclusion, it has been understood that the relationship between *PTGS2* and *NRF2* is essential in maintaining the oxidative balance in the testis tissue. Although it has been reported that food restriction suppresses oxidative stress that may occur in various tissues and or-

Table 3. Correlations between studied parameters

Parameters	PTGS2	NRF2	MDA
PTGS2	-	0.629*	0.070
NRF2	-	-	0.380
MDA	-	-	-

**: P<0.05*

gans, more studies are needed to investigate the effects of diets with different calorie content on testis.

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