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Research Article
Effect of Antioxidants on Testicular iNOS and eNOS after High-Fat Diet in Rat

[1,2]Maryam Sohrabi, [1]Mahnaz Hosseini, [3]Sevinc Inan, [1,5]Zohreh Alizadeh, [1]Mehrangiz Vahabian, [4]Ali Asghar Vahidinia and [1]Hosein Lahoutian

[1]Department of Anatomical Sciences, Faculty of Medicine, Hamadan University of Medical Sciences, Shahid Fahmideh Street, 65178-3-8736 Hamadan, Iran
[2]Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Shahid Fahmideh Street, 65178-3-8736 Hamadan, Iran
[3]Department of Histology and Embryology, Faculty of Medicine, Celal Bayar University, Manisa, Turkey
[4]Department of Biochemistry and Nutrition, Faculty of Medicine, Hamadan University of Medical Sciences, Shahid Fahmideh Street, 65178-3-8736
Hamadan, Iran
[5]Endometrium and Endometriosis Research Center, Hamadan University of Medical Sciences, Shahid Fahmideh Street, 65178-3-8736 Hamadan, Iran

Abstract

Background and Objective: Spermatogenesis is a process by which germ cells produce spermatozoa and can be disturbed at every level. Nitric Oxide Synthases (NOS), implicate in interactions with Oxidative Stress (OS) which is one of the main factors in the etiology of male infertility. The High Fat Diet (HFD) is a major factor of obesity which in turn is important for enhancing OS. Antioxidants and garlic could attenuate or reverse effects of HFD. The aim of the study was to investigate the effects of dietary antioxidants and garlic on testicular inducible NOS (iNOS) and endothelial NOS (eNOS) in Wistar albino rats fed on HFD. **Materials and Methods:** Groups (each n = 8) were: SD (100% access to standard diet), F-HFD, (100% access to HFD) and R-HFD (70% access to HFD), F-HFD +antioxidants, F-HFD+garlic and R-HFD+antioxidants. The HFD consisted of a 60% fatty diet in 3 forms: Without antioxidants, with antioxidants and with garlic. The testicular iNOS and eNOS were studied by immunohistochemical (IHC) method. Also used ANOVA, repeated measures ANOVA, t-tests and Tukey's test (where necessary) to analyze the data ($p<0.05$). **Results:** The iNOS increased in the F-HFD and R-HFD+antioxidants groups. The eNOS increased in R-HFD,F-HFD and F-HFD+garlic groups. The H-E evaluation in R-HFD group showed a decrease in spermatogenesis score count and seminiferous tubules diameters (μm) in comparison with the SD and F-HFD groups. R-HFD+antioxidants group had lower score than F-HFD+antioxidants and F-HFD+garlic groups. **Conclusion:** Restricted fat diet consumption causes increase in weight and impairs spermatogenesis. Results of this study reveal that adding the antioxidants can't improve histological changes of testis. The iNOS expression in seminiferous tubules in restricted fat diet along with antioxidants, suggest a potential role of iNOS in spermatogenesis and male infertility.

Key words: Obesity, testis, antioxidants, infertility, endothelial nitric oxide synthase, Inducible nitric oxide synthase, immunohistochemistry

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Corresponding Author: Maryam Sohrabi, Department of Anatomical Sciences, Faculty of Medicine, Hamadan University of Medical Sciences, Shahid Fahmideh Street, 65178-3-8736 Hamadan, Iran Fax: +988138380462

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Spermatozoa are produced by germ cells in spermatogenesis, a multi-phase process capable of being disrupted at any phase[1]. Magnusdottir *et al.*[2], also showed the relationship between obesity and reduction of sperm count. Obesity can have genetic roots but based on some evidence it can also be attributed to environmental factors such as increase in the amount of fat in the diet of the world's population in the recent decades[3,4]. Additionally, research has shown that High Fat Diet (HFD) is the main cause of obesity[4].

Human body is normally exposed to endogenous and exogenous free radicals. However excessive production of free radicals and inability of the body to neutralize them as well as underproduction of antioxidant lead to Oxidative Stress (OS)[5]. Reactive Oxygen Species (ROS) including free radicals damage cell's DNA in the process of oxidation and in its turn causes many disease[5]. Up to now, some clinical studies have been carried out on the correlation between OS and male infertility but few done on the destructive effect of HFD on spermatogenesis[6,7]. On the contrary, the impact of HFD on female fertility has been already researched[8]. Previous studies showed the obesity increase OS in men[9] and male infertility is an adverse consequence of increasing OS[7]. The OS lead to the decline of leydig cells steroidogenic capacity and loss of germinal epithelium capacity to differentiate normal spermatozoa. Chronic hypoxia and normal oxygen supply had opposing effect on physiological mechanisms of spermatogenesis and male fertility. Chronic hypoxia induced a state of oligospermia and the normal oxygen supply resulted in the normalization of spermatogenesis. Ischemic reperfusion damage to the testis was observed due to the generation of ROS under the condition of OS which is considered the main cause of male infertility. Damage to the antioxidant mechanisms increase in obese patients and animal models are clinically documented[10].

Supplying subjects with antioxidant prevents many HFD-related disease which increase OS in different tissues[11]. Antioxidant along with the main enzymes processing ROS, protect testes against oxidative damage. The antioxidants and a large number of free radical scavengers challenge ions. Spermatogenesis is reactivated in subjects taking dietary vitamins and semen quality is promoted in those receiving fat-and water soluble vitamins[12,13]. According to a study, OS resulting from lack of vitamin E and C disturbs spermatogenesis while a diet containing them reactivate the process[14]. Another study showed that vitamin E and C supplementation decreased testicular toxicity although it did not completely reverse the damage[15]. Garlic and many garlic preparations also have antioxidant properties. Garlic has been proved to be efficient in aiding endogenous antioxidant defense in different animals[16]. Previous study has reported contrary effects of garlic on spermatogenesis[17]. A report showed positive effect of garlic on the process while the other claimed that different doses of crude garlic in a month could reduce spermatogenesis[18].

Nitric oxide (NO), a ubiquitous free radical and freely diffusible water and lipid-soluble gaseous molecule with a short half-life, is synthesized in all mammalian cells by nitric oxide synthases (NOS). With regard to the effect of both deficiency and excess of NO production in the dysfunction of cells and tissues, studying the role of diet in the regulation of NO synthesis in humans and animals to keep them healthy and protected against diseases is critical[19]. There are three isoforms of the NOS: nNOS (originally in neuronal tissue), iNOS (originally as being inducible by cytokines) and eNOS (originally in endothelial cells). In the testis, eNOS is expressed in leydig cells, sertoli cells, spermatocytes and spermatids while various cytokines inducing transcription of iNOS is required for its expression[19]. In the recent decades, previous study has shown the direct relationship of dietary factors such as cholesterol, fatty acids and vitamins with constitutive and inducible NO production in mammalian cells[20].

Many assisted reproduction techniques have been recently devised to study spermatogenesis[21]. Based on a study, the role of obesity in infertility of men was 3 times more than idiopathic and female factors in the couples[22]. The aim of the present study was to measure immunohistochemical expression of testicular iNOS and eNOS after treatment with different types of diets: standard diet, high-fat diet, high-fat diet with crude garlic, high-fat diet with antioxidants, for a 12 weeks period. The study was designed to test the hypothesis that antioxidant supplementation could attenuate or partially reverse the effects of a high-fat diet on expression of iNOS and eNOS in testis.

MATERIALS AND METHODS

Experimental animals: Forty eight 2 months old Wistar Albino rats were utilized in this study. They were divided into five mice in each cage under standard conditions, i.e. 12 h light, 12 h dark, 60±5% humidity, 30±2°C temperature and providing with food and water for 1 week. Research ethics approval from the Ethics Committee of the School of Medicine, Hamadan University (Hamadan, Iran) was obtained. Then rats were divided into six (each n = 8) groups: 3 controls: SD (100% access to standard diet), F-HFD (100% access to HFD) and R-HFD (70% access to HFD) and 3 treatments:

Table 1: Holstein's scores for spermatogenesis classification

Scores	Seminiferous tubules characteristics
10	Intact spermatogenesis: Many mature spermatids and zones of spermiation
9	Modest reduced spermatogenesis: Reduced number of mature spermatids, a few zones of spermiation
8	Distinct reduced spermatogenesis: Few mature spermatids, no spermiation
7	Considerably reduced spermatogenesis: No mature spermatids, only immature spermatids, no spermiation
6	Severely reduced spermatogenesis: Only few immature spermatids, reduced height of germinal epithelium
5	Arrest of spermatogenesis at the stage of primary spermatocytes: Many spermatocytes border the lumen of the seminiferous tubule
4	Arrest of spermatogenesis at the stage of primary spermatocytes: A few primary spermatocytes are present
3	Arrest at the stage of spermatogonia: A type spermatogonia multiplicate but do not develop to maturing cells of spermatogenesis
2	No germ cells, only sertoli cells are present
1	No germ cells, no sertoli cells. The seminiferous tubule is replaced by connective tissue ground substance (shadow of tubule)

Table 2: Holstein's normal spermatogenesis characteristics

• Presence of A-pale type, A dark type, B type-spermatogonia
• Presence of primary and secondary spermatocytes
• Differentiation of spermatids
• Zones of spermiation
• Score count of 8 at the minimum
• Lumen of the seminiferous tubule
• Normal lipid distribution in the sertoli cell cytoplasm
• Presence of stages of spermatogenesis
• Formation of clones of germ cells
• Normal structure and distribution of leydig cells
• Diameter of the seminiferous tubule 180μ in human at the minimum

F-HFD+antioxidants, F-HFD+garlic and R-HFD+ antioxidants for 12 weeks. The received energy was measured weighing of all groups every day during the study.

High fat diet and antioxidants composition: The ingredients for every 100 g of HFD were milk fat (31.65 g), soya oil (3.22 g), casein (25.84 g), maltodextrin (16.14 g), sucrose (8.9 g), alpha (α)-cellulose (6.5 g), potassium citrate (2.1 g), dicalcium phosphate (1.7 g), L-cysteine (0.37 g), calcium carbonate (0.7 g), choline chloride (0.24 g), mixed vitamins (1.27 g) and mixed minerals (1.27 g), mixed minerals (1.25 g), mixed vitamins (1.29 g). Mixed vitamins composition was: 2 g kg^{-1} vitamin E, 2 g kg^{-1} vitamin C and 0.6 g kg^{-1} astaxanthin)[23,24].

Histochemical evaluation: The procedure was ended 12 weeks after feeding the groups with the diets. Under sterile conditions, the animals were anesthetized by general method of intramuscular injection of ketamine and xylazine hydrochloride. All left testes were dissected and cranial halves were put in buffered formalin 10% fixative solution 72 h at +4°C for histological and immunohistological evaluation. Next, specimens were washed in 70% ethanol and immersed in a graded series of ethanol and cleared in xylene and then were embedded in paraffin. Approximately 5 μm thick sections were cut. Slides stained with Hematoxylin-eosin (HE) were used for morphological diagnosis and also the comparison of seminiferous tubule widths and scoring of spermatogenesis. Using an ocular micrometer, the mean diameter of seminiferous tubules was estimated in 10 randomly selected fields by measuring three diagonal diameters of 10 tubules (60 tubules in total) based on Holstein's score Table 1[22].

To assess the stages of spermatogenesis quantitatively, abnormalities were evaluated for each testis using light microscopy and the spermatogonium, sertoli and interstitial cells have been considered. Afterwards, the characteristics of normal spermatogenesis were assayed adopting Holstein's classification (Table 2)[22]. Diameter of the seminiferous tubule was 65 μ in the control group of the present study for rat (180 μ for human according to Holstein's study)[22] at the minimum.

Immunohistochemical evaluation: For immunohistochemical staining, sections (3 μm thick) were cut and then deparaffinization at 60°C during the night, immersion in PBS and treatment with 0.3% hydrogen peroxide in PBS for 10 min to block endogenous peroxidase activity was done successively. The sections were washed in PBS and exposed in 10% normal horse serum to suppress non-specific antigen. A mouse monoclonal iNOS antibody (Cat. 905-385 Neomarkers Inc. Fremont, CA, USA) and a polyclonal eNOS antibody (Cat. RB-1711-P, Neomarkers, Inc. Fremont, CA, USA) were used for immunohistochemistry. The incubation of the sections with eNOS (1:100) or iNOS (1:100) antibodies were done for the whole night at +4°C (To show specificity of the antibodies, normal IgG have been used instead of the primary antibodies for negative control sections). The sections were washed in PBS, then, the addition and incubation of appropriate biotinylated secondary antibodies (Cat.85-9043 Secondary kit Invitrogen Histostain plus kit, Broad spectrum, CA, USA) were performed for 30 min at room temperature and after that the avidin-biotin-peroxidase complex was added. The reagents of DAB kit (Spring Bioscience Inc. CA, USA) developed immunostaining. The sections were dehydrated and mounted (Surgipath, USA) after

counter-staining with Hematoxylin Mayer (JJ Baker, Holland). The specimens were analyzed by B× 40 light microscope (Olympus, Tokyo). The staining scores evaluation were blindly performed by 2 observers separately. The iNOS and eNOS positive IHC stained cells in sections turn brown and their intensity was considered as negative (-), mild (+), moderate (++) and intense (+++).

Estimation of average expression of iNOS and eNOS (%) was carried out by counting positive cells (at least 500 cells in each testis) in 5 random medium-power fields (400×) including 100 cells and finally the total was divided by five.

Statistical analysis: Data was analyzed using Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) program, version 16.0. One-way analysis of variance (ANOVA), repeated measures ANOVA, t-tests and Tukey's test were used to analyze the data. A p-value≤0.05 indicated statistical significance[25].

RESULTS

In this study animals were fed with one of the four types of HFDs including: Standard diet, high-fat diet with or without antioxidants and high-fat diet with crude garlic for 12 weeks.

Animal weight determinations are shown in Table 3. All rats gained weight significantly throughout the experimental period (p<0.001). However, R-HFD+antioxidants rats achieved a final lower body weight as compared with the other groups (Table 3).

The daily weight gain by the treated rats with highest value from F-HFD (1.81±0.54 g) followed by F-HFD+antioxidant in relation to the R-HFD and R-HFD+antioxidants groups, respectively. The feed efficiency ratio of rats fed with R-HFD increased significantly (p<0.05) compared to those with other groups. Interestingly, R-HFD+antioxidants have shown the lowest feed efficiency ratio among all groups (Table 4).

In the SD group, normal spermatogenesis was observed and the mean Holstein's score was 10. In F-HFD and R-HFD groups, some sections of testes contained a few tubules with poor spermatogenesis and the score (9 and 8, respectively) were slightly lower than in the SD group. There were no differences between the scores in F-HFD+antioxidants and F-HFD+garlic groups (7 for both). The R-HFD+antioxidants group showed a more decrease in the mean Holstein's score (6). The results of Holstein's scoring are depicted in Table 1.

The results showed that in F-HFD group, the average diameter of seminiferous tubules was significantly lower than the SD and R-HFD groups (p<0.05). There was also significant reduction in the average diameter in R-HFD+antioxidants compared to F-HFD+antioxidants and F-HFD+garlic groups (p<0.05) (Table 5).

The presence of a continuous Basal Lamina (BM), spermatogonia in the basal compartment of seminiferous epithelium and spermatocytes I, spermatocytes II, spermatids and spermatozoa in the adluminal compartment in testes is shown in Fig. 1. The results from the immunohistochemical staining (Fig. 1) generally detected the eNOS and the iNOS in the cytoplasm of spermatogonia, spermatocyte I, spermatocyte II, spermatid and leydig cells in all experimental groups. There was, however, a considerable variability in the intensity of staining between testicular cells of them: (1) Intense immunoreactivity (+++) was shown for eNOS spermatocytes II and spermatids of F-HFD+garlic

Table 3: Body weight of rats after 12 weeks on the experimental diets

Groups	Initial body weight (g)	Final body weight (g)	[1]Delta body weight gain (g)	p-value*
F-HFD	115.42±15.59	284.42±45.99**	169.00±47.86**	p<0.001
R-HFD	115.67±18.09	258.50±13.39	142.83±20.01	p<0.001
F-HFD+antioxidants	115.50±14.73	267.92±47.77	152.42±47.27	p<0.001
R-HFD+antioxidants	115.25±17.06	232.17±23.19**	116.82±25.63**	p<0.001
p-value	(NS) p>0.05	p = 0.007	p = 0.007	

[1]Delta body weight gain was calculated by subtracting the body weight of the rat the day of the final day from that of the first day of the experiment. The values are shown as Mean±SE. (*Paired t-test and **student t-test) (p<0.05), NS: Non-significant

Table 4: Mean weight gain, food consumption, daily received energy and feed efficiency ratio of rats after 12 weeks on the experimental diets

Groups	Weight gain (g day^{-1})	Food consumption (g day^{-1})	Received energy (kcal per rat day^{-1})	*Feed efficiency ratio
F-HFD	1.81±0.54^{a}	11.72±1.29^{a}	58.78±6.47^{a}	0.153±0.03^{a}
R-HFD	1.48±0.28^{a}	8.31±0.00^{b}	41.68±0.00^{b}	0.179±0.03^{a}
F-HFD+antioxidants	1.51±0.56^{c}	11.5±1.49^{c}	58.56±7.60^{c}	0.131±0.04^{b}
R-HFD+antioxidants	1.04±0.22^{b}	8.16±0.00^{d}	41.57±0.00^{d}	0.127±0.128^{b}

*Feed efficiency ratio was determined as the ratio of body weight gained per unit (g) of feed consumed over a period of time (day), The values are shown as Mean±SE. Values with different superscripts within the column are significantly different (p<0.05)

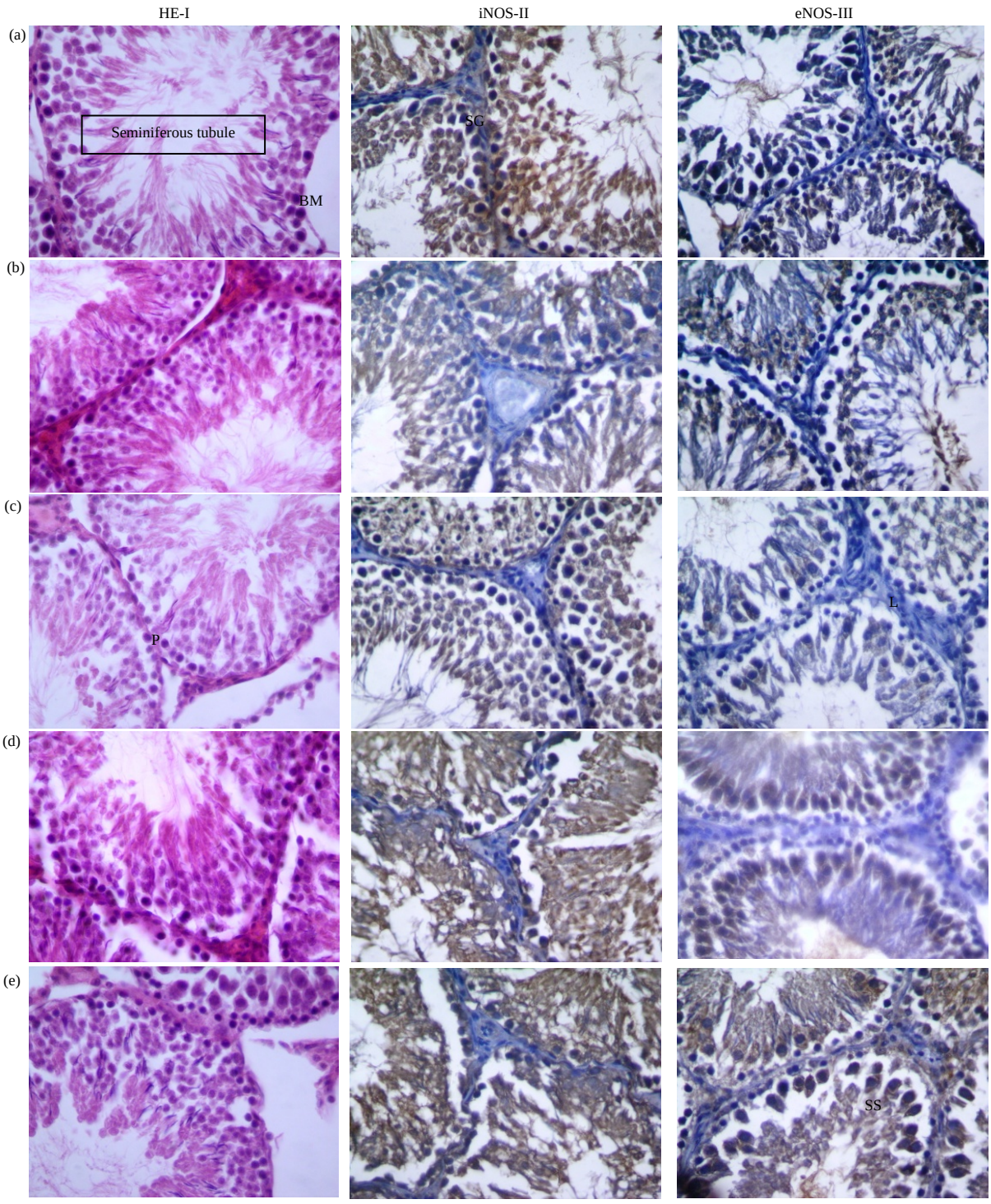

Fig. 1: Photomicrographs of testis. HE-I: H and E staining showed intact basement membrane (BM), pycnotic nuclei cells (P) in the HFD+antioxidants group. iNOS-II and eNOS-III: iNOS and eNOS-III immunoreactivities, intense secondary spermatocytes (SS) and spermatids(S) in the R-HFD+antioxidants and the F-HFD+garlic groups, respectively. (a) F-HFD, (b) R-HFD, (C) F-HFD+antioxidants, (d) F-HFD+garlic and (e) R-HFD+antioxidants

group and (2) iNOS in these cells were intense in F-HFD group. (3) Moderate immunoreactivity (++) was shown for eNOS in spermatocytes I of R-HFD, R-HFD+antioxidants and F-FHFD+garlic groups, spermatocytes II of F-HFD, R-HFD,

F-FHFD+garlic and R-HFD+antioxidants groups, spermatids of F-HFD, R-HFD and R-HFD+antioxidants groups, (4) iNOS was moderate in spermatogonia of F-FHFD group, spermatocytes I of F-FHFD and R-HFD+antioxidants groups, spermatocytes II of R-HFD and F-FHFD+antioxidants and R-FHFD+antioxidants groups, spermatids of F-HFD+antioxidants and F-HFD+garlic groups. (5) Mild immunoreactivity (+) was shown for eNOS in spermatogonia of F-HFD, R-HFD, F-HFD+antioxidants, F-HFD+garlic and R-HFD+antioxidants groups, spermatocytes I of F-HFD, F-HFD+antioxidants groups, spermatocytes II of F-FHFD+antioxidants group, spermatids of F-HFD+antioxidants and R-HFD+antioxidants groups, leydig cells of F-HFD and F-HFD+garlic groups. (6) iNOS was mild in spermatogonia of R-HFD, F-HFD+antioxidants, F-HFD+garlic and R-HFD+antioxidants groups spermatocytes I of R-HFD, F-FHFD+antioxidants F-HFD+garlic groups, spermatids of R-HFD+antioxidants groups and leydig cells of F-HFD and R-HFD groups (7). The expression of eNOS was negative (-) in leydig cells of R-HFD, F-HFD+antioxidants and R-HFD+antioxidants groups. (8) iNOS was negative in leydig cells of F-HFD+antioxidants, R-HFD+antioxidants and F-HFD+garlic groups. There were two rats in R-HFD and one rat in F-HFD+antioxidants groups with hypogonadism. In the rats that were fed with HFD after 12 weeks, the evaluation of staining intensity scores is shown in Table 6 and differences between expression of iNOS and eNOS were compared in all experimental groups (Table 7). Expression rate of iNOS was compared with eNOS (Table 8).

Table 5: Seminiferous tubular diameter of rat after 12 weeks on the experimental diets

Groups	Diameter of seminiferous tubules (μm)
SD	62.33±2.23
F-HFD	57.00±1.85
R-HFD	59.50±2.42
FHFD+antioxidants	56.58±1.95
R-HFD+antioxidants	51.67±1.46
F-HFD+garlic	56.33±2.19

Values are shown as Mean±SE ($p<0.05$)

Table 6: Evaluation of staining intensity scores (by two observers blinded to the study information independently)

Testis-groups	iNOS	eNOS
F-HFD		
Spermatogonium	+/++	+
Primary spermatocyte	++	+
Secondary spermatocyte	+++	+/++
Spermatid	+++	+/++
Leydig cell	+	+
R-HFD		
Spermatogonium	-/+	+
Primary spermatocyte	+	++
Secondary spermatocyte	+/++	++
Spermatid	++	-/+
Leydig cell	-/+	
F-HFD+antioxidants		
Spermatogonium	-/+	-/+
Primary spermatocyte	-/+	-/+
Secondary spermatocyte	+/++	+
Spermatid	+/++	+
Leydig cell	-	-
F-HFD+garlic		
Spermatogonium	+	+
Primary spermatocyte	+	+/++
Secondary spermatocyte	++	++/++
Spermatid	++	++/+++
Leydig cell	-	-/+
R-HFD+antioxidants		
Spermatogonium	+	+
Primary spermatocyte	++	++
Secondary spermatocyte	++	++
Spermatid	+	++
Leydig cell	-	-

Intensity was graded as, -: Negative, +: Mild, ++: Moderate and +++: Intense

DISCUSSION

The results of this study revealed that spermatogenesis function was disturbed in rats fed with a HFD, characterized as decreased body weight, decreased seminiferous tubule diameter, reduced spermatogenesis score and increased iNOS expression. Based on some evidence in the recent decades has of the world's population, obesity can be attributed by environmental factors such as increase in the amount of fat in the diet, High Fat Diet (HFD), is the main cause of obesity[3,4]. Environmental factors, some diseases and

Table 7: Comparison with expression of iNOS and eNOS intensity of rats that were fed with HFD after 12 weeks

	Groups									
	F-HFD		R-HFD		F-HFD+antioxidants		F-HFD+garlic		R-HFD+antioxidants	
Parameters										
Expression	eNOS	iNOS	eNOS	iNOS	eNOS	iNOS	eNOS	iNOS	eNOS	iNOS
Spermatogonium	Mil	Mod	Mil	Mil	Mil	Mil	Mil	Mil	Mil	Mil
Primary spermatocyte	Mil	Mod	Mod	Mil	Mil	Mil	Mod	Mil	Mod	Mod
Secondary spermatocyte	Mod	Int	Mod	Mod	Mil	Mod	Int	Mod	Mod	Mod
Spermatids	Mod	Int	Mod	Mil	Mil	Mod	Int	Mod	Mil	Mod
Leydig cell	Mil	Mil	Neg	Mil	Neg	Neg	Mil	Neg	Neg	Neg

Intensity was graded as Neg: Negative, Mil: Mild (+), Mod: Moderate (++), Int: Intense (+++)

Table 8: Evaluation of expression rate of iNOS and eNOS in rat that were fed with HFD after 12 weeks

Testis-Groups	iNOS expression rate (%)			eNOS expression rate (%)		
	+	++	+++	+	++	+++
F-HFD	17	17	20	40	15	-
R-HFD	32	23	-	32	23	-
F-HFD+antioxidants	32	17	-	33	20	-
F-HFD+garlic	32	23	-	21	21	13
R-HFD+antioxidants	32	23	-	13	38	-

Intensity was graded as, -: Negative, +: Mild, ++: Moderate and +++: Intense

different nutritive substances may directly or indirectly influence spermatogenesis.

In the present study, body weight increased in F-HFD more than R-HFD group. These results demonstrate the positive effect of calorie restriction on prevention of body weight raise and support the results obtained by Hall *et al.*[26]. Hariri *et al.*[27], Park *et al.*[28] and Mu *et al.*[29] also observed overweight in animals fed with a high fat diet, regardless of the lipid quality. Unexpected results of present study showed that antioxidants treatment cannot improve increased body weight. These results were in disagreement with the Mu *et al.*[29].

One of the parameters used for the evaluation of spermatogenesis is the seminiferous tubule diameter[30]. In present study, the seminiferous tubule diameter was lower in restricted HFD than the free HFD group. Campos-Silva *et al.*[31] also reported that long-term administration diet rich in saturated fatty acids alters the testicular morphology with reductions of seminiferous tubule diameter and cell proliferation which could be related to a disturbance of spermatogenesis.

Antioxidants administration in the both F-HFD and R-HFD groups caused more reduction in seminiferous tubule diameter. With respect to the spermatogenic cell layers and mature sperms and spermatogenesis score RHFD+antioxidants and HFD+antioxidants groups also showed decrease compared with the R-HFD and F-HFD groups respectively, indicating a decrease in spermatozoa production. Some studies revealed the protective effect of antioxidants against sperm damage in rats fed with HFD which contradicts present results. It seems unexpected results regarding to antioxidents effect in the present study were due to that the effects of antioxidants on the oxidation of foods are dependent on their concentration[32], polarity and the medium[33,34] and also the presence of other antioxidants[35]. Moreover when there are 2 or more antioxidants together, interaction occurs such as synergism, antagonism and simple addition[36]. Results of Adewoyin *et al.*[37] showed normal functioning of the spermatozoa may require a balance between ROS and antioxidant.

Nitric oxide synthases (NOS) isoforms producing nitric oxide (NO) that is a free radical involved in physiologic and pathologic interactions with ROS[11]. Expression of eNOS and iNOS showed in leydig cells, primary spermatocytes and spermatids by IHC method in the testis of pigs. Seemingly, these NOS isoforms contribute to the process of spermatogenesis[14].

In the present study, eNOS immunoreactivity was mild in spermatogonia of F-HFD group. Moderate in primary spermatocytes and secondary spermatocytes of the R-HFD and RHD+antioxidants groups, spermatids in F-HFD, F-HFD+antioxidants and R-HFD+antioxidants, leydig cells of F-HFD+antioxidants and R-HFD+antioxidants groups, intense secondary spermatocytes and spermatids were in the R-HFD+antioxidants groups. It was shown that normal germ cells were not eNOS stained, it was detectable in some degenerating germ cells, also presence of eNOS protein was shown at all stages of spermatogenesis. The eNOS was localized in leydig cells, sertoli cells and degenerating or apoptotic intraepithelial germ cells. Moreover, eNOS overexpression happened in prematurely shed spermatocytes and spermatids[14]. Bayatli *et al.*[38] showed that oxidative stress induced by torsion gives rise to serious damage in testis and increases in eNOS expression level. Grape seed proanthocyanidin extract as a potent antioxidant agent decreased eNOS expression and improved testicular morphology. Present study were agreed with these findings.

The results of iNOS immunoreactivity in this study were evaluated as following: mild in spermatogonia of F-HFD group, moderate in primary spermatocytes and secondary spermatocytes of the R-HFD and RHD+antioxidants groups, spermatids of F-HFD, F-HFD+antioxidants and R-HFD+antioxidants, leydig cells of F-HFD+antioxidants and R-HFD+antioxidants groups, intense in secondary spermatocytes and spermatids of the R-HFD+antioxidants group. The over expression of iNOS has reported in other tissues in the HF diet condition. Park *et al.*[28] showed liver iNOS expression pattern in the HF group.

These findings were in accordance with Uzun *et al.*[15] study that up-regulation of iNOS occurred in the damaged seminiferous epithelium resulting from LPS-induced inflammation. Atta *et al.*[39] also reported that thymoquinone treatment exerted a protective effect against reproductive dysfunction induced by diabetes not only through its powerful antioxidant and hypoglycemic effects but also through its down regulation of testicular iNOS expression levels in diabetic rats.

The iNOS was a critical factor in harming spermatogenesis in adult rats[16]. In idiopathic varicocele, over expression of iNOS was observed in leydig cells[17]. The results of Asgharzade *et al.*[40] study also showed that iNOS may play a functional role in spermatogenesis via apoptosis.

The present study showed that restricted HFD+antioxidants had higher disturbing effect on spermatogenesis than free HFD access. Restriction of high fat diet and adding antioxidants seems have not therapeutic effect for disturbing changes in testis. It is suggested to do research on basic causes of oxidative stress in the reproductive tract of male subjects and also prepare optimized antioxidants to treat conditions due to an imbalance in the redox status of testis. This could develop the knowledge about male fertility in obese men.

CONCLUSION

Results of this study revealed that restricted fat diet consumption caused increase in weight, although the restriction in high fat diets a beneficial way in weight loss. In this study impaired spermatogenesis could not improve by adding antioxidants. HFD could induce testicular iNOS expression. Since the intense iNOS was expressed in secondary spermatocytes and spermatids of seminiferous tubules in R-HFD+antioxidants group, confirms a potential role of iNOS in disturbing changes of spermatogenic layer and possible roles in male fertility.

SIGNIFICANCE STATEMENT

High and restricted fat diet consumption impairs spermatogenesis and could not improve by adding antioxidants. Over expression of iNOS in seminiferous tubules in restricted fat diet+antioxidants condition, confirms a potential role of iNOS in destructive changes of testis and its possible role in male fertility.

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