Polish Journal of Veterinary Sciences Vol. 20, No. 3 (2017), 467–475

DOI 10.1515/pjvs-2017-0056

Original article

Spirulina platensis affects factors involved in spermatogenesis and increases ghrelin receptors in testis tissue of rats fed a high-fat diet

O.B.B. Esener[1], E. Gurel-Gurevin[2], B. Isbilen-Basok[3], F. Yigit[1], T. Bilal[4], A. Altiner[5], N. Yilmazer[6], E.I. Armutak[1]

[1] Istanbul University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Avcilar Campus, Avcilar, Istanbul, 34320, Turkey
[2] Istanbul University, Faculty of Science, Department of Biology, Vezneciler, Fatih, Istanbul, 34134, Turkey
[3] University of Health Sciences, Tepecik Training and Research Hospital, Guney Mah Yenisehir, Konak, Izmir, 1140/1, Turkey
[4] Istanbul University, Faculty of Veterinary Medicine, Department of Animal Nutrition of Nutritional Disease, Avcilar Campus, Avcilar, Istanbul, 34320, Turkey
[5] Istanbul University, Faculty of Veterinary Medicine, Department of Biochemistry, Avcilar Campus, Avcilar, Istanbul, 34320,Turkey
[6] Namik Kemal University, Faculty of Arts and Sciences, Department of Biology, Degirmenalti Campus, Tekirdag, 59030, Turkey

Abstract

Ghrelin is a peptide hormone which plays important role in maintaining growth hormone release and energy homeostasis in vertebrates. *Spirulina platensis* (SP) has antioxidant and hypolipidemic effects due to its ingredients. In this study we aimed to investigate the effects of SP on the testicular structure and relation between ghrelin and testosterone in the testis of rats fed a high fat diet (HFD). Sixty four young adult male rats were used and divided to 8 equal groups. Experimental groups received addition of 10% cholesterol (CHL), 43% hydrogenated vegetable oil (HVO) and 3% SP alone or in combination to basal diet while the control group received only basal diet. Serum ghrelin and testosterone levels were measured with ELISA. Receptors for ghrelin and androgen were detected with immunohistochemistry. For histomorphometric investigation, tubulus seminiferus, intertubular area, tubulus seminiferus lumen, Leydig cell nucleus, Sertoli cell nucleus, germ cell nucleus, spermatocyte nucleus and elongated spermatid volume densities were determined stereologically. Serum ghrelin level was increased especially in HVO and CHL combination group compared to the control while serum ghrelin levels were close to control levels in SP-received groups. Ghrelin receptor level was increased in tubulus seminiferus with HVO+CHL administration but this effect was, however, limited in HVO+CHL and SP challenged groups. HVO+CHL administration caused a significant decrease in Leydig cell nucleus volume density, as well as in all SP-received groups, compared to the control. Significantly increased spermatocyte nucleus volume density in cholesterol-receiving groups was decreased to control level with SP alone and its combinations.

Key words: Ghrelin, *Spirulina platensis*, testis, apoptosis, proliferation

Correspondence to: E.I. Armutak, e-mail: elif@istanbul.edu.tr, tel.: +90 212 473 70 70-17179

Introduction

Obesity, which is common due to excessive consumption of high fat foods, is associated with a deficiency of antioxidant defense capacity and an increase in oxidative stress (Meineri et al. 2009). This medical condition has been defined as a risk factor for certain reproductive and metabolic disorders (Mayes and Watson 2004). It has been known for some time that obese men exhibit decreased levels of total testosterone (Glass et al. 1977) and high fat diet (HFD) has been reported to cause decreased testosterone levels in rats (Erdemir et al. 2012).

Ghrelin discovered by Kojima et al. (1999) is a peptide hormone and is mainly produced by the oxyntic cells in the gastric mucosa, whereas it is also expressed in minor amounts in other organs including gonads (Gualillo et al. 2003, Kojima and Kangawa 2005). Ghrelin plays important roles for maintaining GH release and energy homeostasis in vertebrates (Kojima and Kangawa 2005). Ghrelin has several effects including regulation of reproductive functions, pregnancy and lactation (Repaci et al. 2011). It was also proven to significantly inhibit, in a dose-dependent manner, stimulated testosterone secretion (García et al. 2007). However, there are few data on reproductive outcomes in the obese male. Although studies reporting the relation between obesity and male infertility are available, the exact mechanisms that underlie infertility have not been documented well (Vigueras-Villaseñor et al. 2011).

Spirulina is a microscopic filamentous blue-green alga (photosynthesizing Cyanobacterium) that is rich in proteins, vitamins, essential amino acids, minerals and essential fatty acids (Dartsch 2008). The antioxidant (Dartsch 2008) and hypolipidemic (Small 2011, Strasky et al. 2013) properties of *Spirulina* have been reported previously.

In this study we aimed to investigate the relationship between ghrelin and dietary *Spirulina platensis* with an emphasis on the effects on the testis tissues of rats fed a high fat diet.

Materials and Methods

Animals

Our experiment was approved by the Animal Care and Use Committee of Istanbul University (Ethics committee number: 2011/20). All animals were housed in polypropylene cages in a controlled environment and under a 12 h dark/12 h light cycle. Animals were fed *ad libitum*.

In the present study 64 young adult male Sprague-Dawley rats were randomly divided into 8 equal groups including control and seven experimental groups. The control group received only a basal diet while experimental groups received the basal diet supplemented with 10% cholesterol (CHL), 43% hydrogenated vegetable oil (HVO) and 3% *S. platensis* alone or in combination. The experimental groups were called as HVO group (Group 2), CHL group (Group 3), HVO+CHL (Group 4), SP group (Group 5), HVO+SP group (Group 6), CHL+SP group (Group 7), and HVO+CHL+SP group (Group 8), designated G2 to G8, while the control group as G1. Rats had access to food and water *ad libitum* during 60 days. At the end of this period, blood samples and testes were collected at slaughter after cervical dislocation. Testis tissues were removed immediately, and after recording testis weights they were fixed with Bouin's solution. Powdered *S. platensis* was purchased from Algbiyoteknik Dis Ticaret Ltd. (Istanbul, Turkey).

Hormone Analyses

Serum Testosterone Levels: The blood tubes were centrifuged at 2.500 x *g* for 15 min, the serum samples were aliquoted in 1.5 mL microcentrifuge tubes and then stored at -80°C until use. All the samples were measured together to avoid inter-assay variation. A commercial ELISA kit was used for the quantitative determination of testosterone in rat serum (Catalog no. YHB1031Ra; Shanghai Yehua Biological Technology Co. Ltd, China). The lowest limit of the assay was 0.25 nmol/L. Test's intra-assay and inter-assay coefficients of variations (CVs) were under 10% and 12%, respectively.

Serum Ghrelin Levels: Serum ghrelin levels were determined by enzyme immunoassay using a commercial kit (A05118, SPI-BIO Bertin Pharma, F-78180 Montigny le Bretonneux, France).

Immunohistochemistry (IHC): Ghrelin receptor (GR), androgen receptor (AR) and proliferating cell nuclear antigen (PCNA) levels were examined immunohistochemically. The streptavidin-biotin complex (StrepABC) method was applied. 4 µm thick sections were collected on positively charged slides, deparaffinized in xylene and rehydrated in a descending series of ethanol. The sections were subjected to antigen retrieval in citrate buffered solution (pH 6.0) for 10 min in a microwave oven at 700 Watts. Endogenous peroxidase was eliminated by incubation in 3% H_2O_2 (Merck, 1.08597) in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) for 10 min. They were blocked in a blocking buffer (Thermo Scientific, TA-125-UB; Deutsch, Germany) for 10 min at room

temperature. Afterwards, they were incubated with GR; 1:300 (Abcam, ab85104), AR; 1:200 (Santa Cruz, sc-816) and PCNA; 1:100 (Santa Cruz, sc-7907) antibodies overnight (18 hours) at 4°C. The sections were incubated with biotinylated goat anti-rabbit antibodies (Thermo Scientific, TP-125-BN) for 30 min at room temperature. They were then incubated with streptavidin-peroxidase label reagent (Thermo Scientific, TS-125-HR) for 30 min at room temperature. Colored product was developed by incubation with AEC (3-amino-9-ethyl carbazole) substrate kit (Thermo Scientific, TA-004-HAC) for 10 min. The sections were counterstained with Mayer's haematoxylin for 1 min and mounted in vision mount. Control procedures were performed on adjacent sections of the same slides. No immunolabelling was detected when the primary antibody was omitted or replaced with PBS.

DNA Fragmentation: A commercially available terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (S7101, ApopTag Plus Peroxidase In Situ Apoptosis Kit, MILLIPORE, Massachusetts, USA) was used to detect apoptosis in testis tissue. After being deparaffinized in xylene, the sections were digested with 20 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) at room temperature for 15 min, incubated at room temperature for 5 min in 3% H_2O_2 in PBS to quench endogenous peroxidase. They were then incubated with equilibration buffer at room temperature for 30 min, followed by the application of TdT enzyme for 1 h at 37°C. Anti-digoxigenin peroxidase was applied for 30 min at room temperature. Methyl green was used for counterstaining. Distilled water was used instead of TdT for negative controls (Taskin et al. 2009).

Evaluation of GR Immunostaining: Immunostaining for GR was quantified using a histological score (HSCORE) (Senturk et al. 1999, Taskin et al. 2008). Briefly, tissue sections were stained with antibody against GR, and then observed under an Olympus microscope equipped with a special ocular grid. Cells were counted in eight different fields at 400x magnification. Based on staining intensity, positively stained cells were scored as: 0, no staining; 1, weak staining; 2, distinct staining; 3, intense staining. For each slide, an HScore value was calculated as: HScore = Pi (i + 1) where "i" is the intensity score and "Pi" is the corresponding percentage of cells with that score.

Evaluation of AR Immunostaining: Semi quantitative evaluation was carried out in 7-8 fields in every section for androgen receptor immunostaining, and positively stained cells were scored as: 0, no staining; 1+, weak staining; 2+, distinct staining; 3+, intense staining (McKinnell et al. 2001).

Proliferation and Apoptotic Index: Positively stained cells from each slide prepared for IHC and TUNEL techniques were considered to calculate proliferation and apoptotic indices. Eight microscopic fields with around 800-1000 cells were counted per each slide at 400x magnification (Taskin et al. 2008).

Statistical Analysis: All evaluations were performed independently by two blinded observers. All slides were observed and photographed using a Leica DM4000 B microscope (Leica). Cell count results were statistically analyzed by the one way ANOVA and Duncan tests, whereas ghrelin and testosterone levels, IHC, ghrelin HSCORE and testosterone distribution results by the ANOVA and Kruskal Wallis methods, by using SPSS (version 13.0) program for all analyses. The results were expressed as mean ± SE. A value of $p<0.05$ was considered statistically significant.

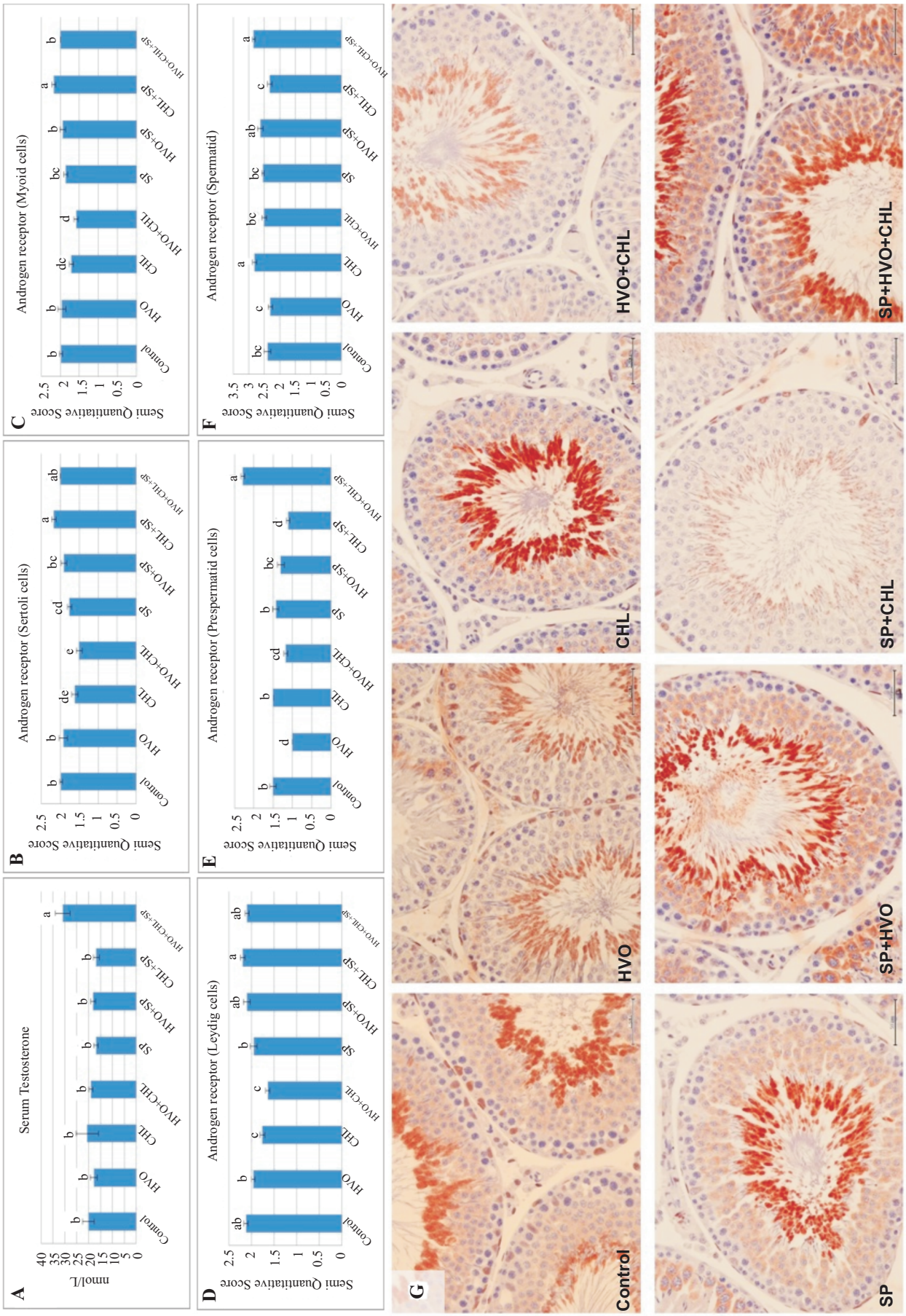
Histomorphometric Evaluation: Tubulus seminiferus volume density, tubulus seminiferus lumen volume density and volume densities of intertubular area, Leydig cell nucleus, Sertoli cell nucleus, germ cell nucleus, spermatocyte nucleus and elongated spermatid were measured using stereological methods based on point counting. A total of 30 random fields from three sections taken from three different parts of the left testis of each animal were selected for measurements.

Results

There was no statistically significant difference between groups in terms of right testis weight (RTW), left testis weight (LTW), total testis weights (TTW) and gonado-somatic indices.

Serum Testosterone Levels: Serum testosterone level was only increased in HVO+CHL+SP group (G8) ($p<0.01$), while its levels in other groups were close to that of the control group (Fig. 1A).

Androgen Receptor Levels: A decrease ($p<0.001$) in AR level in Sertoli cells, Leydig cells and myoid cells was observed in HVO (G2), CHL (G3), and especially in HVO+CHL (G4) groups (Fig. 1B,1C,1D). Similarly, a significant decrease ($p<0.001$) in AR level occurred in pre-spermatid cells of HVO group (G2) (Fig. 1E), while spermatid cells of CHL group (G3) showed a significant increase ($p<0.001$) in AR level (Fig. 1F). These levels became close to the control level with SP combination (G6-7). In HVO+CHL+SP group (G8) AR level was significantly increased in pre-spermatid cells ($p<0.001$) (Fig. 1G), in parallel to the increase in serum testosterone level.


A
Serum Testosterone
nmol/L
B
Androgen receptor (Sertoli cells)
Semi Quantitative Score
C
Androgen receptor (Myoid cells)
Semi Quantitative Score
D
Androgen receptor (Leydig cells)
Semi Quantitative Score
E
Androgen receptor (Prespermatid cells)
Semi Quantitative Score
F
Androgen receptor (Spermatid)
Semi Quantitative Score
Control
HVO
CHL
HVO+CHL
SP
HVO+SP
CHL+SP
HVO+CHL+SP
G
Control
HVO
CHL
SP+CHL
SP+HVO
SP+HVO+CHL
SP

Serum Ghrelin Levels: Serum ghrelin level was increased especially in HVO+CHL group (G4) compared to the control (G1) ($p<0.05$), while there was no difference in HVO group (G2). Serum ghrelin levels were close to control levels in SP receiving groups (G5-8) (Fig. 2A).

Ghrelin Receptor Levels: HScore results showed that the quantity of ghrelin receptor was elevated in tubulus seminiferus in all groups compared to the control ($p<0.001$). The highest increase was in HVO+CHL group (G4) ($p<0.001$). The increase of ghrelin receptor level in tubulus seminiferus in HVO and CHL groups (G2-4) was inhibited in SP groups (G6-8) ($p<0.001$) (Fig. 2B, Fig. 2D). In Leydig cells, the quantity of ghrelin receptor was elevated in HVO and CHL groups (G2-4) while HVO+CHL group (G4) exhibiting the highest increase, compared to the control ($p<0.001$) (Fig. 2C). However, this increase was inhibited in CHL+SP and HVO+CHL+SP groups (G7-8) (Fig. 2D) ($p<0.001$).

Apoptotic Index: Increased apoptotic index was seen in HVO and CHL receiving groups (G2-4, respectively) ($p<0.001$). There was no statistical difference between SP group (G5) and the control. In SP combination groups (G6-8), increased apoptotic index dropped close to the control value, but there was still a statistical difference compared to the control ($p<0.001$) (Fig. 3A).

PCNA: Proliferation was low in SP (G5) and HVO+CHL+SP (G8) groups, compared to the control ($p<0.001$). A significant decrease in proliferation index ($p<0.001$) was also seen in HVO (G2) and CHL (G3) groups, whereas the increase was not significant ($p>0.05$) in HVO+CHL group (G4) when compared to the control (Fig. 3B).

Histomorphometric Results: While there was a significant decrease of cell volume in SP groups (G6-8) compared to the control, a similar decrease in Leydig cell nucleus volume density ($p<0.05$) was found in HVO+CHL group (G4) (Fig. 4A). Spermatocyte nucleus volume density was significantly higher ($p<0.05$) in CHL receiving groups (G3-4) in comparison with the control, but it has dropped to the control value when CHL was combined with SP (G7-8) or given alone (G5) (Fig. 4B). There was no statistical difference between the groups in terms of other histomorphometric parameters investigated.

Discussion

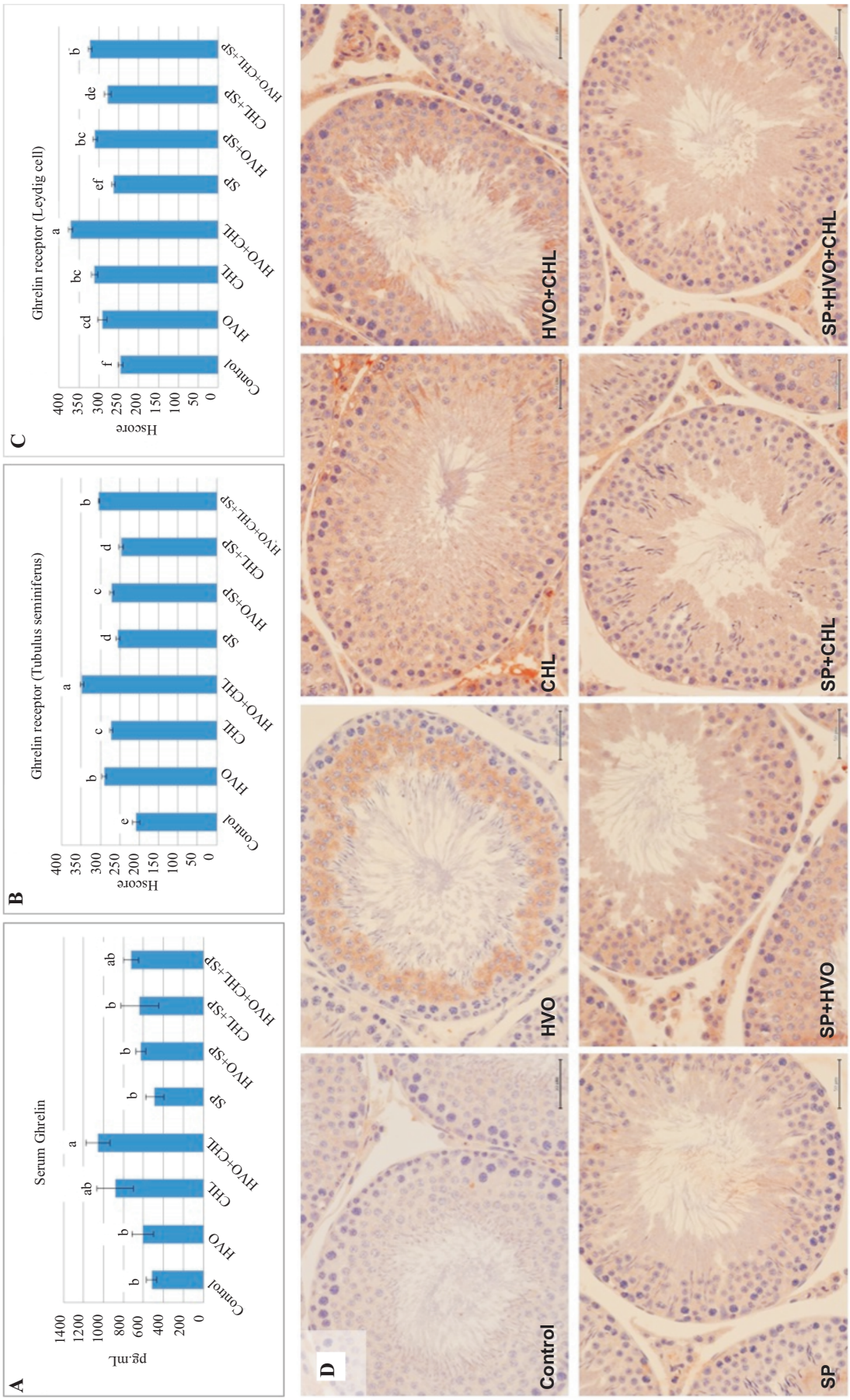
Several papers are available, which report a significant decrease in testicular weight of rats fed with a HFD (Yan et al. 2015, Gujjala et al. 2016), beside those reporting no significant difference in testicular mass (Hu et al. 2013, Campos-Silva et al. 2015). A previous study (El-Desoky et al. 2013) stated that administration of SP, suspended in water, by gavage for 60 consecutive days did not cause any significant difference in testis weights. Likewise, when compared to the control, HFD supplemented with SP (G6-8) did not make any statistical difference in the results of RTW, LTW, TTW and total testis weight/body weight ratio in our study.

Previously, some studies reported that HFD causes a decrease in plasma ghrelin levels in male mice (Briggs et al. 2010) and in Sprague Dawley rats (Lee et al. 2002). Yet, our study showed that serum ghrelin levels increase with the HFD, especially supplemented with combined HVO and CHL (G4). Increased serum ghrelin levels observed in the groups receiving CHL-supplemented diet were inhibited with SP supplementation (G8). This inhibiting effect was not seen in HVO+SP group (G6) compared to HVO group (G2). Our results which are discordant with those of the previous studies may arise from the different experimental periods, animal's species, and the source of HFD. It is so obvious from our results that *S. platensis* is effective in decreasing serum ghrelin level in rats fed a cholesterol-based HFD by means of its hypocholesterolemic effect reported elsewhere (Nagaoka et al. 2005).

The amount of dietary fat and its composition, as well as the period during which the animals are fed a diet containing fat significantly affect the secretion and metabolism of androgens (Gromadzka-Ostrowska 2006). A significant decrease in serum testosterone level has been reported in male rats fed a HFD in a study by Erdemir et al. (2012) although a mouse study stated that HFD does not cause any significant difference in serum testosterone level (Bakos et al. 2010). On the other hand, serum testosterone level is in negative relation with ghrelin expression in Leydig cells (Ishikawa et al. 2007), and ghrelin inhibits stimulated testosterone release, depending on the dose (Garcia et al. 2007). In our study the only statistically significant increase in testosterone level was seen in

←

Fig. 1. Serum testosterone levels (nmol/L) in groups (A). Androgen receptor level (arbitrary units of density staining) in; Sertoli cells (B), myoid cells (C), Leydig cells (D), prespermatid (E) and spermatid cells (F). Androgen receptor staining can be seen in the cytoplasm of the cells (G) (Scale bars: 50 μm). Columns that do not share a common letter are significantly different (A: $p<0.01$) (B, C, D, E, F: $p<0.001$).


A
Serum Ghrelin
pg.mL
0
200
400
600
800
1000
1200
1400
Control
HVO
CHL
HVO+CHL
SP
HVO+SP
CHL+SP
HVO+CHL+SP
B
Ghrelin receptor (Tubulus seminiferus)
Hscore
C
Ghrelin receptor (Leydig cell)
Hscore
D
Control
HVO
CHL
HVO+CHL
SP
SP+HVO
SP+CHL
SP+HVO+CHL

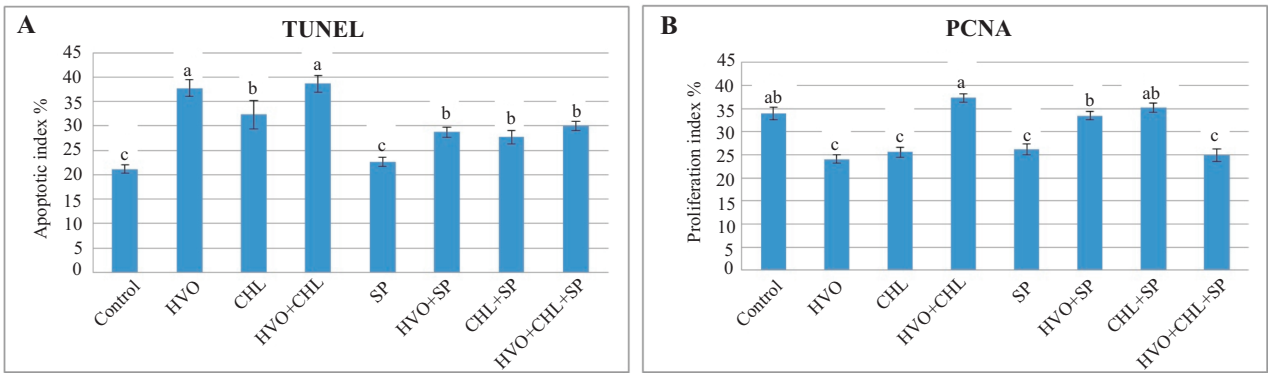


Fig. 3. Apoptotic index (%) (A) and proliferation index (%) (B) values in groups. Differences between columns that do not share a common letter are statistically significant ($p<0.001$).

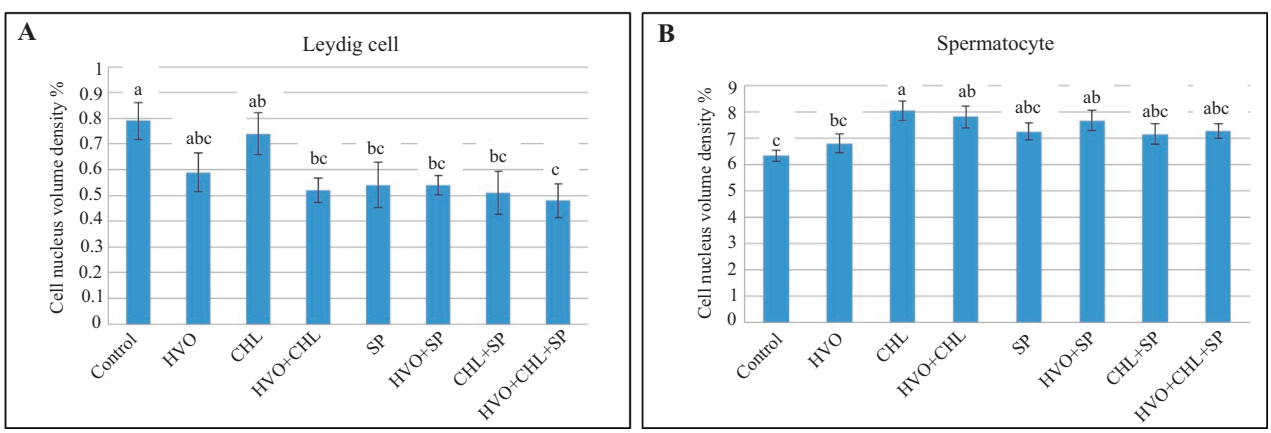


Fig. 4. Leydig cell nucleus volume density (A) and spermatocyte cell nucleus volume density (B) in groups. Differences between columns that do not share a common letter are statistically significant ($p<0.05$).

HVO+CHL+SP group (G8), but there was no decrease in ghrelin receptor levels in tubulus seminiferus and Leydig cells. On the contrary, increased ghrelin receptor levels were observed. However, the significant increase in serum ghrelin level did not cause any difference in serum testosterone level in HVO+CHL group (G4).

A PCNA immunohistochemistry study reported a significant decrease in cell proliferation in the testis of Wistar rats fed a HFD rich in saturated fatty acids (Campos-Silva et al. 2015). In a previous study using TUNEL method, apoptotic index was reported to significantly increase in testis of rats fed a HFD (Yan et al. 2015). In accordance with these studies, apoptotic index was significantly higher in HVO+CHL group (G4), and cell proliferation was significantly decreased in HVO (G2) and cholesterol (G3) groups in our study. By contrast, there was a nonsignificant increase in cell proliferation in HVO+CHL group (G4). Our results on apoptotic index demonstrated that SP supplementation inhibited the significantly increased apoptosis in HVO+CHL group (G4). Moreover, SP supplementation caused the significantly decreased cell proliferation in HVO+CHL group (G4) to become close to the control. However, SP alone and combined HVO, CHL and SP significantly decreased cell proliferation.

From the previous studies ghrelin is known to inhibit cell death in cardiomyocytes and endothelial cells (Baldanzi et al. 2002), increase cell proliferation

←

Fig. 2. Serum ghrelin levels (pg/ml) in groups (A). Ghrelin receptor Hscore (arbitrary units) values in; Tubulus seminiferus (B) and Leydig cells (C) were increased in HVO, CHL and their combination groups. Addition of *S. platensis* suppresses this increase. Cytoplasmic staining of Ghrelin receptor was seen in the cells. Scale bars: 50 µm (D). The strongest immunostaining in Tubulus seminiferus and Leydig cells was seen in HVO+CHL combination group. Columns that do not share a common letter are significantly different (A: $p<0.05$) (B, C: $p<0.001$).

in PC3 prostate cancer cell line (Jeffery et al., 2002) and in some human breast carcinomas cell lines (Cassoni et al. 2001). In our study both serum ghrelin level and its receptor level in tubulus seminiferus and Leydig cells increased in parallel to the significant increase of apoptotic index in HVO+CHL group (G4). However, increased levels of both serum ghrelin and its receptor in CHL group (G3) were inhibited with SP supplementation.

An *in vivo* study (Fan et al. 2015) demonstrated that the level of androgen receptor expression in testis was reduced and accompanied by decline in testosterone levels in mice fed a HFD. Similarly, our study indicated a decreased androgen receptor level in Leydig cells and Leydig cell nucleus volume density in negative relation with serum ghrelin hormone levels. Even though an increase was, regarding a HFD, observed in serum ghrelin level and ghrelin receptor level in tubulus seminiferus and Leydig cells, there was no statistical difference in serum testosterone levels compared to the control in our study.

Kheradmand et al. (2009) reported that subcutaneous ghrelin administration led to a decrease in spermatogenic cell, Leydig cell and Sertoli cell numbers besides a decrease in tubulus seminiferus diameter and germinal epithelium thickness. Although an increase was present in serum ghrelin levels in the HFD groups of our study, differences in tubulus seminiferus, intertubular area, lumen of tubulus seminiferus, Sertoli cell nucleus, germ cell nucleus and elongated spermatid volume densities were not statistically significant. Moreover, a HFD caused a decrease in Leydig cell volume density and an increase in spermatocyte volume density in our study. These conflict findings may result from different ways of ghrelin application in the studies. Yet, the increased ghrelin level in the serum caused by HFD and the increase in the level of serum ghrelin resulting from ghrelin injection may exert different effects on the body due to the complexity of energy and ghrelin mechanism.

In conclusion, *S. platensis* was determined to have a positive effect on factors involved in spermatogenesis in testis tissue of animals fed on a high fat diet, especially cholesterol-rich diet. Further *in vitro* and *in vivo* studies, which will examine mechanisms of action of S. platensis on factors involved in spermatogenesis in detail, may contribute significantly to the prevention and treatment of nutritional causes of infertility problems.

Acknowledgement

This study was supported by the Scientific Research Projects Coordination Unit of Istanbul University (Project No. 45753), Istanbul, Turkey.

References

Bakos HW, Mitchell M, Setchell BP, Lane M (**2010**) The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse. Int J Androl 34: 402-410.

Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A (**2002**) Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 159: 1029-1037.

Briggs DI, Enriori PJ, Lemus MB, Cowley MA, Andrews ZB (**2010**) Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. Endocrinology 151: 4745-4755.

Campos-Silva P, Furriel A, Costa WS, Sampaio FJ, Gregório BM (**2015**) Metabolic and testicular effects of the long-term administration of different high-fat diets in adult rats. Int Braz J Urol 41: 569-575.

Cassoni P, Papotti M, Ghe C, Catapano F, Sapino A, Graziani A, Deghenghi R, Reissmann T, Ghigo E, Muccioli G (**2001**) Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. J Clin Endocrinol Metab 86: 1738-1745.

Dartsch PC (**2008**) Antioxidant potential of selected *Spirulina platensis* preparations. Phytother Res 22: 627-633.

El-Desoky GE, Bashandy SA, Alhazza IM, Al-Othman ZA, Aboul-Soud MA, Yusuf K (**2013**) Improvement of mercuric chloride-induced testis injuries and sperm quality deteriorations by *Spirulina platensis* in rats. PLoS One 8: e59177.

Erdemir F, Atilgan D, Markoc F, Boztepe O, Suha-Parlaktas B, Sahin S (**2012**) The effect of diet induced obesity on testicular tissue and serum oxidative stress parameters. Actas Urol Esp 36: 153-159.

Fan Y, Liu Y, Xue K, Gu G, Fan W, Xu Y, Ding Z (**2015**) Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. PLoS One 10: e0120775.

García MC, López M, Alvarez CV, Casanueva F, Tena-Sempere M, Diéguez C (**2007**) Role of ghrelin in reproduction. Reproduction 133: 531-540.

Glass AR, Swerdloff RS, Bray GA, Dahms WT, Atkinson RL (**1977**) Low serum testosterone and sex-hormone-binding-globulin in massively obese men. J Clin Endocrinol Metab 45: 1211-1219.

Gromadzka-Ostrowska J (**2006**) Effects of dietary fat on androgen secretion and metabolism. Reprod Biol 6 (Suppl 2): 13-20.

Gualillo O, Lago F, Gomez-Reino J, Casanueva FF, Dieguez C (**2003**) Ghrelin, a widespread hormone: insights into molecular and cellular regulation of its expression and mechanism of action. FEBS Lett 552: 105-109.

Gujjala S, Putakala M, Gangarapu V, Nukala S, Bellamkonda R, Ramaswamy R, Desireddy S (**2016**) Protective effect of Caralluma fimbriata against high-fat diet induced testicular oxidative stress in rats. Biomed Pharmacother 83: 167-176.

Hu GX, Lin H, Lian QQ, Zhou SH, Guo J, Zhou HY, Chu Y, Ge RS (**2013**) Curcumin as a potent and selective inhibitor of 11f-hydroxysteroid dehydrogenase 1: improving lipid profiles in high-fat-diet-treated rats. PLoS One 8: e49976.

Ishikawa T, Fujioka H, Ishimura T, Takenaka A, Fujisawa M (**2007**) Ghrelin expression in human testis and serum testosterone level. J Androl 28: 320-324.

Jeffery PL, Herington AC, Chopin LK (**2002**) Expression and action of the growth hormone releasing peptide ghrelin and its receptor in prostate cancer cell lines. J Endocrinol 172: R7-11.

Kheradmand A, Roshangar L, Taati M (**2009**) The role of ghrelin on the morphometry and intracellular changes in the rat testis. Tissue Cell 41: 105-111.

Kojima M, Kangawa K (**2005**) Ghrelin: structure and function. Physiol Rev 85: 495-522.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (**1999**) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402: 656-660.

Lee HM, Wang G, Englander EW, Kojima M, Greeley GH Jr (**2002**) Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. Endocrinology 143: 185-190.

McKinnell C, Saunders PT, Fraser HM, Kelnar CJ, Kivlin C, Morris KD, Sharpe RM **(2001)** Comparison of androgen receptor and oestrogen receptor beta immunoexpression in the testes of the common marmoset (*Callithrix jacchus*) from birth to adulthood: low androgen receptor immunoexpression in Sertoli cells during the neonatal increase in testosterone concentrations. Reproduction 122: 419-429.

Mayes JS, Watson GH (**2004**) Direct effects of sex steroid hormones on adipose tissues and obesity. Obes Rev 5: 197-216.

Meineri G, Ingravalle F, Radice E, Aragno M, Peiretti PG (**2009**) Effects of high fat diets and *Spirulina platensis* supplementation in New Zealand white rabbits. J Anim Vet Adv 8: 2735-2744.

Nagaoka S, Shimizu K, Kaneko H, Shibayama F, Morikawa K, Kanamaru Y, Otsuka A, Hirahashi T, Kato T (**2005**) A novel protein C-phycocyanin plays a crucial role in the hypocholesterolemic action of *Spirulina platensis* concentrate in rats. J Nutr 135: 2425-2430.

Repaci A, Gambineri A, Pagotto U, Pasquali R (**2011**) Ghrelin and reproductive disorders. Mol Cell Endocrinol 340: 70-79.

Senturk LM, Seli E, Gutierrez LS, Mor G, Zeyneloglu HB, Arici A (**1999**) Monocyte chemotactic protein-1 expression in human corpus luteum. Mol Hum Reprod 5: 697-702.

Small E (**2011**) 37. Spirulina-food for the universe. Biodiversity 12: 255-265.

Strasky Z, Zemankova L, Nemeckova I, Rathouska J, Wong RJ, Muchova L, Subhanova I, Vanikova J, Vanova K, Vitek L, Nachtigal P (**2013**) *Spirulina platensis* and phycocyanobilin activate atheroprotective heme oxygenase-1: a possible implication for atherogenesis. Food Funct 4: 1586-1594.

Taskin EI, Kapucu A, Akgün-Dar K, Yagci A, Caner M, Firat UB, Firat I, Dogruman H (**2008**) Effects of an oral treatment with concentrated grape juice (ENT) on cell NOS (eNOS and iNOS) expression and proliferation in experimentally induced carcinoma in mice. Revue Med Vet 159: 123-129.

Taşkin EI, Akgün-Dar K, Kapucu A, Osanç E, Doğruman H, Eraltan H, Ulukaya E (**2009**) Apoptosis-inducing effects of *Morinda citrifolia* L. and doxorubicin on the Ehrlich ascites tumor in Balb-c mice. Cell Biochem Funct 27: 542-546.

Vigueras-Villaseñor RM, Rojas-Castañeda JC, Chávez-Saldaña M, Gutiérrez-Pérez O, García-Cruz ME, Cuevas-Alpuche O, Reyes-Romero MM, Zambrano E (**2011**) Alterations in the spermatic function generated by obesity in rats. Acta Histochem 113: 214-220.

Yan WJ, Mu Y, Yu N, Yi TL, Zhang Y, Pang XL, Cheng D, Yang J (**2015**) Protective effects of metformin on reproductive function in obese male rats induced by high-fat diet. J Assist Reprod Genet 32: 1097-1104.