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The effects of dietary restriction and administration of β-glucan from *Euglena gracilis* on the sperm characteristics and reproductive organs of rats

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Abstract

The aim of this study was to demonstrate the possible individual and/or synergistic effects of β-glucan and dietary restrictions on the reproductive parameters of rats. For this purpose, forty male *Sprague-Dawley* rats were randomly divided into four equal groups (n = 10 per group). The first group was the control, the second group was kept under dietary restriction (DR), the third group was kept under a dietary restriction and given β-glucan (DR + βG) and the fourth group was supplemented only with β-glucan (βG; 20 mg/kg) intragastrically for 14 days. Motility, vitality and morphology of spermatozoa, reproductive organ weights (testis, vesicula seminalis and epididymis) and seminiferous tubule diameters were evaluated in experimental rats. β-glucan had excellent effects on motility, live spermatozoa rate and the acrosome integrity when compared to the control group ($p < 0.05$). We also observed that β-glucan administration to rats having dietary restriction could improve sperm motility and acrosome integrity ($p < 0.05$). While the β-glucan improved seminiferous tubule diameter ($p < 0.05$), weights of the reproductive organs did not change positively as a result. This study demonstrated that β-glucan treatment significantly improved some spermatological characteristics in rats. Therefore, treatment with β-glucan could be used for its positive effects on motility, spermatozoa vitality rate and acrosome integrity for infertile men.

KEYWORDS

dietary restriction, rat, spermatozoa, testis, β-glucan

1 | INTRODUCTION

Nutrition plays a significant role in human and animal health. The metabolic state of a mammal is known to be tightly linked to its reproductive capacity. The proper maintenance of reproductive function requires a considerable amount of free available energy (Martin et al., 2008).

Reduced energy intake, or dietary restriction (DR), is known to extend lifespan and retard age-related health decline in a number of different species, including worms, flies, fish, mice and rats. Dietary restriction has been shown to reduce oxidative stress, improve insulin sensitivity and alter different neuroendocrine responses and central nervous system functions in animals. Dietary restriction plays an especially profound role in reproductive actions (Martin et al., 2008). Although the reproductive system is sensitive to changes in energy status, the physiological mechanisms that may explain the link between energy balance and reproduction are not clear (Kumar & Kaur, 2013; Luo, Li, Li, Zhang, & Zhang, 2016).

For many years, researchers have been working to increase animal yields. Some feed additives are used for just this purpose; however, antibiotics, hormones and other substances leave detectable residues in animals that are in turn creating health problems in

people. For this reason, researchers have started to work on new natural resources that will not negatively affect animal and human health. β-glucan (βG) is one of the most well-known of these natural sources.

β-glucans are soluble fibres located in the endosperm cell walls of cereals, baker's yeast, certain fungi, mushrooms, algae, brown seaweeds and bacteria (Baldassano, Accardi, & Vasto, 2017; Rieder, Ballance, Böcker, & Knutsen, 2018). β-glucan is a polysaccharide comprised of β-linked glucose molecules (Kofuji et al., 2012.). Glucans have been reported to scavenge free radicals (Sener, Toklu, & Cetinel, 2007; Tsiapali et al., 2001) and exhibit antioxidant activity (Babincova, Baci Ova, Machova, & Kogan, 2002; Krizková et al., 2003).

Reactive oxygen species (ROS) formation and membrane lipid peroxidation have been recognised as major problems for sperm survival and fertility (Guthrie & Welch, 2012). Spermatozoa spontaneously produce a variety of ROS, which is functionally important for driving the tyrosine phosphorylation cascades associated with spermatozoa capacitation. However, when ROS production exceeds the spermatozoa's limited antioxidant defenses, a state of oxidative stress is induced, characterised by peroxidative damage to the spermatozoa plasma membrane and DNA strand breakage in the spermatozoa nucleus. This oxidative stress not only disrupts the fertilising potential of the spermatozoa itself, but also the ability of these cells to create a normal healthy embryo (Aitken, Iuliis, Finnie, Hedges, & McLachlan, 2010).

Despite what is known about βG, no detailed studies have been executed to evaluate any effects caused by adding βG to rat diets, or what effects the combination of βG and DR may have on rat reproductive parameters. For this purpose, this study tested the quality of motility, vitality and morphology of spermatozoa, reproductive organ weights (testis, vesicula seminalis and epididymis) and seminiferous tubule diameters in experimental rats.

2 | MATERIALS AND METHODS

2.1 | Animals and treatment

Forty adult, male, *Sprague-Dawley* rats, 3 months of age and 200 g in weight were used for these experiments. Animals were procured from Experimental Animals Breeding and Research Centre of Uludag University (DEHYUAM), Bursa and were housed three or four per cage under a light cycle with 12-hr light and 12-hr dark (light: 07:00–19:00 hr), at a stable temperature (22 ± 2°C) and humidity level (55% ± 5%). Rats were given free access to tap water and were fed a laboratory chow diet (MBD Laboratory Animal Food Company, Kocaeli, Turkey). The composition of the diet was as follows: protein 18% (min), lipid 2.5% (min), fibre 4% (max), ash 5.5% (max), nitrogen-free extract 57.0% (max), metabolic energy 2,650 kcal/kg (min), water 13% (max) plus various amino acids, minerals and vitamins (data obtained from the supplier). Animal care and procedures were in accordance with the guidelines of the Animal Ethical Committee at Uludag University (permit number for this study: 2012–14/05).

All rats were acclimatised for one week prior to experiment. The rats were randomly divided into four equal groups (n = 10 in each group). The control and βG feeding groups were given tap water and a standard laboratory chow diet ad libitum. The DR and DR + βG groups were fed by dietary restriction (Monday, Wednesday and Friday mornings—the food hoppers were placed the following morning on an every-other-day feeding schedule) and were given tap water ad libitum during the 24-week experimental period (Elal Mus & Ak Sonat, 2015). Previous studies have demonstrated that rats and mice that are maintained on an every-other-day feeding regimens consume approximately 40% less calories over time than animals fed ad libitum (Kumar & Kaur, 2013). Animals in the βG and DR + βG groups were administered 20 mg/kg of βG intragastrically for 14 days following the 24-week housing with dietary restriction (DR + βG group) and ad libitum feeding (βG group). Pure βG was purchased from Sigma-Aldrich (89,862–5G-F).

At the end of experiment, all animals were anesthetised with sevoflurane (2%–4%/100%O_2). Under the sevoflurane anaesthesia, testis, vesicula seminalis and epididymis samples were excised for motility, vitality and morphology of spermatozoa, measurement of these organ weights and seminiferous tubule diameters. Then, overdose pentobarbital sodium (200 mg/kg; i.p.) was injected to sacrifice the animals.

2.2 | Semen collection and evaluation of spermatological parameters

Semen was collected from the epididymis of the rats using the diffusion method (Erkan et al., 2015). The epididymis was placed in a 37°C petri dish (on heat attachment of phase-contrast microscope) containing 1,000 μl TI HEPES, and the cauda epididymis was chopped with a scalpel blade. The lumen of the duct and blood vessels were avoided. The tubule segment was immersed in buffer to avoid exposure of spermatozoa to air and to facilitate dispersion into the buffer. The spermatozoa were allowed to disperse into the medium for 3 min after which, the tissue was removed, and the spermatozoa were left on heat attachment of phase-contrast microscope until for analysis (Seed et al., 1996). Each of the studied semen parameters was measured by the same person throughout the study.

2.3 | Motility evaluation

For sperm motility, a slide that had been warmed to 37°C was placed on a phase-contrast microscope (Nikon Alpha phot YS, Japan), and 5 μl of the epididymal sperm solution was placed on it. The motility was evaluated at a magnification of 400×, and the results were expressed as a percentage. Motility estimations for each sample were performed using three different zones (Erkan et al., 2015). Sperm motility was expressed as %.

2.4 | Vitality (Dead/live spermatozoa)

The vitality of spermatozoa was performed using eosin/nigrosine stain (Hafez, 1993). The live spermatozoa were unstained while the dead spermatozoa absorbed the stain. The stained and the unstained 200 spermatozoa were counted using 1000× microscope objectives and percentage of dead spermatozoa was calculated.

2.5 | Morphology

The sperm smears were prepared (with 5 µl semen sample) on histological slides and stained using Giemsa for morphological analysis (Hafez, 1993). After staining, the slides were dried for at least 90 min, at which time 200 spermatozoa were evaluated under a phase-contrast microscope (Nikon Alpha phot YS) using 1000× magnification. Morphological abnormalities were measured as total defected acrosomes and other morphological defects (OMD; head, midpiece and tail) (Perobelli et al., 2012). Morphological defects were expressed as %.

2.6 | Reproductive organ weights

Under the sevoflurane anaesthesia, all samples, the right and left of testis, vesicula seminalis and epididymis, were excised for measurement of organ weights.

2.7 | Morphometric analysis of the testis

Testes samples were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Five micrometer thick sections were cut from paraffin blocks, mounted on slides and dried overnight. After dewaxing and rehydration, sections were stained with Crossman's triple staining for morphometric examination (Mehraein & Negahdar, 2011).

Tubulus seminiferous contortus that were rounded or nearly rounded were chosen randomly, and the diameters of 30 seminiferous tubules were measured for each group. Micrographs were taken with a light microscope (Nikon 80i).

2.8 | Statistical analysis

Statistical analysis was performed using IBM SPSS version 20. Shapiro–Wilk test was used as normality test. Statistical significance of differences between subdivided groups was analysed with Kruskal–Wallis test followed by Mann–Whitney *U*. Differences were considered significant with p values <0.05.

3 | RESULTS

Differences in the percentages of motility, vitality (via Eosin–Nigrosine), defective acrosomes and other morphological defects (via Giemsa) are presented in Table 1.

Higher motility percentages were observed in the βG group (74.0 ± 2.85) when compared with the other groups ($p < 0.05$). The motility values for the control group were the lowest among the groups ($p < 0.05$).

Eosin–Nigrosin is a staining technique that assesses the vitality of a sperm sample. Although there were no apparent differences in dead spermatozoa rates among the DR, βG and DR + βG groups, the lowest dead spermatozoa rate was observed in the βG group ($p > 0.05$). However, the high dead spermatozoa rate of the control group was apparent ($p < 0.05$).

The rate of defective acrosomes of βG (5.93 ± 0.42) and DR + βG (6.93 ± 0.51) groups was significantly lower than control (11.40 ± 0.42) and DR (9.13 ± 0.46) groups. When the other morphological defect rates were compared, there were no differences among the groups ($p > 0.05$).

The values of the reproductive organ weights (testis, vesicula seminalis and epididymis) are presented in Table 2. The results showed that the weights of the both of right and left testes of control group were significantly higher than the other groups ($p < 0.05$). While the weights of the right and left vesicula seminalis of control group were significantly higher than the DR and DR + βG groups ($p < 0.05$), weights of this organ in βG group were not different compared to control group ($p > 0.05$). The weight of the right epididymis of control, DR + βG and βG groups was significantly different from each other ($p < 0.05$). When the left epididymis was evaluated, only significantly difference was observed between control and DR + βG groups ($p < 0.05$).

The seminiferous tubule diameters of rat testes are presented in Table 3. The diameters of the seminiferous tubules were assessed using Crossman's triple stain (see Figure 1). There was no significant difference observed in the seminiferous tubule diameters between the βG and the DR + βG groups ($p > 0.05$). It did appear that the seminiferous tubule diameters decreased in the DR group

TABLE 1 The effect of DR, DR + βG and βG on spermatological characteristics in rats (Mean ± *SEM*)

Groups	*n*	Motility (%)	Dead spermatozoa rate (%)	Acrosome defect (%)	OMD (%)
Control	10	49.87 ± 3.09^{c}	49.77 ± 1.66^{b}	11.40 ± 0.42^{c}	1.58 ± 0.19
DR	10	64.38 ± 3.16^{b}	32.20 ± 3.80^{a}	9.13 ± 0.46^{b}	1.67 ± 0.14
DR + βG	10	67.50 ± 2.21^{b}	35.33 ± 3.91^{a}	6.93 ± 0.51^{a}	1.29 ± 0.18
βG	10	74.0 ± 2.85^{a}	30.0 ± 3.34^{a}	5.93 ± 0.42^{a}	1.86 ± 0.25

Notes. OMD, other morphological defect rate.
a, b and c: Values with different superscripts in the same column are significantly different ($p < 0.05$).

TABLE 2 The effect of DR, DR + βG and βG on reproductive organ weights in rats (Mean ± *SEM*)

		Testis weight (g)		Vesicula seminalis weight (g)		Epididymis weight (g)	
Groups	*n*	Right	Left	Right	Left	Right	Left
Control	10	1.84 ± 0.04[a]	1.84 ± 0.04[a]	0.74 ± 0.05[a]	0.77 ± 0.06[a]	0.30 ± 0.01[c]	0.29 ± 0.01[a]
DR	10	1.66 ± 0.03[b]	1.67 ± 0.03[b]	0.50 ± 0.04[b]	0.50 ± 0.04[b]	0.27 ± 0.02[abc]	0.27 ± 0.01[ab]
DR + βG	10	1.65 ± 0.04[b]	1.64 ± 0.04[b]	0.50 ± 0.05[b]	0.53 ± 0.04[b]	0.24 ± 0.01[a]	0.25 ± 0.01[b]
βG	10	1.67 ± 0.03[b]	1.68 ± 0.03[b]	0.67 ± 0.04[a]	0.70 ± 0.05[a]	0.27 ± 0.01[b]	0.27 ± 0.01[ab]

Note. a, b and c: Values with different superscripts in the same column are significantly different ($p < 0.05$).

TABLE 3 The effect of DR, DR + βG and βG on seminiferous tubule diameter (μm) in rats (Mean ± *SD*)

Groups	*n*	Means ± *SD*
Control	10	248.43 ± 29.25[b]
DR	10	221.22 ± 40.18[a]
DR + βG	10	337.48 ± 30.62[c]
βG	10	347.08 ± 34.62[c]

Note. a, b and c: Values with different superscripts in the same column are significantly different ($p < 0.05$).

($p < 0.05$). However, βG supplementation to the DR group significantly improved the diameter of the seminiferous tubules ($p < 0.05$). Seminiferous tubule diameters were observed to be very low in the control group compared to groups that were given βG ($p < 0.05$).

4 | DISCUSSION

The goal of this study was to determine whether a dietary restriction combined with βG as an antioxidant has any effect on sperm characteristics, reproductive organ weights and the diameters of seminiferous tubules in adult male rats.

It has been indicated that antioxidant agents positively affect sperm parameters (motility, integrity of plasma membrane and acrosome) and the actions of antioxidants have been confirmed by Turk, Sonmez, Cereribası, Yuce, and Atessahin (2010). Koc, Erısgın, Tekelıoglu, and Takır (2018) also noted that βG has a protective effect on rat testicular tissue damage resulting from chemotherapeutic agents.

Motility is the best indicator of sperm functionality (D'alessandro, 2001). Reactive oxygen species (ROS) can trigger serious damage to various sperm functions, including motility or DNA integrity (Martorana, Klooster, & Meyers, 2014). It is accepted that βG has antioxidant activity (Kofuji et al., 2012) and therefore may be responsible for increased motility. Motility was also improved in our DR group. Positive affect of DR on sperm quality may be due to reduce oxidative stress and effect on neuroendocrine responses. These results are consistent with Vahidinia, Rahbar, and Mahmoodabadi (2017) and Pascual et al.. (2016), who observed the same trend in rats and rabbits respectively. However, Wu, Wan, Sun, and Liu (2002) and Kabir, Oni, Akpa, Adeyinka, and Rekwot (2007) reported that DR has adverse effects on sperm motility in mice and *Rhode Island* chicken sperm. These varying results may be due to species diversity and differences in the percentages of restricted diets in the experimental studies. There is no study involving the effects of βG and βG + DR may have on rat spermatological characteristics. In the present study, βG further strengthened the beneficial effects that DR has on motility.

Rate of dead/live spermatozoa is important for successful sperm activation, acrosome reaction and oocyte binding (Sitzmann et al., 2010). The structure of the plasma membrane consists of high levels of polyunsaturated fatty acids (PUFAs) that improve membrane flexibility, but also make the spermatoza vulnerable to ROS (Eskenazi et al., 2005; Rao, Soufir, Martin, & David, 1989; Sheweita, Tilmisany, & Al-Sawaf, 2005). Increase in lipid peroxidation can seriously impair the functional integrity of the cell membrane, decrease sperm motility and subsequently reduce fertility. Our study showed that there was a higher live spermatozoa rate in DR, βG and βG + DR groups than the control group ($p < 0.05$). There was also a slight decrease in the dead spermatozoa rate of the βG groups compared to the DR group, which may be a result of the antioxidant capacity of βG. Aydilek, Varisli, Kocyigit, Taskin, and Kaya (2015) and Sitzmann et al. (2010) reported no significant differences in the spermatozoa vitality rate of control and DR groups of rats and *Rhesus Macaques*. According to Gredilla et al. (2004), these differences indicate that results from DR depend on many variables such as age, duration and degree of restriction.

Acrosomal enzymes are essential factors for fertilisation, and tight bonds exist between acrosome intactness and the presence of enzymes in the acrosomal cap. Therefore, acrosome integrity is an indicator of the fertilisation ability of the spermatozoa (Watson, 2000). Similar to the motility and dead spermatozoa rate results, βG had a positive effect on the integrity of the acrosome. In present study, the acrosome integrity rates of the DR and control groups were higher than those reported by Aydilek et al. (2015). However, Aydilek et al. (2015) observed that there was no difference in acrosome integrity between the DR and control groups in rats. In present study, there were no significant differences among groups for other morphological defect rates (OMD; $p > 0.05$). Pascual et al. (2016), Sitzmann et al., (2010) and Wu et al. (2002) reported that in rabbits, *Rhesus Macaques* and mice, DR had no effect on OMD.

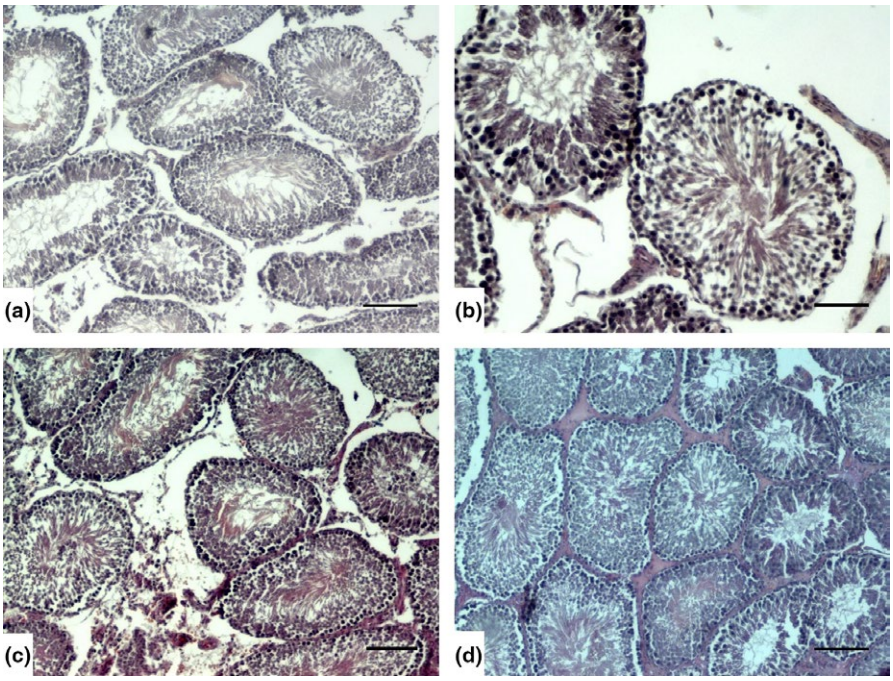

FIGURE 1 Tubulus seminiferous contortus, Testis, (a) Control group, Bar; 100 μm.; (b) DR group, Bar; 50 μm (c) DR + βG group, Bar; 100 μm (d) βG group Bar; 100 μm

To date, there are no studies regarding on the effects of βG administration on sperm parameters and reproductive organ weights (testis, vesicula seminalis and epididymis) of rats. In this study, DR or β-glucan did not beneficially affect testis weight when compared to the control group. Dietary restriction alters other biochemical and metabolic processes that may affect reproductive organ weights (Rehm et al., 2008). Our results are similar to those of Aydilek et al. (2015), who noted that rat testis weight decreased with DR. In this research, testis weights and vesicula seminalis weights were reduced by DR. It is known that decrease testosterone and luteinising hormone (LH) levels caused by dietary restriction, leading to effects on androgen-dependent male rat reproductive organs, including reduced organ weights and testicular degeneration. Also, the greater sensitivity of vesicula seminalis to DR compared with other accessory glandular structures is likely because of its larger proportion of glandular luminal contents relative to organ mass (Rehm et al., 2008). In addition, βG did not adversely affect the organ weight of the vesicula seminalis as DR did.

The results of epididymis weights in DR group of the present study are consistent with the findings of Vahidinia et al. (2017) who reported no difference in the weight epididymis in diet restriction compared with control group. There was only significant difference observed between control and DR + βG groups in terms of left epididymis ($p < 0.05$). Additionally, Ghanayem, Bai, Kissling, Travlos, and Hoffler (2010) reported that diet in male mice was not associated with changes in the average weight of testes or epididymis. These findings show that the different results may be due to the influence of DR and antioxidant supplementation on ROS and consequently sperm quality is independent of weight of the reproductive organs.

It was hypothesised that improving spermatological characteristics ran in parallel to histological parameters (seminiferous tubule diameter). In this study, the widest seminiferous tubule diameter was observed in the βG group. It is also observed that βG reduced the negative effects of DR on seminiferous tubule diameters. These results are consistent with the findings published by Melo, Almeida, Caldeire-Brant, Parreira, and Chiarini-Garcia (2014) who reported that seminiferous tubule diameters were reduced after DR. In this context, we conclude that that βG administration may scavenge directly free radical and protect the testicular structure and increase sperm quality.

In conclusion, βG treatment was found to be highly effective in improving certain spermatological characteristics: motility, vitality and acrosome integrity and seminiferous tubule diameter. Additionally, DR with supplement of βG seems to have positive effects on these parameters. Therefore, treatment with βG can effectively be used to elicit positive effects on the reproductive performance of males and people who are infertile. The reproductive performance impacts of coadministering βG and DR in humans have yet to be determined and warrant further investigation.

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