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# Probiotic-Green Tea Yoghurt on Improving Testicular Histology of High-fat and Fructose Diet Mice

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## ABSTRACT

An unhealthy lifestyle can cause changes in the body's metabolism, leading to obesity. The development of obesity is supported by a disturbance in gut microbiota balance that triggers visceral fat deposition in organs such as the testes. Excess fat deposition triggers inflammation, dysfunction, and high ROS production that can damage testicular tissue. Yoghurt, a fermented milk product fortified with green tea, is high in antioxidants that can help reduce excess ROS. Adding encapsulated probiotics in yoghurt can stabilize the gut microbiota in obesity so that dysbiosis can be resolved. This study was conducted to determine the potential of green tea-probiotic yoghurt (GTY) on testicular tissue repair in mice fed a high-fat and fructose diet (HFFD). The research procedure includes feeding HFFD for 3 months, calculating the Lee index, lactic acid bacteria preparation, microencapsulated probiotics, yoghurt preparation, treatment, data collection, including relative weight of testes, the diameter of seminiferous tubules (DST), the epithelium thickness (ET), the number of Leydig cells (LC), and the number of spermatogenic cells. The mice groups were divided into normal (P0), HFFD (P1), HFFD + simvastatin 1.3 mg/Kg BW (P2), HFFD + plain yoghurt 5 g/Kg BW (P3), HFFD + GTY 2.5 g/Kg BW (P4), HFFD + GTY 5 g/Kg BW (P5), and HFFD + GTY 10 g/Kg BW (P6). The results showed that green tea infusion yoghurt with encapsulated probiotics could improve the structure of testicular tissue in mice after HFFD administration. The most effective dose is green tea yoghurt 5 g/Kg BW.

***Keywords*:** Obesity; ROS; Spermatogenesis; Yoghurt

## INTRODUCTION

The impact of COVID-19 has given rise to unhealthy behavior, starting from a sedentary lifestyle to minimizing the spread of COVID-19. During 24 hours, the activities most frequently carried out were sedentary behavior and sleeping. A sedentary lifestyle, short sleep duration, and late nights can trigger a decline in cardiometabolic health and weight gain.[1]

Weight gain can be caused by a lifestyle that involves consuming salty and sweet snacks and drinks high in sugar over a long

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period.[2] An unhealthy lifestyle can trigger changes in the body's metabolism, leading to obesity. Excessive consumption of fat and fructose causes lipid accumulation, which leads to obesity.[3] Obesity is defined as a body condition with a body mass index (BMI) ≥ 30 kg/m$^2$.[4]

Based on Riskesdas reports, the prevalence of obesity in Indonesia (adults aged 18 years and over) has more than doubled, from 10.5% in 2007 to 21.8% in 2018.[5] Obesity is associated with systemic and chronic inflammation, oxidative stress, increased lipogenesis, and decreased lipolysis. The development of obesity can also be triggered by changes in the composition and activity of the gut microbiota that can affect intestinal barrier defense, decreased thermogenesis, and adipose tissue browning.[6]

High ROS production indicates oxidative stress in the body would disrupt cell activity due to increased protein carbonylation and lipid peroxidation.[789] Oxidative stress affects the reproduction of obese men by exacerbating cell oxidative damage in the form of inhibition of germ cell proliferation and spermatozoa apoptosis.[10] HFFD causes variable seminiferous tubule size, tubule membrane atrophy, and decreased germ cells. Cellular damage due to high ROS can be alleviated by the consumption of exogenous antioxidants.[11,12]

Obesity impacts various dimensions of health. Therefore, requires comprehensive prevention and control interventions. The main interventions required at an individual level may be lifestyle and dietary interventions.[13] Nutrient-dense foods play an important role in a balanced diet.[14] Yoghurt is a nutrient-dense product because it contains a variety of macro and micro nutrients.[15]

Yoghurt can be combined with plants high in polyphenols, such as green tea, to increase its antioxidant content.[16] Adding *L. paracasei* can overcome the gut microbiota dysbiosis caused by obesity.[17] To maintain the physiological effectiveness of *L. paracasei* against adverse conditions while passing through the digestive tract, encapsulation can be carried out.[18] This study was conducted to determine the potential of green tea infusion yoghurt with encapsulated probiotics on the structural repair of testicular tissue in mice fed an HFFD.

## METHODS

### Time and Research Subjects

The research was conducted from May to December 2023 at the Laboratory of Animal Physiology, Structure, and Development, Brawijaya University. The research design used was a completely randomized design (CRD) with a true-experimental research type and a post-test-only control group design.

The experimental animals used were 35 male *Balb/C* mice (*Mus musculus*), five to six weeks old, with 20 g BW (body weight). Induction of HFFD was carried out for 12 weeks. Obese mice were categorized by calculating the Lee index > 0.3 at the end of week 12. Treatment was given in weeks 13 to 16. The treatment of experimental animals was under ethical standards by the Research Ethics Committee of Brawijaya University with Reg No. 155-KEP-UB-2023.

Susu PAP (SP) pellet (Japfa Comfeed Indonesia) was fed to the normal diet group, while HFFD feed was fed to the HFFD induction group with the addition of beef tallow, egg yolk, oxidized oil, and 10% fructose. The diet was modified from the American Institute of Nutrition 93-Growth (AIN93G, Inotiv 2023) and High-Fat Diet-32 (HFD32, CLEA Japan 2015). HFFD mice were given an additional drink with 10% fructose.

The mice were categorized into the following groups.

1. Normal mice (P0; $n = 5$)
2. Mice induced with HFFD for 16 weeks (P1; $n = 5$)
3. Mice induced with HFFD for 16 weeks and 1.3 mg/kg BW simvastatin for the last 4 weeks (P2; $n = 5$)
4. Mice induced with HFFD for 16 weeks and 5 g/kg BW plain yoghurt for the last 4 weeks (P3; $n = 5$)

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5. Mice induced with HFFD for 16 weeks and 2.5 g/kg BW green tea yoghurt for the last 4 weeks (P4; *n* = *5*)
6. Mice induced with HFFD for 16 weeks and 5 g/kg BW green tea yoghurt for the last 4 weeks (P5; *n* = *5*)
7. Mice induced with HFFD for 16 weeks and 10 g/kg BW green tea yoghurt for last 4 weeks (P6; *n* = *5*)

**Lactic Acid Bacteria (LAB) Preparation**

*Streptococcus thermophilus* FNCC 0040, *Lactobacillus bulgaricus* FNCC 0041, *Lacticaseibacillus paracasei* E1 were each taken one ose from agar media and inoculated on each MERCK brand MRS broth media. The bacteria were incubated on a shaker incubator at 150 rpm for 48 hours at 45°C for *S. thermophilus* and 37°C for *L. bulgaricus* and *L. paracasei.*[19]

**Microencapsulation of *Lacticaseibacillus paracasei***

*L. paracasei* with $10^9$ log CFU/mL was mixed with 1.5% alginate (Sigma-Aldrich 71238-250G) in a 1:5 (v/v) ratio. The alginate-bacteria mixture was homogenized and spray-dried at 130°C. The alginate-bacteria powder was mixed in 100 mL $CaCl_2$ and stirred for 30 minutes. The alginate-bacteria mixture was centrifuged at 3500 rpm for 15 minutes. The supernatant was discarded, and the pellet was mixed with 100 mL of 0.5% chitosan (Sigma-Aldrich 448877-250G). Alginate-chitosan was stirred for 40 minutes. The mixture of bacteria-alginate-chitosan was centrifuged and washed with NaCl twice. The pellets were mixed with 100 mL/gram distilled water. The alginate-chitosan coating mixture was spray-dried at 130°C.[20]

**Yoghurt Preparation**

UHT full cream milk (mL) and 6% sugar were placed in a beaker glass. The components were homogenized, and the glass was covered with aluminum foil. The milk mixture was pasteurized in a water bath at 85°C for 30 minutes. The milk was allowed to stand at room temperature. The yoghurt starter bacteria (*L. bulgaricus* and *S. thermophilus*) 3% in a ratio of 1:2, and *L. paracasei* 3% was added to the milk. The milk mixture was incubated overnight at 37°C. Plain yoghurt was sub-cultured two times until the texture of the yoghurt became solid.[19]

Plain yoghurt with added probiotics was prepared by standing the pasteurized milk at room temperature. A total of 9.2 g of yoghurt starter was taken and incorporated into the milk. 2% encapsulated probiotic *L. paracasei* was added and incubated for 30 hours at 37°C.

Green tea yoghurt was made by adding 4% green tea (*Camellia sinensis* L.) dried leaves to pasteurized milk and left to stand overnight. Green tea was obtained from Bird Tea Gallery. The resulting infusion can be warmed in a water bath at 37°C for 30 minutes, and the green tea leaves can be filtered. Green tea-infused milk was supplemented with yoghurt starter, and 2% encapsulated *L. paracasei* probiotics. The milk mixture was incubated for 30 hours at 37°C. The yoghurt was stored at 4°C.

**Testicular tissue isolation and observation**

Mice were injected with ketamine-xylazine and dissected after week 16.[21] Testicular organs were isolated, weighed, and washed in PBS. Testicular organs were fixed in Bouin's solution (25 mL 37% formaldehyde, 75 mL saturated picric acid, and 5 mL glacial acetic acid).[22] Histology preparations were made with hematoxylin-eosin (HE) staining.

Testicular tissue observations include the number of spermatogenic cells, the number of Leydig cells, and the diameter and thickness of the seminiferous epithelium. The tissue was observed using Olympus microscope magnification 200x and 400x. Photographing the preparations was done with OptiLab Viewer 3.0 software. Measurement of DST and ET was performed on Image Raster software. Data on the number of spermatogenic cells, LC, DST, and ET were obtained from the

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average number of five testicular pieces in the preparations for each group.[23]

**Data Analysis**

Testicular relative weight results, ET, and DST measurements were analyzed in parametric tests with normality (Shapiro-Wilk) followed by homogeneity (Levene test). One-way ANOVA was performed to obtain the conclusion that the treatment given is significant or not. The data obtained was continued with Duncan's multiple range test (DMRT) to see the most effective treatment group with $P < a\ 0.05$. The number of spermatogenic cells and LC were tested using non-parametric statistics.

## RESULTS AND DISCUSSION

### Effect of Green Tea Yoghurt on Testicular Relative Weight

The results of measuring the relative weight of the testes in the HFFD group (P1) were lower (significantly different) than the normal group (P0). Meanwhile, the P5 group had the highest value compared to the treatment groups (Table 1). Detailed data on organ weight and organ index are presented in Table 1. The results of simvastatin and yoghurt treatment showed an increase in testicular organ weight after HFFD induction, so the treatment successfully repaired the damage in the testicular organs caused by HFFD induction for 3 months. The treatment that can increase the weight of the testes after HFFD is highest by the GTY at a dose of 5 g/Kg BW.

Based on these results, it is known that giving green tea yoghurt can repair damage to the testes due to HFFD induction. The relative testicular weight of the treatment group is greater than that of the HFFD group. Other studies have shown that the testicular weight in normal mice is higher than in HFFD mice.[24] Testicular weight is an effective indicator of testicular health that can indirectly describe any changes in seminiferous tubule retention or changes in germ cell number.[25]

The decreased testicular weight of HFFD may be due to the decreased population of germ cells caused by cell death under oxidative stress conditions. Green tea may increase testicular weight if consumed long-term (1 month).[26] Antioxidant compounds can neutralize free radicals and prevent them from damaging cells and tissues.[27] Flavonoids have antioxidant and anti-obesity activity.[2829] GTY can reduce ROS and repair damaged testicular tissue.

The results of calculating the relative weight of testes (Table 1) show that the heavier the mice body weight, the lighter the testicular weight, and the smaller the testicular index. The GTY treatment given could cause the relative weight of the testicles to be lower compared to the normal group (P0) and higher than the HFFD group (P1). The decrease in testicular index due to high-fat diet intervention is an indicator of decreased testicular tissue, disrupted endocrine function, loose tissue structure in mice, and decreased sperm count.[30]

**Table 1.** Effect of GTY on testicular weight and relative testicular weight

| Group | Testicular Weight (g) ± SD | Relative Weight (%) ± SD |
|---|---|---|
| P0 | $0.14^{b}$±0.01 | $0.46^{b}$±0.02 |
| P1 | $0.08^{a}$±0.03 | $0.16^{a}$±0.06 |
| P2 | $0.14^{b}$±0.03 | $0.37^{b}$±0.07 |
| P3 | $0.17^{bc}$±0.03 | $0.41^{b}$±0.09 |
| P4 | $0.15^{b}$±0.02 | $0.41^{b}$±0.06 |
| P5 | $0.19^{c}$±0.02 | $0.45^{b}$±0.03 |
| P6 | $0.15^{b}$±0.01 | $0.39^{b}$±0.05 |

Different superscript letters indicate the significant results in each group based on the DMRT test ($p<0.05$).

### Effect of Green Tea Yoghurt on Diameter and Thickness of Seminiferous Tubule Epithelium

The results of measuring DST and ET showed that all groups given treatment (simvastatin and yoghurt) succeeded in increasing the DST and ET of mice induced by HFFD. The data showed that the DST and ET of HFFD mice (P1) were significantly different compared to the normal mice group (P0) (Table 2). The average results showed that DST and ET in

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P1 were lower than P0 ($p \leq 0.05$). Feeding a high-fat diet can reduce DST, which is indicated by a decrease in spermatogenesis activity due to decreased testosterone.[31]

**Table 2.** Effect of GTY on DST and ET

| Group | Diameter (µm) ± SD | Epithelial thickness (µm) ± SD |
|---|---|---|
| P0 | 191.31$^{b}$± 6.67 | 50.28$^{b}$±2.72 |
| P1 | 177.07$^{a}$± 7.28 | 45.40$^{a}$±0.77 |
| P2 | 197.57$^{bc}$± 1.17 | 52.15$^{bcd}$±2.12 |
| P3 | 197.45$^{bc}$± 4.57 | 55.62$^{cd}$±0.62 |
| P4 | 204.63$^{cd}$± 5.05 | 51.68$^{bc}$±2.24 |
| P5 | 218.08$^{e}$± 7.48 | 55.94$^{d}$±2.83 |
| P6 | 211.70$^{de}$± 2.69 | 54.96$^{cd}$±2.07 |

Different superscript letters indicate the significant results in each group based on the DMRT test (*p<0.05*).

The average DST and ET in the treated HFFD group had higher values than the HFFD group (P1). The HFFD group given GTY (P5-P6) significantly differed from the HFFD group (P1). This shows that the treatment given was successful in repairing tissue damage. Based on the group treatment, the green tea yoghurt that provided the best improvement was GTY 5 g/Kg BW.

Green tea leaves contain many polyphenolic flavonoids, dominated by catechins.[32] Green tea contains flavonoids that can transfer hydrogen atoms or chelate metals so they can reduce free radicals. Antioxidants in green tea can donate hydrogen ions ($H^+$) to ROS, resulting in the inactivation of the oxidant compounds' activity.[33] Consuming foods containing flavonoids can protect lipophilic antioxidants so that cellular antioxidants can be increased.

**Effect of Green Tea Yoghurt on Leydig Cell Number**

The results of calculating the number of LC showed that the treatment group could increase the number of LC compared to the HFFD group (P1). The average LC in HFFD mice (P1) was not significantly different from that in normal mice (P0) (Table 3). Based on the results, P1 is lower than P0. The results of the average number of LC in the treatment group were significantly different and had a higher number than HFFD (P1). This proves that the treatment given has a positive impact on the LC. The HFFD group that was given GTY had the highest average number of LC with 5 g/Kg BW.

Oxidative stress created by HFFD can reduce Sertoli cells, Leydig cells, and the number of spermatogenic cells. A decrease in the number indicates the death of LC, thereby affecting a decrease in spermatogenesis.[34] Mice on a high-fat diet for a long period cause hyperlipidemia. Hyperlipidemia conditions can reduce the number of LC. The adverse effects of hyperlipidemia can disrupt the hypothalamic-pituitary axis, increase ROS levels, decrease LH secretion, and interfere with the activation of LC to produce testosterone. The polyphenols contained in green tea have been proven to have more effective antioxidant activity than vitamins C and E.

**Table 3.** Effect of GTY on LC

| Group | Leydig Cell Number ± SD |
|---|---|
| P0 | 14$^{ab}$±3 |
| P1 | 12$^{a}$±1 |
| P2 | 15$^{b}$±1 |
| P3 | 17$^{b}$±2 |
| P4 | 18$^{b}$±2 |
| P5 | 25$^{b}$±3 |
| P6 | 19$^{b}$±3 |

Different superscript letters indicate the significant results in each group based on the DMRT test (*p<0.05*).

**Effect of Green Tea Yoghurt on the Number of Spermatogenic Cells**

The results obtained showed that spermatogenic cells (spermatogonia, spermatocytes, round and elongated spermatids) in HFFD (P1) were significantly different from the normal group (P0). Spermatogenic cells in HFFD mice had the lowest average. After giving green tea yoghurt treatment at each dose, there was an improvement marked by an increase in the number of cells in each

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spermatogenic stage after HFFD (Table 4). The most effective dose in correcting the negative impact of HFFD was green tea yoghurt at a dose of 5 g/Kg BW (Table 4).

The testicular histology of mice from groups P1 to P0 shows the shedding of spermatogenic cells towards the lumen so that a partition layer appears to be formed (red arrow). In addition, the interstitial area of the testis was also reduced when compared with the normal group (Figure 1). HFFD can cause changes in the structure of testicular tissue. The low number of spermatogenic cells can be caused by developmental disorders from the round spermatid stage to elongated spermatids due to oxidative stress. Low proliferation activation of spermatogonia can be caused by testicular conditions unsuitable for spermatogenesis.[35] Green tea contains EGCG, which can suppress metabolic syndrome, obesity, and fatty liver after being induced by a high-fat diet.[36]

**Table 4.** Effect of GTY on Spermatogenic Cells

| Group | Sg | Scyt | RS | ES |
|---|---|---|---|---|
| P0 | $49^{b}$±4 | $46^{ab}$±10 | $73^{bc}$±5 | $29^{bc}$±5 |
| P1 | $42^{a}$±3 | $35^{a}$±3 | $48^{a}$±4 | $17^{a}$±3 |
| P2 | $51^{b}$±1 | $55^{cd}$±9 | $82^{c}$±8 | $28^{b}$±4 |
| P3 | $48^{b}$±2 | $53^{c}$±4 | $65^{ab}$±2 | $33^{bc}$±6 |
| P4 | $47^{ab}$±5 | $56^{cd}$±8 | $66^{b}$±10 | $30^{bc}$±2 |
| P5 | $46^{ab}$±3 | $60^{d}$±4 | $95^{d}$±5 | $37^{c}$±1 |
| P6 | $55^{b}$±3 | $47^{b}$±2 | $89^{cd}$±8 | $36^{c}$±2 |

Different superscript letters indicate the significant results in each group based on the DMRT test ($p<0.05$). Spermatogonia (Sg); Spermatocyte (Scyt), Round spermatids (RS); Elongated spermatids

**Correlation of the Number of Spermatogenic Cells with Relative Testicular Weight**

The relationship between two variables (spermatogenic and testicular index) is classified as a moderate correlation (Table 5). The interpretation of the correlation coefficient is very strong (0.90-1.00), strong (0.7-0.89), moderate (0.40-0.69), weak (0.10-0.39), and correlation negligible (0.00-0.10).[37] The Pearson correlation value has no negative sign before the number, so the correlation formed is positive. A positive correlation means that the size of the testicular index is directly proportional to the number of spermatogenic cells.

Based on the correlation test results between spermatogenic cells and relative testicular weight, the significance value is <0.05, so it can be concluded that the two variables correlate. Pearson correlation (r) between two variables has an asterisk indicating a correlation between them with a value of 0.664. The coefficient of the Pearson correlation scale ranges from -1 to +1.[37]

**Table 5.** Correlation Between Spermatogenic Cells and Relative Testicular Weight

| Correlation | Spermatogenic cells | Relative Testicular Weight |
|---|---|---|
| Pearson Correlation | 0,664** | 0,664** |
| Sig. | 0,001 | 0,001 |

## CONCLUSION

Green tea infusion yoghurt with encapsulated probiotics could improve the structure of testicular tissue in mice after HFFD administration. The best dose is GTY 5 g/kg BW, which can increase the number of spermatogenic and Leydig cells the most after HFFD induction.

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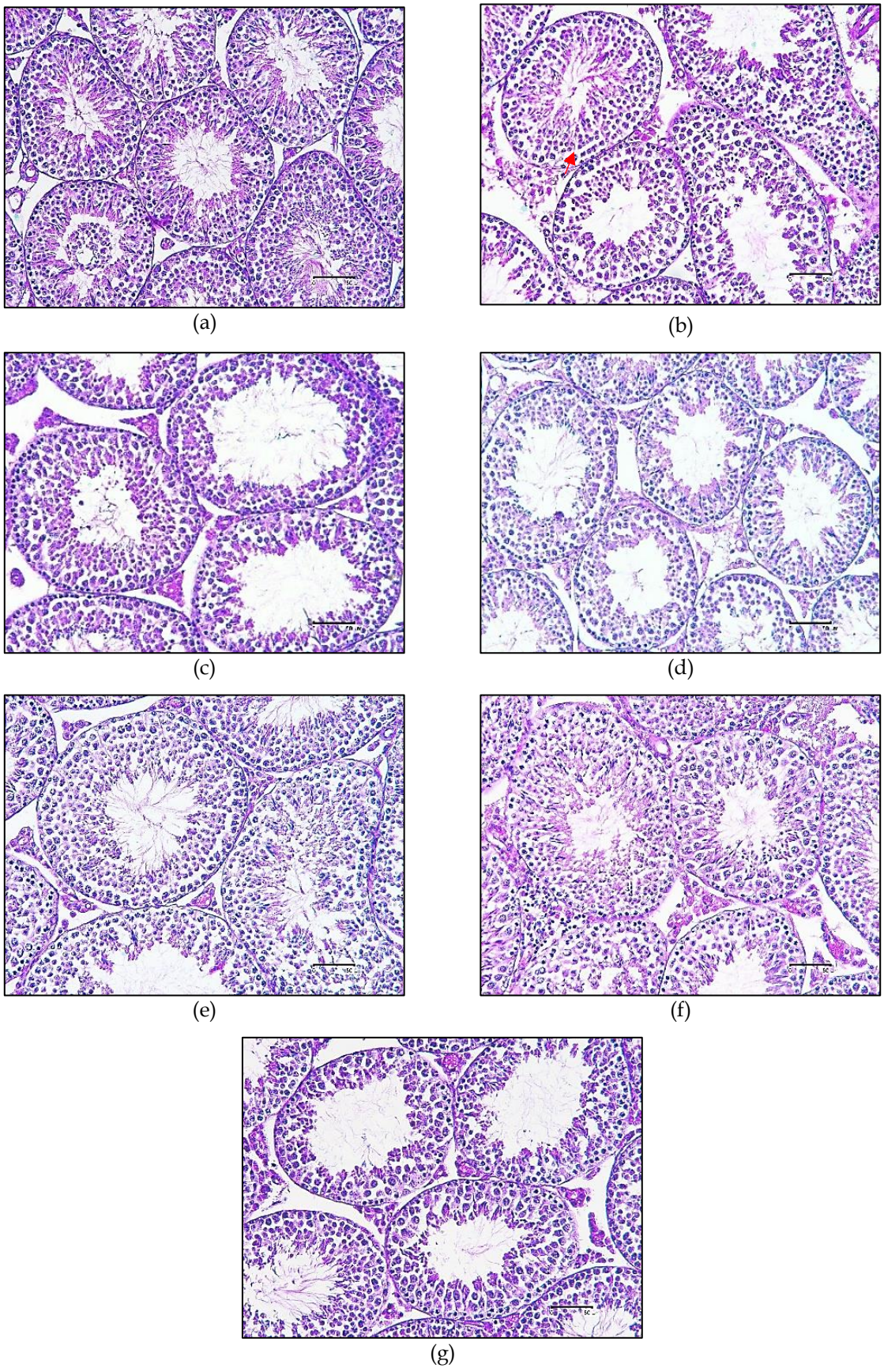


**Figure 1.** Histology of seminiferous tubules (HE, 200x magnification, 50µm scale). a) P0= normal, b) P1= HFFD, c) P2= HFFD+Simvastatin 1.3 mg/Kg BW, d) P3= plain yoghurt 5 g/Kg BW, e) P4= GTY 2.5 g/ Kg BW, f) P5= GTY 5 g/Kg BW, g) P6= GTY 10 g/Kg BW, a red arrow (septum)

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**Conflict of Interest**

The authors declare no conflict of interest.

**Authors' Declaration**

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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