

Evaluation of Sperm Parameters, Reproductive Hormones, Histological Criteria, and Testicular Spermatogenesis Using Turnip Leaf (*Brassica Rapa* L.) Hydroalcoholic Extract in Male Rats: An Experimental Study

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Abstract

Turnip leaves are rich in vitamins, contain large amounts of various substances with biological properties, and contain various flavonoids, some of which have phytoestrogens properties. The effect of hydroalcoholic extract of turnip leaf (TLE) on pituitary-gonad axis and testicular tissue changes in adult male rats was investigated. Forty Wistar rats were used in 5 groups of 8. The control group used water and standard laboratory feed and did not receive any TLE. The placebo group received orally a certain amount of distilled water as an extracting solvent. Experimental groups 1, 2, and 3 used TLE included 500, 1000, and 2000 mg/kg, respectively, for 28 days. Finally, blood samples were collected from all examined groups to measure the serum concentrations of testosterone, LH, and FSH by the ELISA method. The testes were removed from the animals, and the diameter of the seminiferous tubules (STD) was measured by DinoCapture software. The results revealed that BW, left and right testes did not exhibit significant changes. The results of hormonal tests showed that the TLE in experimental groups increased the level of testosterone and had no significant effect on the levels of LH and FSH. Histological studies showed that the number of spermatogonia, spermatocytes, spermatids, spermatozoa, Leydig cells, STD, and germinal epithelium diameter (GED) in experimental groups showed significance. The effects of TLE were not dose-dependent and the value of 1000 mg/kg is recommended for effectiveness and margin of assurance. This research article is taken from the master's thesis in the field of animal biology.

Keywords: Plant extract, Phytoestrogens, Spermatogenesis, Testosterone, Histomorphometry

Introduction

The hypothalamic-pituitary-gonad axis plays an important role in regulating reproductive activities through hormonal control. Since the synthesis and secretion of androgens is the result of the activity of this axis. It is effective in creating differentiation and secondary sexual traits. Due to the high sensitivity of the reproductive process, it is important to know the factors that affect the activity of the hypothalamic-pituitary-gonad axis, and since reproduction is an important and sensitive process in human societies, recognizing the factors that somehow affect gonad pituitary, has always been considered by researchers.

Today's, infertility, or low fertility is one of the medical problems in the world that can affect about 8-12% of men and women [1,2]. According to the reports, the fertility of a normal couple is about 30-50% of the mentioned range, which in this regard, the defect in the factors of both sexes is equal to about 40% [3,4]. It is quite clear that 10% of infertility is due to common factors between the two sexes and the leftover, 10% is due to unknown factors [2]. In general, factors such as genetic [5], testicular prolapsed [6], testicular cancer [7], some chemical medicines [8], environment [9,10], testicular venous congestion [11], and other factors can be causes of infertility in men [12].

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There is a great interest in recognizing and applying medicinal plants in the world [13], and herbal plants have established their place as medicine in human life, over time. Herbal ingredients contain phytoestrogens with a natural formula that causes fewer side effects than chemical medicine [14]. One of these plants is turnip (*Brassica rapa*), which has been used not only because of its acceptable nutritional value but also medicinal components in roots, leaves, and stems [15,16]. Turnip with an annual production of about 44.8 million tons [17] has large amounts of biologically active substances beneficial to health that the antioxidant properties of leaf extract, due to chemical compounds such as phenolic compounds and organic acids are vital.

Turnip leaves are rich in vitamins, contain large amounts of various substances with biological properties [18], and contain various flavonoids, some of which have phytoestrogens properties and at high doses of leaf extract compounds (Phytoestrogens), it can decrease estrogen [19]. The phytoestrogens in leaf extract can attach to estrogen receptors in the anterior pituitary gland and inhibits estrogen secretion by reducing FSH levels through exerting negative feedback control [20], consequently, can reduce FSH and LH hormone [21]. This study was conducted to investigate the effect of TLE on sperm parameters, reproductive hormones, histological criteria, and testicular spermatogenesis in male rats.

Material and Methods

Laboratory Animals and Rearing House

In this experiment, 40 adult male Wistar rats aged 80-90 days and weighing approximately 150-200 g were purchased from the Laboratory Animal Breeding Center of the Faculty of Veterinary Medicine, Shiraz University. To adapt to the environment, animals were kept in the animal house of Darab branch, Islamic Azad University, two weeks before the start of the experiment. Pelleted feed was used on the animals. Ambient temperature and the humidity were 22 °C and 50-55%, respectively. The periods of 12 hours of light and 12 hours of darkness were provided. The air in the room was ventilated by two ventilators embedded on either side of the room, and the floor of the equipment was disinfected. Water and feed were available to the animals without any restrictions (*ad libitum*). The animal cages were made of transparent Macrolon at dimensions of 55 × 30 × 20 cm with a mesh roof. The floors of the cages were covered with sawdust and wood chips, and the cages were cleaned and disinfected once every two days. Before the start and during the experimental period, the weights of the rats were recorded twice a week. After calibrating the digital scale, the rats were weighed individually.

Plant Material

The leaves of *B.rapa* were obtained in the required quantity during April 2019 from Fars province, Darab, Iran. The turnip leaves were distinguished by an expert botanist in Shiraz University, and a voucher specimen (221) was kept in the herbarium of the agricultural faculty of Darab branch, Islamic Azad University, Darab, Iran.

Extraction Method

After cleaning and washing in the laboratory of Islamic Azad University, the leaves of *B. rapa* were dehydrated in the presence of air and shade, milled, and transferred for extraction. For each preparation of the extract, 100 grams of turnip leaf powder, 700 ml of 50% hydroalcoholic solution (50% ethanol, 50% distilled water) was added and after 72 hours, the above solution was placed in a percolator. The solution was filtered and the excess solvent of the extract was taken using a rotary apparatus. Then, the dried extract was weighed and the result was 10 g of dried extract for 100 g of powdered turnip leaves with 10% efficiency. To prepare the solvent, the resulting dry matter was dissolved in distilled water at doses of 500, 1000 and 2000 mg.

Animal Grouping

To determine the dose of the extract, the LD₅₀ method was used. The animals were 40 adult Wistar male rats that randomly divided into 5 groups of 8 in control, placebo and experimental groups (1, 2, and 3) for 28 days. The experimental treatments were given to rats by nasogastric tube or NG tube (gavage). The treatment groups were included: 1. Control group: used only pelleted feed and water daily and did not receive any extract or medicine. 2. Placebo group: In addition to the feed consumed by the control group, only solvent (distilled water) daily. 3- Experimental group 1: 500 mg/BW_(kg)/day of TLE. 4. Experimental group 2: 1000 mg/BW_(kg)/day of TLE. 5. Experimental group 3: daily 2000 mg/BW_(kg)/day of TLE.

Sampling and Determination

On the 28th day of the experiment, all rats were placed individually in a jar containing ether-impregnated cotton until anesthetized, due to little effect of ether on blood flow. After anesthesia with a 5 ml syringe, blood was taken from the animal's heart and a separate syringe was used for each blood draw. The concentrations of LH and FSH in the serum of rats were measured using the specific kits (Pishtaz Teb Company), and the concentration of testosterone was estimated using a testosterone kit (DRG) by the ELISA method.

The testes were withdrawn from the rats and placed in a container containing 10% formalin. After tissue passage, paraffin blocks and tissue sections with a thickness of 5 microns were developed by a microtome device. The

tissue samples were analyzed under a light microscope using hematoxylin-eosin staining. Slides fixed from the cross-sections of the left and right testes of rats were studied separately by light microscopy at X40 magnification. In each slide, the number of spermatogonia, spermatocytes, spermatids, spermatozoa, Sertoli, and Leydig cells were counted with a magnification of 40. Then, the diameter of the seminiferous tubules, the LD, and the thickness of the GED were computed by Dinocapture software equipped with a microscopic measuring system that involves a ruler and a graticule lens and a Nikon camera microscope was operated to prepare the photomicrograph.

Ethical Consideration

In all stages of the experiment, the principles of working with laboratory animals in accordance with the Law on Care and Working with Laboratory Animals were observed by the Research Ethics Committee of the Islamic Azad University (IAU).

Statistical Analysis

The collected data from serum concentrations of LH, FSH, and testosterone, as well as germ cell count and measurement of tube and LD in different groups, were analyzed by SPSS software using proc ANOVA and the mean values were compared by Duncan test.

Table 1 Changes in BW, left and right testicular weight (g) using different treatments

Parameter	Control	Placebo	TLE (mg/kg)			C.V	P value
			500	1000	2000		
BW ¹	217.00 ± 26.72	194.00 ± 12.01	211.25 ± 7.07	217.00 ± 11.52	203.87 ± 12.47	1.011	0.433
LTW ²	1.293 ± 0.129	1.306 ± 0.117	1.307 ± 0.062	1.483 ± 0.063	1.262 ± 0.061	1.388	0.562
RTW ³	1.219 ± 0.076	1.325 ± 0.115	1.325 ± 0.065	1.461 ± 0.067	1.275 ± 0.065	1.171	0.640

¹ Body weight, ² Left testicular weight, ³ Right testicular weight

Table 2 Changes in serum LH, FSH, and testosterone concentrations (mg/ml) using different treatments

Parameter	Control	Placebo	TLE (mg/kg)			C.V	P value
			500	1000	2000		
SLHC ¹	0.157 ± 0.067	0.124 ± 0.006	0.114 ± 0.015	0.122 ± 0.012	0.089 ± 0.010	2.715	0.699
SFSHC ²	0.430 ± 0.039	0.456 ± 0.048	0.379 ± 0.057	0.841 ± 0.272	0.587 ± 0.172	3.744	0.498
STC ³	1.404 ± 0.176 b	2.163 ± 0.609 b	2.400 ± 0.673 b	5.283 ± 0.832a	3.066 ± 0.746b	2.605	0.01

¹ Serum LH concentration, ² Serum FSH concentration, ³ Serum testosterone concentration

*Columns with at least one common letter do not differ significantly

Data are expressed as Mean±SE. One-way ANOVA test and the mean values were compared by Duncan test.

Data are expressed as Mean±SE. One-way ANOVA test and the mean values were compared by Duncan test.

Based on the results of Table 3 and Figures 1-9, a significant difference was recognized between the mean number of spermatogonia (P=0.01), spermatocytes (P=0.05), spermatids (P=0.04), spermatozoa (P=0.02), and Leydig cells (P=0.03). Therefore, the use of TLE had a significant influence on the quantity of these cells. Consumption of the TLE had no significant effect on the number of Sertoli cells. Consumption of TLE, despite the dose of the extract, recorded a significant improvement in the GED. But the use of the extract had no statistical notable effect on the LD.

Table 3 Changes in germ cells parameters and testicular tissues diameters (mm) using different treatments

Parameter	Control	Placebo	TLE (mg/kg)			C.V	P value
			500	1000	2000		
Spermatogonia	74.58 ± 1.434 ab	73.002 ± 1.287 bc	73.72 ± 0.177 bc	77.58 ± 1.111 a	74.497 ± 1.163 ab	0.251	0.007
Spermatocytes	109.74 ± 3.191 b	117.78 ± 1.729 ab	122.19 ± 2.344 a	125.24 ± 2.977 a	124.38 ± 5.698 a	0.394	0.049
Spermatid	121.64 ± 4.814 b	128.05 ± 4.240 ab	129.31 ± 5.604ab	131.08 ± 6.747 a	129.26 ± 4.437 ab	0.642	0.035
Spermatozoa	79.16 ± 5.872 b	88.94 ± 3.676 ab	84.91 ± 2.137 ab	98.70 ± 4.480 a	92.45 ± 6.293 ab	0.794	0.024
Sertoli cell	18.96 ± 0.554	19.05 ± 0.362	20.49 ± 0.674	20.61 ± 0.495	20.66 ± 0.638	0.432	0.108
Leydig cell	11.14 ± 0.310 b	12.16 ± 0.874 ab	12.01 ± 0.426 ab	13.46 ± 0.314 a	11.99 ± 0.719 ab	0.694	0.026
STD1	0.361 ± 0.006 b	0.361 ± 0.005 b	0.363 ± 0.007 b	0.387 ± 0.013 a	0.378 ± 0.01 b	0.342	0.028
LD2	0.224 ± 0.011	0.232 ± 0.006	0.215 ± 0.009	0.208 ± 0.006	0.211 ± 0.009	0.609	0.209
GED3	0.143 ± 0.006 b	0.147 ± 0.004ab	0.148 ± 0.003 ab	0.161 ± 0.006 a	0.150 ± 0.004 ab	0.470	0.026
LD4	0.050 ± 0.002	0.054 ± 0.001	0.046 ± 0.001	0.059 ± 0.009	0.064 ± 0.014	1.982	0.630

¹ Seminiferous tubules diameter, ² Lumen diameter, ³ Germinal epithelium diameter, ⁴ Leydig diameter

*Columns with at least one common letter do not differ significantly

Data are expressed as Mean±SE. One-way ANOVA test and the mean values were compared by Duncan test.

Results

Table 1 revealed that there was no significant difference between BW, right, and left the testicular weight of rats in all 5 groups of experimental treatments. The concentration of LH and FSH hormones did not exhibit a significant variation with the use of different treatments, but with the applying of TLE, the testosterone concentration of experimental rats and 100 mg/kg group was significantly higher than other groups ($P < 0.01$) (Table 2).

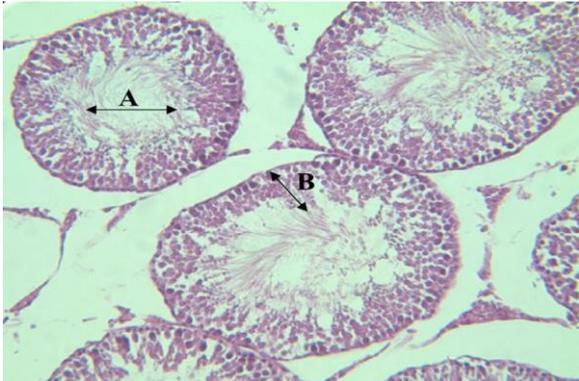


Fig. 1 Photomicrograph of testicular tissue in the control group. H&E staining, 100x (A: LD, B: GED)

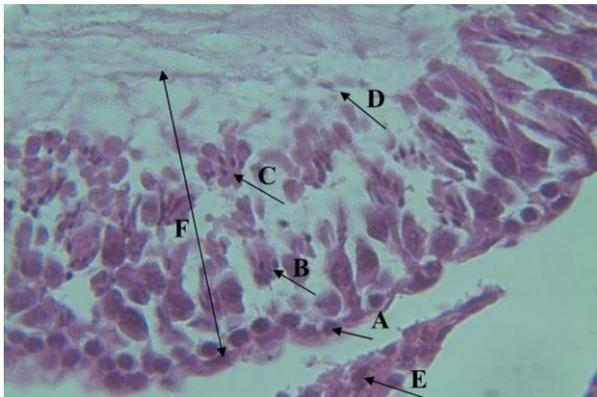


Fig. 1 Photomicrograph of testicular tissue in the control group. H&E staining, 400x (A: spermatogonia, B: spermatocytes, C: spermatids, D: spermatozoa, E: Leydig and F: GED)

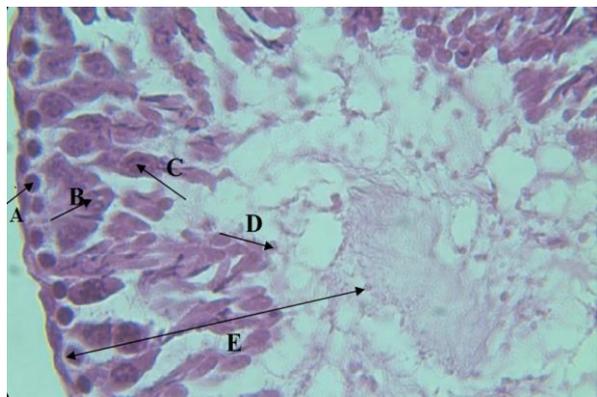


Fig. 2 Photomicrograph of testicular tissue in the placebo group. H&E staining, 400x (A: spermatogonia, B: spermatocytes, C: spermatids, D: spermatozoa, E: GED)

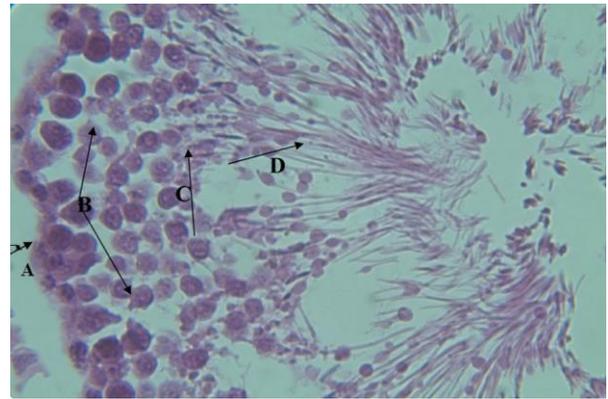


Fig. 3 Photomicrograph of testicular tissue in the 500 mg/kg TLE group. H&E staining, 400x (A: spermatogonia, B: spermatocytes, C: spermatids, D: spermatozoa)

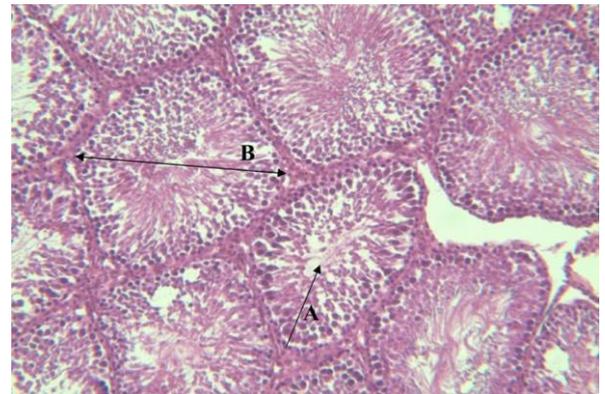


Fig. 4 Photomicrograph of testicular tissue in the 1000 mg/kg TLE group. H&E staining, 100x (A: GED, B: STD)

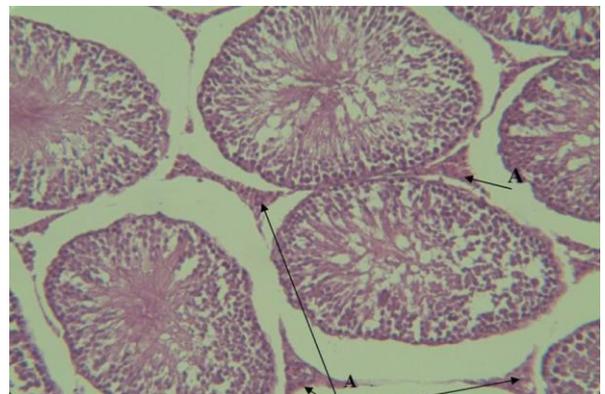


Fig. 5 Photomicrograph of testicular tissue in the 1000 mg/kg TLE group. H&E staining, 100x (A: Leydig cell)

Discussion

Weight variations are influenced by heredity, diet quality, feed intake, and factors influencing digestion. Due to the stability of all research conditions and providing the same quantity of diet, no difference in weight gain was observed. Therefore, changes in the number of spermatogonia, spermatocyte, spermatid, spermatozoid, Sertoli and Leydig cells, cannot be due to differences in BW or testicular weight of rats, and only hormonal changes can be effective.

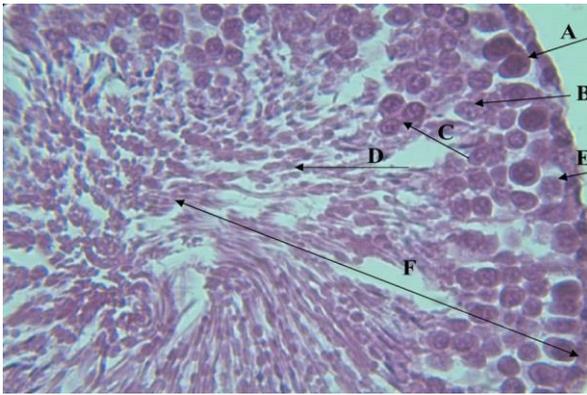


Fig. 6 Photomicrograph of testicular tissue in the 1000 mg/kg TLE group. H&E staining, 400x (A: spermatogonia, B: spermatocytes, C: spermatids, D: spermatozoa, E: sertoli cell and F: GED)

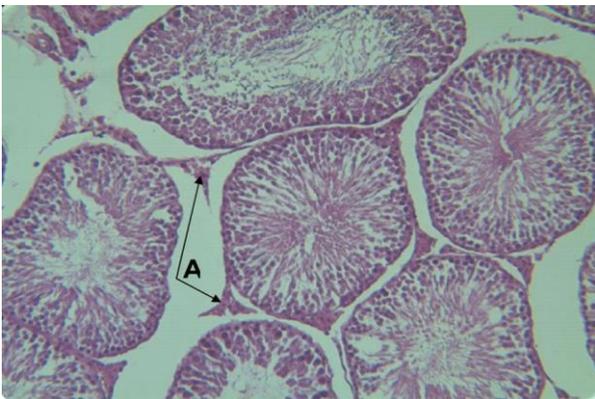


Fig. 7 Photomicrograph of testicular tissue in the 2000 mg/kg TLE group. H&E staining, 100x (A: Leydig cell)

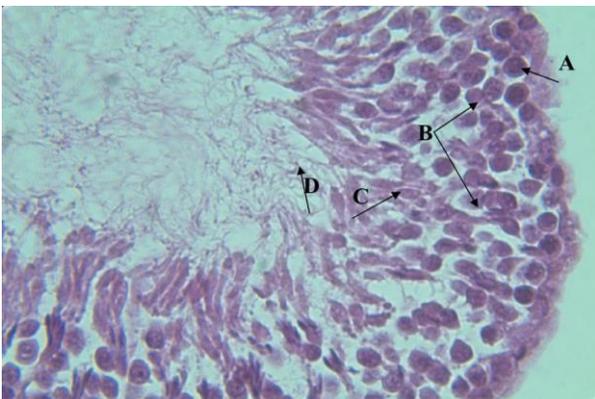


Fig. 8 Photomicrograph of testicular tissue in the 2000 mg/kg TLE group. H&E staining, 400x (A: spermatogonia, B: spermatocytes, C: spermatids, D: spermatozoa)

The results of this experiment revealed that consumption of TLE had no significant effect on LH and FSH hormones but statistically raised the serum testosterone concentration. The flavonoid compounds in turnips inhibit the production of Arachidonic acid by inhibiting the lipoxygenase and cyclooxygenase pathways and in turn inhibit the production of prostaglandins [22, 23]. As a result, due to the effect of prostaglandins on the production of gonadotropins, these compounds affect the secretion of testosterone with a negative self-regulatory

effect [24]. This hormone can also be secreted by interstitial cells. So increasing the number of interstitial cells can be a factor for increasing hormone secretion. Various flavonoids such as kaempferol, quercetin and lutein in TLE, which have phytoestrogens properties (Estradiol-like structure) has antiestrogenic properties in females [25, 26].

The effect of TLE on LH was not statistically significant, but consumption of TLE reduced the amount of LH, numerically. The consumption of the extract raised the amount of testosterone and this in turn can reduce the serum LH level by affecting the cells that produce LH in the anterior pituitary gland and through creating a negative self-regulatory mechanism. Therefore, reducing the rate by GnRH can reduce the rate of LH. Researchers have pointed that lower testosterone secretion, based on negative feedback control, allows the hypothalamus to secrete high amounts of GnRH, thereby increasing LH secretion from the anterior pituitary gland and increased testosterone secretion from the testis [27]. Testosterone acts as an inhibitory agent for the enzyme monoamine oxidase. This enzyme is involved in dopamine catabolism and reduces the amount of dopamine in the synaptic space [28-30]. Therefore, testosterone directly increases dopamine levels by reducing this enzyme. With a decrease in testosterone, the inhibitory effect on monoamine oxidase (MAO) decreases and the concentration of dopamine decreases. By acting on the arc nucleus, dopamine inhibits the production of luteinizing hormone-releasing hormone (LHRH), and decreasing dopamine increases LHRH and prolactin. Finally, as dopamine decreases, the rate of LH increases indirectly [31].

Consumption of TLE created a significant increase in the number of spermatogonia cells. Following the progress in the secretion of hormone FSH, the rate of generation of spermatogonia cells also increased [32, 33]. There is a direct relationship between secretion of FSH and the number of spermatogonia cells and their generation. The researchers noted that GH increased the synthesis of EGF in the kidneys and liver. The EGF, developed receptors EGF in granulosa cells [34] and increased EGF, stimulated the generation of cells spermatogonia and caused the synthesis of DNA [35]. Concentration of EGF in the testes increased spermatogenesis, which coincided with the mitotic division of spermatogonia type A [36, 37]. Sertoli cells, on the other hand, secrete IGF1, which caused spermatogonia to proliferate during spermatogenesis [38]. According to the results of this study, it is likely that the increase in the number of spermatogonia cells was due to an increase in testosterone.

The results confirmed that the use of TLE increased the number of spermatocytes. High concentrations of testosterone affect the division of spermatocytes [39, 40].

The relationship between Leydig cell function and germ cells in sperm tubes was reported by Ge *et al.* [41]. A significant increase in stem cells due to testosterone increases the function of Leydig cells. On the other hand, the level of FSH has a direct effect on spermatocyte production in Leighton and purity [42]. This hormone caused spermatogonia to reproduce and differentiate into primary spermatocytes. This study was shown a significant increase in testosterone and a numerical increase in hormone FSH.

Flavonoid compounds have antioxidant properties and eliminate free radicals [43]. Antioxidant properties prevent cells from damage and fractures and cause mitotic and meiotic divisions to continue [44]. In this experiment, increasing the number of interstitial cells could be a factor in increasing testosterone secretion, which led to the development of spermatogenesis and spermatid proliferation. Due to the increase in the number of spermatogonia and spermatocytes can also be justified the increase in spermatozoa.

In this study, TLE did not have a significant effect on the number of Sertoli cells and the lack of change was due to the high resistance of these cells to environmental factors [45].

Research has shown that sperm tubes control secretory function, number, size and differentiation of Leydig cells. Injection of androgenic hormones changed the testicular circulation, lymphatic flow and increased the permeability of blood vessels. In addition, there was a very close relationship between Leydig cells and germ cells in sperm-producing tubes. Significant increase in germ cell types increased the number and function of Leydig cells [46].

There was a positive correlation between the seminiferous tubes and spermatogenesis activity [47]. With increasing testosterone and spermatogenesis activity, increasing the diameter of seminiferous tubes was reasonable. The number of spermatogonia, spermatocytes, and spermatozoa was also influenced by the consumption of TLE and consequently, reduced the LD. The decrease in LD was due to the buildup of lumen space from germ cells, and this decrease was not unexpected. While, the increase in the GED was affected by the increase in spermatogenesis and the number of spermatogonia, spermatocytes, and spermatozoa cells.

Conclusion

The homogenous results of this study revealed that TLE, without adhering to the extract dose, led to an increase in serum testosterone. The increase of this hormone increased the process of spermatogenesis. Regarding Figures 1 to 9, it was determined that consumption of 1000 mg/BW_{kg}/day can increase the process of spermatogenesis without damaging the tissue in the

testicles. Therefore, consumption of TLE in moderate amounts is recommended to increase male fertility. However, it seems that to prescribe TLE, it is necessary to examine other tissues such as the liver, kidney, and stomach.

Conflict of Interest

We declared that no conflict of interest exists.

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