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Original Research Article

The Effects of Methotrexate, *Moringa oleifera* Leaf Extract, and *Andrographis paniculata* Leaf Extract on the Testes of Hyperglycemic Wistar Rats

Muhammad H.F. Nasution[1,2], Viskasari P. Kalanjati[3]*, Abdurachman Abdurachman[3], Dwi M. N. Aditya[1,4], Dimas B. B. Pamungkas[1,5], Maya R. Syamhadi[1,5]

[1]*Master's Study Program Basic Medical Sciences in Anatomy and Histology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia*
[2]*Department of Anatomy, Faculty of Medicine, Universitas Malikussaleh, Aceh, Indonesia*
[3]*Department of Anatomy, Histology, and Pharmacology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia*
[4]*Department of Anatomy and Histology, Faculty of Medicine, Universitas Surabaya, Indonesia*
[5]*Department of Anatomy, Faculty of Medicine, Universitas Muhammadiyah Surabaya, Indonesia*

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ABSTRACT

It has been reported that MTX can lower blood glucose levels in type 1 diabetes mellitus (T1DM), while MO and AP can lower blood glucose levels in hyperglycemia. This study investigates the effects of administering MTX, MO leaf extract, AP leaf extract, and their combinations on the testicular tissue of diabetic rats. A total of 49 male Wistar rats were divided into seven groups, namely control (K1), STZ-NA (K2), STZ-NA+MO (K3), STZ-NA+AP (K4), STZ-NA+MTX (K5), STZ-NA+MTX+MO (K6), and STZ-NA+MTX+AP (K7). Out of 49 Wistar rats, 42 Wistar rats were intraperitoneally injected with streptozotocin and nicotinamide with a dose of 50 mg/kg and 110 mg/kg respectively. MTX was administered once a week (7 mg/kg), while MO and AP leaf extracts were given every day (500 mg/kg) for 28 days. Blood glucose levels were tested using a glucometer and body weight was measured using an Ohaus Triple Beam balance. Meanwhile, IL-6 levels in the testes were measured using ELISA. Testicular tissue was collected and analysed for histology using hematoxylin and eosin (H&E) staining. The results revealed a significant decrease in body weight in all STZ-NA-induced groups. In addition, the results revealed a significant increase in blood glucose levels in all STZ-NA-induced group. On the other hand, the administration of MO, AP, MTX, and their combinations significantly reduced the expression of IL-6 in the testicular tissue of diabetic rats. Therefore, it can be concluded that the administration of MO and AP can mitigate the adverse effects of hyperglycemia on the testicular tissue.

Keywords: Diabetes mellitus, methotrexate, *Moringa oleifera*, *Andrographis paniculata*, testes

Introduction

Hyperglycemia in patients with type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) has adverse effects, one of which is male infertility due to testicular inflammatory pathways.[1] The incidence of T1DM in children and adolescents and T2DM in patients aged under 40 increases the risk of prolonged exposure to hyperglycemia and subsequent long-term complications.[2] The prevalence of diabetes mellitus in men of childbearing age has been reported to increase and will continue to rise until 2045.[3] This increase in prevalence is closely linked to infertility rates in men.[4] Infertility can cause significant financial loss and emotional distress, affecting approximately 49 to 72 million people worldwide, or one in seven people. In 50% of childless couples, abnormal sperm is a factor in male infertility.[5]

One of the reported developments of methotrexate (MTX) as a potential antihyperglycemic agent is that low doses of MTX can lower blood glucose levels in rheumatoid patients with diabetes mellitus.[6]

*Corresponding author. E mail: viskasari-p-k@fk.unair.ac.id
Tel: +6231 5020251

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MTX has the potential to stimulate 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), activate AMP-activated protein kinase (AMPK), and regulate glucose uptake, and promote lipid oxidation in skeletal muscle.

Pharmacological activation of AMPK in skeletal muscle plays a role in enhancing glucose uptake without insulin mediation. As a result, it is very beneficial for patients with T1DM.[6] *Moringa oleifera* (MO) and *Andrographis paniculata* (AP) leaf extracts have been reported to be very beneficial for diabetes mellitus patients in terms of controlling blood sugar levels.[7–9] Previous studies have reported that MO and AP leaf extracts reduce the negative effects of chronic hyperglycemia on various tissues.[10,11] In addition, it has been reported that the potential anti-inflammatory effects of MO and AP leaf extracts is due to the suppression activity of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway.[12–15]

This study investigates the potential antihyperglycemic and anti-inflammatory effects of MTX, MO, and AP on the testes of male rodents with STZ-NA-induced hyperglycemia. We examined the individual and combined effects of these agents on the interleukin-6 (IL-6) levels in testicular tissue, number of Leydig cells, diameter and epithelial thickness of seminiferous tubules, and Johnsen score.[16–18]

Materials and Methods

Drugs and chemicals

This study used the following materials: streptozotocin (STZ) made in the United States by BioWorld with a batch number 41910012-2, nicotinamide (NA) made in India by Jubilant Ingrevia with a batch number B-2208-NIA049, methotrexate made in Indonesia by PT Kalbe

Farma (6°10'06.2"S 106°52'20.8"E) with a batch number TRHTA80001, *Moringa oleifera* leaf extract made in Indonesia by PT Sido Muncul (7°19'48.7"S 112°45'30.1"E) with a batch number EH00012, *Andrographis paniculata* leaf extract made in Indonesia by PT Jamu Iboe Jaya (7°22'19.1"S 112°38'40.7"E) with a batch number SB1081A, and IL-6 kit made in Indonesia by Bioenzy with a catalog number BZ- 08185310-EB.

Animal preparation
A total of 49 male Wistar rats aged between two and three months and weighing between 150 and 250 grams were acclimatized prior to the experiment. They were provided with *ad libitum* standard rodent diet (Pokphand CP 593, PT Charoen Pokphand, Indonesia) and drinking water during the experiment. The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Airlangga with a certificate of ethical approval number No.1/EC/KEPK/FKUA/2022.

Induction of diabetes with streptozotocin and nicotinamide
Streptozotocin (50 mg/kg) and nicotinamide (110 mg/kg) dissolved in citrate buffer (pH 4.5) was injected intraperitoneally to induce diabetes. The injection of NA was administered 15 minutes earlier than the injection of STZ. One or two drops of blood from the lateral vein of the rats were used to test their blood glucose levels 72 hours after the administration of SN-NA using a glucometer.[19]

Animal experimental design
The animals were acclimatized for seven days prior to the experiment (n = 49). All animals were randomly divided into K1, K2, K3, K4, K5, K6, and K7 groups which consisted of seven rats per group. Animals in group 1 (K1) served as the control group and were not induced with STZ-NA. Meanwhile, the other animals with blood glucose levels of 250 mg/dl and above were assigned into K2, K3, K4, K5, K6, and K7 groups. On the third day following the injection of STZ-NA, treatments with MTX, MO, AP, and their combinations were given. Animals in K3 group were given *Moringa oleifera* leaf extract with a daily dose of 500 mg/kgBW for 28 days (STZ-NA+MO). Animals in K4 group were given *Andrographis paniculata* leaf extract with a daily dose of 500 mg/kgBW for 28 days (STZ-NA+AP). Animals in K5 group were given methotrexate with a daily dose of 7 mg/kgBW for 28 days (STZ-NA+MTX) . Animals in K6 group were given methotrexate with a daily dose of 7 mg/kgBW and *Moringa oleifera* leaf extract with a daily dose of 500 mg/kgBW for 28 days (STZ-NA+MTX+MO). Animals in K7 group were given methotrexate with a daily dose of 7 mg/kgBW and *Andrographis paniculata* leaf extract with a daily dose of 500 mg/kgBW for 28 days (STZ-NA+MTX+AP). The administered dose of methotrexate was based on a previous study by Koyama et al.[20] The administration of *Moringa oleifera* leaf extract was based on a previous study by Jamil et al.[21] The administration of *Andrographis paniculata* leaf extract was based on a previous study by Ogunlana et al.[18] The animals were euthanized using an overdose of ether inhalation and decapitation following the end of experiment. Finally, the samples were collected for analysis.

Measurement of body weight
Body weight was measured on the day of the injection of STZ-NA and the third of the experiment (day 24) using an Ohaus Triple Beam balance (Shimadzu, Japan).

Measurement of blood glucose level
Blood glucose levels were measured following the injection of STZ-NA and prior to any treatments (day 3 and day 15) using an EasyTouch Glucometer type ET-301F made in Taiwan with a batch number 301F2C007837.

Measurement of testicular IL-6 expression
The IL-6 levels of the right testes was measured using the ELISA technique (day 29).[22]

Histological preparations
On day 29, the left testes of the animals were collected and histologically processed. The histology process began with immersing the testes in a fixative solution, namely 10% buffered formalin. Following fixation were dehydration and embedding. The dehydration used ethanol, while the embedding used paraffin wax to make it easy for cellular extraction. The paraffin block containing the left testis then sliced into 5 µm sections and stained with hematoxylin-eosin. The histological analysis were performed using a light microscope (Olympus CX23, Olympus Corporation, Japan) with an objective magnification of 400x in 20 visual fields. Subsequently, images from 20 seminiferous tubules were processed using analytical software, namely Olympus cellSens (RRID: SCR_014551) and ImageJ.[23,24]

Testicular histopathological evaluation
Testicular histopathology was evaluated in terms of four measurements: average diameter, epithelium thickness, number of Leydig cells, and Johnsen score of 20 seminiferous tubules in each experimental animal. The diameter of the seminiferous tubules was measured by calculating the average diameter of the longest and shortest seminiferous tubules. The epithelium thickness of the seminiferous tubules was measured by calculating the average of four measurements at 90˚, 180˚, 270˚, and 360˚ angles.[23] The Johnsen score criteria provided a quantitative evaluation elements of the seed and the relationship between spermatogenesis and the density of spermatozoa in the seminal fluid.[25]

Statistical analysis
The data were analyzed using SPSS 17.0.[26] Prior to making statistical comparisons, the Shapiro-Wilk test for normality and Levene's test for homogeneity. Body weight, random blood glucose levels, IL-6 levels in the testes, number of Leydig cells, diameter of seminiferous tubules, epithelium thickness of seminiferous tubules, and Johnsen score of the seven groups were compared with significance level of $p < 0.05$. A post-hoc test for Kruskal-Wallis was performed with Mann-Whitney post-test, while one-way ANOVA was performed with Fisher's LSD post-test.

Results and Discussion

The results of this study revealed dynamic changes in the body weight from the beginning of the experiment to day 24, as shown in Table 1. It was found that the body weight of the animals in BW1 did not show any significant differences among all groups ($p = 0.716$). However, after 24 days of experiment, the body weight of the animals in K2, K3, K4, K5, K6, and K7 groups decreased. Therefore, the body weight of the animals in BW2 showed significant differences ($p < 0.001$).
Previous studies have demonstrated significant weight loss in diabetic experimental animals induced with streptozotocin.[27,28] This weight loss is associated with reduced insulin levels, leading to protein degradation for amino acid provision and subsequent gluconeogenesis which can result in muscle mass reduction and weight loss in animals injected with STZ.[29] Several studies have shown that the administration of MO and AP leaf extracts can prevent weight loss in diabetic rats induced with STZ and STZ-NA.[7,29–32] MO leaf extract containing antioxidants and antimicrobial compounds (e.g., phenols, tannins, alkaloids, and coumarins) acts as a growth promoter and inhibits lipase activity, which reduces lipolysis and hepatic conversion of fatty acids to cholesterol.[31,32] Meanwhile, AP leaf extract increases plasma protein levels, thereby preventing weight loss associated with excessive protein breakdown.[8] Furthermore, it has been reported that the administration of MTX in rheumatoid arthritis patients resulted in weight gain.[33] This is probably due to the accumulation of AICAR upon the administration of MTX, which inhibits fructose-1,6-bisphosphatase, a key enzyme in gluconeogenesis, and increases GLUT4 translocation enhancing glucose uptake by insulin target cells.[34] Throughout the study, dynamic changes in blood glucose levels were observed. Table 2 presents the descriptively data on blood glucose levels in the experimental animals. BG1 showed a significant difference among all groups ($p = 0.006$). BG2 also showed a significant difference among all groups ($p < 0.001$). When comparing BG1 to BG2, a downward trend was observed in K3,

K4, K5, K6, and K7 groups. An increase was observed in K2 group, K1 group showed relatively no change.

The administration of MO and AP leaf extracts in previous studies has been reported to significantly reduce blood glucose level.[7,8,35,36] Quercetin in MO extract effectively increases glucokinase activity in the liver like insulin. MO leaf also contains terpenoids, which stimulates cells and promote insulin secretion. Other compounds found in MO leaf, such as isothiocyanates, reduce insulin resistance and hepatic gluconeogenesis.[10,11] On the other hand, the andrographolide compound in AP extract has been reported to enhance glucose utilization by increasing GLUT-4 mRNA and protein levels.[37] The administration of MTX can significantly lower blood glucose levels due to its inhibitory effect on the AICAR transformylase. This triggers intracellular AICAR accumulation and results in an increase in GLUT4 expression and translocation as well as inhibition of fructose-1,6-bisphosphatase as a key enzyme in gluconeogenesis. This further reduces blood glucose levels and insulin concentrations. In addition, MTX involved in the pathogenesis of diabetes mellitus has been reported to suppress the NF-kB pathway.[34,38]

The IL-6 levels in the left testicular tissue for each group are presented in Table 3. A significant difference in the IL-6 levels in testicular tissue was observed among all groups ($p = 0.002$). A significant increase of IL-6 levels was observed in K1 and K2 groups ($p < 0.001$). The IL-6 levels in K3, K4, K5, K6, and K7 groups were significantly lower than K2 group ($p = 0.006$, $p = 0.002$, $p = 0.021$, $p = 0.001$, $p < 0.001$, respectively).

These results are consistent with previous studies which demonstrated an increase in the expression of IL-6 in testicular tissue of diabetes mellitus rats.[39] This is probably due to the activation of inflammatory pathway mediated by NF-kB.[40]

Table 1: Average body weight (gr) of rats

	Groups	BW1 (Mean ± SD)	BW2 (Mean ± SD)
1	K1	244 ± 22.2	314 ± 7.9
2	K2	252 ± 23.0	134 ± 2.4
3	K3	245 ± 29.2	235 ± 28.0
4	K4	248 ± 22.1	239 ± 91.9
5	K5	231 ± 16.1	218 ± 39.0
6	K6	242 ± 21.0	222 ± 37.3
7	K7	241 ±18.4	213 ± 84.9
	p-value	0.716[a]	<0.001[b]

[a]Significant at $p < 0,05$ (ANOVA)
[b]Significant at $p < 0,05$ (Kruskal-Wallis)
BW1 = BW on the day of STZ-NA injection
BW2 = BW after 24 days of MTX, MO, and AP administration

Table 2: Average blood glucose levels (mg/dL)

	Groups	BG1 (Mean ± SD)	BG2 (Mean ± SD)
1	K1	107 ± 9.0	104 ± 11.6
2	K2	565 ± 47.4	597 ± 4.9
3	K3	465 ± 90.9	372 ± 88.6
4	K4	490 ± 121.6	364 ± 47.9
5	K5	451 ± 110.0	394 ± 64.3
6	K6	407 ± 109.0	343 ± 182.1
7	K7	444 ± 51.8	334 ± 154.0
	p-value	0.006*	<0.001*

*Significance with $p<0.05$ (Kruskal-Wallis)
BG1 = BG after 3 days of STZ-NA injection
BG2 = BG after 15 days of MTX, MO, and AP administration

Table 3: Testicular IL-6 (ng / L) expression levels in K1-K7 groups

	Groups	Mean ± SD	p-value
1	K1	10.40 ± 1.40	
2	K2	12.8 ± 1.30	
3	K3	11.23 ± 1.19	
4	K4	11.04 ± 0.53	0.002
5	K5	11.53 ± 0.82	
6	K6	10.73 ± 0.37	
7	K7	10.54 ± 0.62	

*Significant at $p < 0.05$ (one way-ANOVA)

Previous studies have reported that MO extract has the ability to decrease the IL-6 levels in the plasma and also reduce the IL-6 expression in the kidneys.[16,41,42] Other studies have also reported that AP extract has the ability to reduce the IL-6 levels in the testes.[43] In addition, MTX has been shown to decrease the IL-6 levels in the blood and reduce the IL-6 expression in the synoviocytes.[44,45] These effects may be associated with the activity of the NF-kB pathway, which can be suppressed by MO extract, AP extract, and MTX, thereby reducing the IL-6 expression.[12,14,15,46]

Histopathology of the testes was evaluated in terms of four measurements: (1) the average number of Leydig cells analyzed using one way-ANOVA test; (2) the average diameter of seminiferous tubules analyzed using the Kruskal-Wallis test; (3) the average epithelial thickness of seminiferous tubules analyzed using the Kruskal-Wallis test; (4) the Johnsen score analyzed using the Kruskal-Wallis test (Figure 1). The number of Leydig cells in K2 group was significantly lower than in K1 group ($p < 0.001$), while in K3, K4, K6, and K7 groups were significantly higher than in K2 group ($p < 0.001$, $p = 0.001$, $p = 0.001$, $p < 0.001$, respectively). The diameter of seminiferous tubules in K2 group was significantly smaller than in K1 group ($p = 0.003$), while in K4 and K7 groups were significantly higher than in K2 group ($p = 0.022$ and $p = 0.01$, respectively). The epithelial thickness of seminiferous tubules in K2 group was significantly thinner than in K1 group ($p = 0.004$), while in K3, K4, K6, and K7 groups were significantly thicker than in K2 group ($p = 0.025$, $p = 0.015$, $p = 0.022$, $p = 0.007$, respectively). The Johnsen score in K2 group was significantly lower than in K1 group ($p = 0.002$), while in K3, K4, K6, and K7 were significantly higher than in K2 group ($p = 0.010$, $p = 0.003$, $p = 0.038$, $p = 0.003$, respectively). These results are consistent with previous studies which demonstrated a decrease in the average number of Leydig cells, diameter and epithelium thickness of seminiferous tubules, and Johnsen score in the testes of diabetes mellitus rats.[47–53] Diabetes mellitus causes hyperglycemia, which can increase reactive oxygen species (ROS) in all organs. Furthermore, oxidative stress caused by increased ROS can reduce luteinizing hormone (LH) secretion, which stimulates the growth of Leydig cells.[54] Other studies have reported that diabetes-induced oxidative stress can decrease antioxidant enzyme levels in Leydig cells, which can result in reduced testosterone synthesis. Low testosterone levels cause dysfunction of spermatogenesis, which can interfere with cell proliferation.[55] In addition, the inflammatory activity in diabetes mellitus can increase the IL-6 levels, which can affect the number and function of Leydig cells.[40,56,57] IL-6 as an inflammatory cytokine has been reported to participate in spermatogenesis.[13,58] Previous studies have shown that IL-6 deficiency increases daily sperm production, spermatid count, as well as testosterone and dihydrotestosterone levels.[59] IL-6 can also affect p-ERK1/2 expression in Sertoli cells and disrupt the ERK-MAPK pathway, leading to impaired permeability of the blood-testis barrier (BTB) in Sertoli cells, compromising the integrity of Sertoli cells and ultimately affecting spermatogenesis.[13] Both the hormone and IL-6 expression pathway can disrupt the structure of seminiferous tubules, in terms of diameter and epithelium thickness, and the Johnsen Score, which indicates histopathological failure of spermatogenesis in diabetic rats. This is due to germ cell proliferation that plays a role in the growth

of the seminiferous tubules, both in diameter and thickness.[60,61] These findings are supported by the results of this study, which demonstrated a decrease in the number of Leydig cells and an increase in the IL-6 expression in the STZ-NA group (K2) compared to the control group (K1). Furthermore, the results of histopathological evaluation in K3, K4, K5, K6, and K7 groups were consistent with previous studies which demonstrated a significant increase in the number of Leydig cells, epithelium thickness of the seminiferous tubules, and Johnsen score, following the administration of MO or AP.[62–65] Both MO and AP extracts contain antioxidant and antihyperglycemic agents which can reduce oxidative stress and increase testosterone production in experimental animals.[10,66–69] In addition, MO extract has been reported to have anti-inflammatory properties which can reduce the IL-6 levels in the plasma, while AP extract can reduce the IL-6 expression in the testes.[16,43]

The group that received MTX therapy (K5) did not show a significant increase in the four testicular histopathological evaluations when compared to the STZ-NA group (K2) ($p > 0.05$). Low doses of MTX as an anti-inflammatory agent suppress the activity of NF-kB pathway and block gene transcription mediated by NF-kB and IL-6. This decrease in IL-6 expression will increase spermatogenesis.[13,46] However, it should be noted that the results of this study are slightly different from previous studies, where MTX can cause seminiferous tubule atrophy and significantly reduce the Johnsen score in experimental animals when compared to experimental animals that did not receive MTX.[70,71] In addition, MTX can increase ROS and decrease LH secretion, which in turn will decrease the number of Leydig cells and interfere with spermatogenesis.[72] Because of these opposing effects, MTX has not been able to significantly improve testicular histopathology.

Conclusion

The administration of MTX, MO, or AP led to a decrease in the body weight and an increase in blood glucose levels and IL-6 levels in STZ-NA-hyperglycemic-induced rats. MO or AP extracts have been shown to increase the average number of Leydig cells as well as the diameter and epithelial thickness of the seminiferous tubules. Meanwhile, histological evaluations of the testes showed higher Johnsen score in the treated animals. In conclusion, administering MO and AP leaf extracts may prevent infertility complications in diabetic rats.

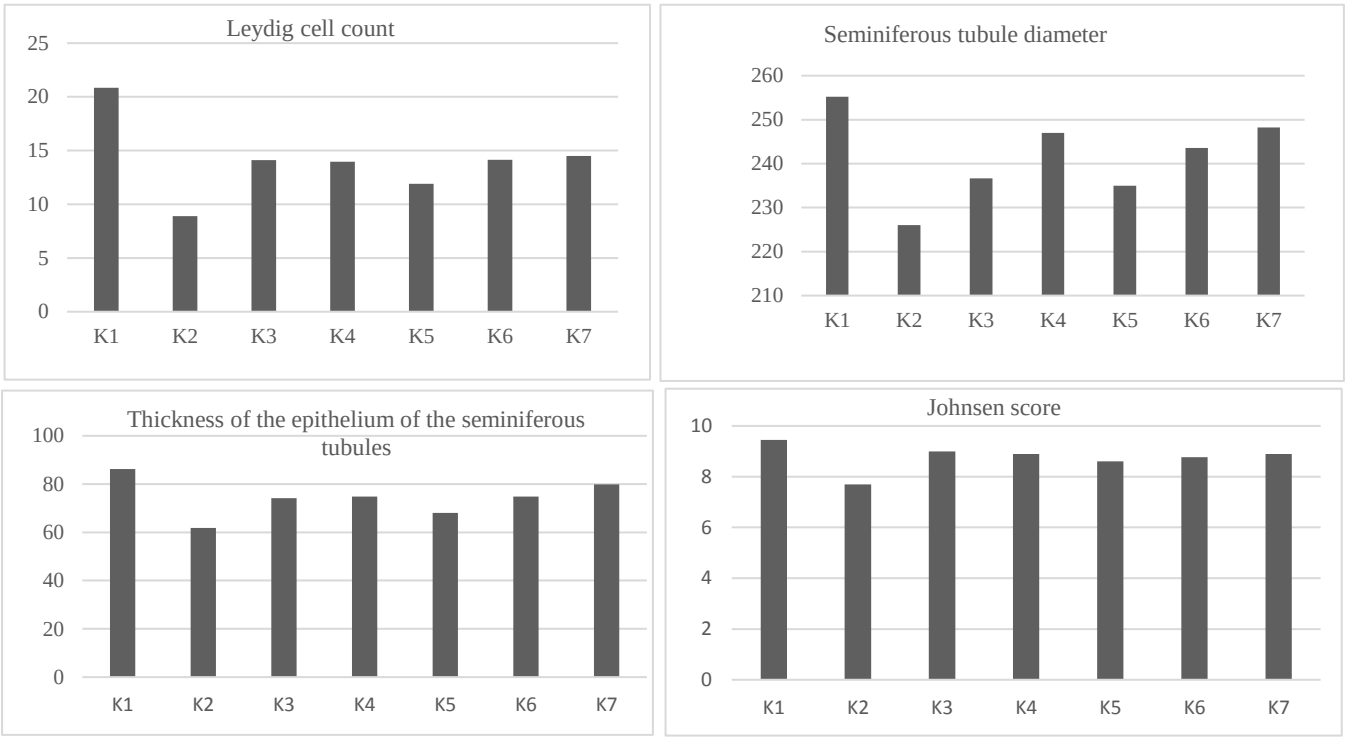

Figure 1: Results of testicular histopathological evaluation of K1-K7 (observed under 400x magnification of light microscope [Olympus, Japan]).

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 regading the content of this article will be borne by them.

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