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Histological and histochemical comparative study on the effects of fasting and a ketogenic diet on the male reproductive system in mice

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ABSTRACT

Diet is one of the most important variables that control reproduction. Fasting is a kind of physical stress and can cause oxidative stress. Fasting can affect the formation of sperm. The ketogenic diet consists of about 70% fat, 25% protein, and 5% carbohydrates. It is similar to fasting as it leads to increased ROS. This study aimed to compare histological and histochemical alterations in the male reproductive system between the fasting and ketogenic diet groups. In this study, 120 male mice were used. They were divided into three groups: (i) the control group, they were given regular diets; (ii) the fasting group, which were given one meal per day; and (iii) the ketogenic diet group which were subjected to the keto diet. Each group consisted of 40 male mice, average age 8-12 weeks, and average weight of 28 g. The experiment lasted 8 weeks. Ten male mice from each group were killed at the end of every two weeks to obtain organs (testes and epididymis) to prepare tissue sections for microscopic examination. In this study, PAS stain was used. The results of the histological study showed the presence of histological alterations in each of testis and epididymis in both groups. The results of the histochemical study showed the interaction of the basement membrane of the seminiferous tubules in the testis with PAS ranged from weak to strong in the fasting group, while in the ketogenic diet ranged moderate to strong, albeit the reaction of the basement membrane of the epididymal tubules with PAS was strong in the two groups for eight weeks.

Keywords: Testis, Epididymis, Fasting, Ketogenic diet, PAS.
Article type: Research Article.

INTRODUCTION

Diet is one of the most important variables that control reproduction, and it has long been known that hunger negatively affects reproductive functions. Fasting is the intentional abstinence from the normal meal (s) including food and drink or abstaining from eating/drinking for an unusual period of time. Fasting has various physiological effects on the body and on growth. Fasting can affect the formation of sperm, and the quality of sperm is an important determinant of male fertility (Samuel *et al.* 2015). It is a kind of physical stress and can cause oxidative stress (Wresdiyati *et al.* 2007; Al-Moussawi 2022; Babashev *et al.* 2023). Oxidative stress occurs due to an imbalance of oxidants and antioxidants, which causes an increase in reactive oxygen species (ROS; Srinivasan *et al.* 2015). Mammalian testicular membranes contain a lot of polyunsaturated fats and are very vulnerable to oxidative stress. So, an increased concentration of ROS may cause infertility and testicular damage (Edlik *et al.* 2014). The ketogenic diet, also knownas the keto diet, is a specific type of diet in which low carbohydrates are consumed, however, the fat and protein contents are high in food, so body weight is reduced with high fat (Ding *et al.* 2019). When a person eats low levels of carbohydrates, the brain begins to use ketone bodies instead of glucose (Morfy *et al.* 2005). The ketogenic diet consists of about 10% fat, 22% protein, and 2% carbohydrates (Fan *et al.* 2018). This application has a harmful effect similar to fasting, taking the whole body into another phase of ketosis (Jeszka-skowron *et al.* 2018). Ketone bodies can act as an alternative fuel for sperm and stimulate sperm

motility (Faure *et al.* 2014). Excessive oxidative stress leading to increased ROS production and a decrease in antioxidant enzymes has been shown to be a possible mediator of male infertility (Marzony *et al.* 2016). The high concentration of ROS easily causes oxidative damage due to the abundance of fatty acids polyunsaturated in sperm membranes and leads to lipid oxidation, sperm DNA damage and apoptosis (Al-Ahmer 2019). The aim of this study was to compare alterations in the histological and histochemical status of male reproductive system between the fasting and ketogenic diet groups.

MATERIALS AND METHODS

Experimental animals

Male mice were obtained from the Iraqi Center for Cancer Research and Medical Genetics and were transferred to the animal house of the Biology Department, College of Science, Maysan University. These mice were free of pathogens. Their average age was 8-12 weeks and their average weight was 28 g. The male mice were left for two weeks to adapt before the start of the experiment and placed in plastic cages covered with a metal net and furnished with sawdust. The cages were cleaned twice a week. This study included 120 male mice divided into three groups (control group 40 mice, fasting group 40 mice and a third group 40 mice on which the ketogenic diet was applied) with a 12:12 hour light: dark cycle. The animals were handled in accordance with institutional guidelines and approved by the local animal ethics committee for all experimental procedures.

Collection of samples

Male mice were euthanized by placing them in a closed cage and placing chloroform on cotton and placing it inside the cage (Blackshaw *et al.* 1988). Small samples of testis and epididymis were obtained and wished with normal saline (Basim 2019), fixed in 10% formalin, and processed by routine histological techniques (Luna 1968). Tissue sections of 5 μm thickness were stained with hematoxylin and eosin and examined by light microscopy. Other tissue sections of the epididymis were also stained with PAS (Bancroft & Stevens 2012), and evaluated at the end of every two weeks of the experiment.

Statistical analysis

The mean and standard deviation of the data were analyzed by SPSS software using One-Way ANOVA (analyses of variance) followed by the LSD test for statistical differences at the level of significance ($p < 0.05$) (Griffith 2007).

RESULTS

The testis

The results of microscopic examination of the histological sections of the testes in the mice of the control group showed that they consist of a normal structure consisting of seminiferous tubules containing a basement membrane, spermatogenic germ epithelial cells (spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids), Sertoli cells, a lumen filled with sperm, and interstitial tissue among the tubules contains Leydig cells (Fig. 1). In the second week, the results of the fasting group showed decrease in the diameter of the lumen, an elevated number of germ layers, and an increase in the space between the seminiferous tubules (Fig. 2). The results of the ketogenic diet group in the same period were the presence of gaps between spermatogenic germ cells, expansion of the lumen, decreased Leydig cells, and drop in sperm (Fig. 3). In the fourth week, the results in the fasting group showed the presence of spaces between spermatogonia and decrease in lumen. Degeneration was found in the interstitial tissue, as happened in the second week (Fig. 4). In the case of the ketogenic diet group, in the same period, the results showed the presence of thickening in parts of the basement membrane of the seminiferous tubule, the presence of spaces between the layers of germ epithelial cells, and decrease in lumen (Fig. 5). In the sixth week, the fasting group showed the same alterations appeared in the fourth week (Fig. 6), while the ketogenic diet group, in the same period, showed a decrease in sperm, the presence of spaces between spermatogonia and spermatid, and the elevated number of germ cells layers (Fig. 7). In the eighth week, the fasting group showed degeneration of the interstitial tissue and a decrease in the diameter of the lumen (Fig. 8), while the ketogenic diet group in the same period showed an elevation in the diameter of the lumen and decrease in its sperm, as was found degeneration in the interstitial tissue and spaces between spermatogonia (Fig. 9).

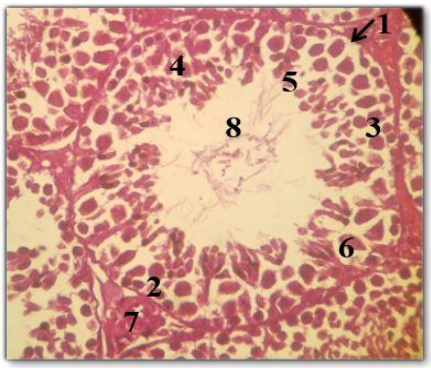

Fig. 1. Histological section of testis of mice of the control group showing normal tissue consisting of seminiferous tubules consisting basement membrane (1), spermatogonia (2), primary spermatocytes (3), secondary spermatocytes (4), spermatid (5) Sertoli cells (6), Leydeck cells (7), medial lumen (8) (H&E stain, 400 X).

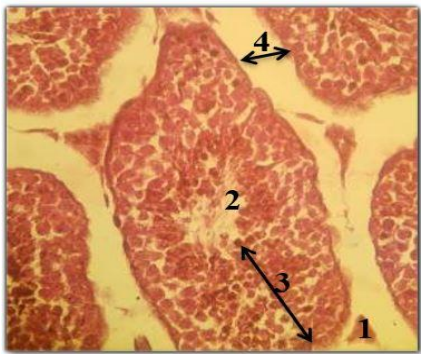

Fig. 2. Histological section of the testes of mice of the fasting group in the second week showing interstitial tissue degeneration (1), decreased lumen (2), an increase in the number of germ cell layers (3), an increase in the distance between the seminiferous tubules (4) (H&E stain, 400 X).

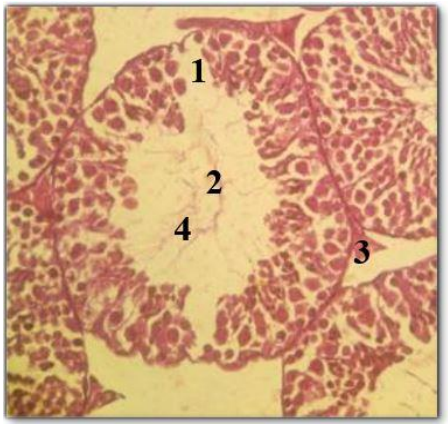

Fig. 3. Histological section of the testes of mice of the ketogenic diet group in the second week showing there is a gap between the germs cells (1), lumen dilatation (2), Leydig cells deficiency (3), sperm deficiency (4), (H&E stain, 400 X).

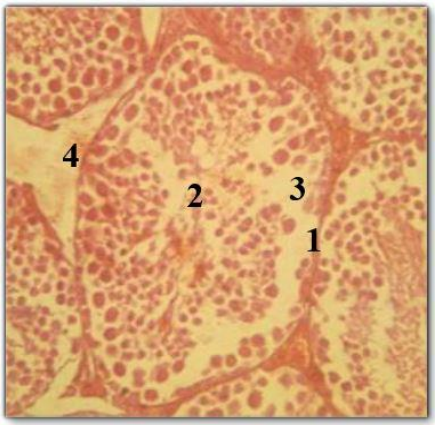

Fig. 4. Histological section in the testes of mice of the fasting group in the fourth week showing the presence of spaces between the spermatogonia (1), the absence of a lumen (2), the presence of spaces between the spermatogonia and the spermatid (3), interstitial tissue degeneration (4; H & E stain, 400X

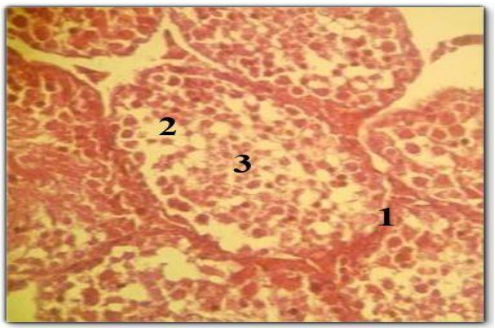

Fig. 5. Histological section of the testes of mice of the ketogenic diet group in the fourth week showing the presence of thickening in parts of the basement membrane of the seminiferous tubule (1), the presence of spaces between the layers of germ epithelial cells (2), lack of lumen (3) (H&E stain, 400X).

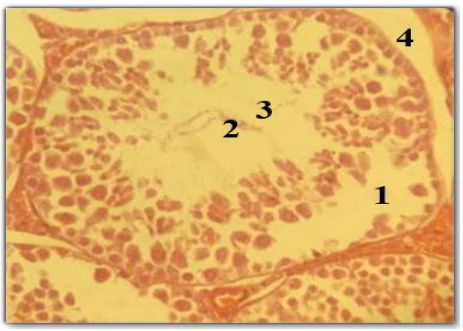

Fig. 6. Histological section of the testis of mice of the fasting group in the sixth week showing spaces between spermatogonia and primary spermatocytes (1), an increase in the diameter of the lumen (2) decrease of sperm (3), interstitial tissue degeneration (4; H&E stain, 400X).

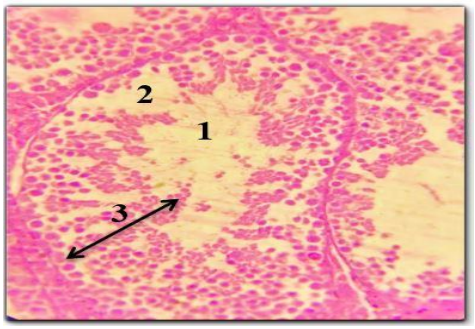

Fig. 7. Histological section of the testis of mice of the ketogenic diet group in the sixth week showing decrease of sperm (1), the presence of spaces between the spermatogonia and spermatids (2), an increase in the number of layers of spermatogenic germ epithelial cells (3) (H&E stain, 400X).

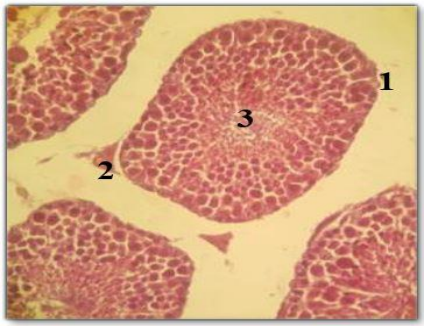

Fig. 8. Histological section of the testis of mice of the fasting group in the eighth week showing interstitial degeneration (1), decrease of Leydig cells (2), decrease in the diameter of the lumen of the seminiferous tubule (3; H&E stain, 400X).

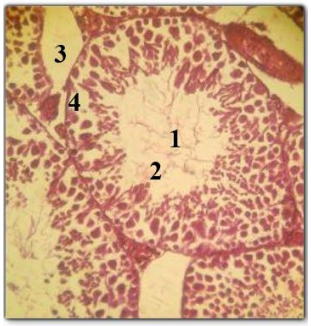

Fig. 9. Histological section of the testis of mice of the ketogenic diet group in the eighth week shows an increase in the diameter of the lumen (1) with a few sperms (2), the presence of spaces between the seminiferous tubules (3), and the presence of spaces between the spermatogonia (4; H&E stain, 400X).

Epididymis

The results of the microscopic examination of the histological sections of the epididymis in the mice of the control group showed the normal tissue consisting of the conducting tubules and the pseudo columnar cells lining the tubule. The lumen filled with sperm, and the presence of smooth muscle fibers surrounding the tubule (Fig. 10). The fasting group in the 2nd week showed the presence of spaces between the tubules, declined diameter of the lumen, decreased sperm, detachment of the epithelial cells from the basement membrane, hyperemia, and the appearance of macrophages in the lumen (Fig. 11), while the ketogenic diet group in the same period showed disintegration of epithelial cells, atrophy of some epididymal tubules, filling of the lumen with sperm, and a decrease in smooth muscle fibers (Fig. 12). In the 4th week, the results showed the same alterations in the fasting group in the second week including the appearance of some macrophages cells in the lumen and the detachment of the epithelial cells from the basement membrane (Fig. 13). The ketogenic diet group in the same period also showed the same alterations in the second week including the epithelial cells dissociation. The lumen was filled with sperm, as shown by the detachment of the epithelial cells from the basement membrane (Fig. 14). In the 6th week, the results showed in the fasting group an elevation in the thickness of the epithelium, decreased tubular lumen, detachment of the epithelial cells from the basement membrane, dissociation of epithelial cells, and drop in sperm number in the lumen (Fig. 15). In the ketogenic diet the tubules were filled with sperm, a drop in the thickness of the epithelium, and the appearance of phagocytic cells in the lumen (Fig. 16). In the 8th week, the results of the fasting group showed an increase in the smooth muscle surrounding the tubules and the tubules being filled with sperm (Fig. 17), while the ketogenic diet group showed the same alterations in the same period as the fasting group, including an upraise in the smooth muscle surrounding the tubules and the presence of sperm inside the lumen. It also showed disintegration of epithelial cells (Fig. 18).

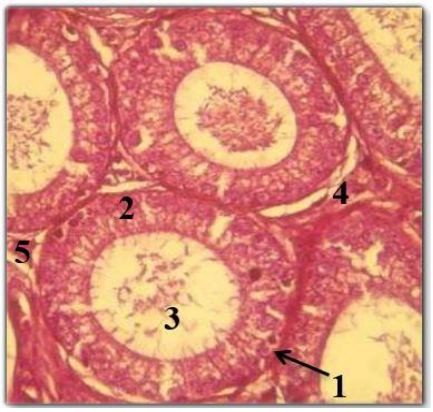

Fig. 10. Histological section of the epididymis of the control group mice showing the normal tissue of epididymal tubules (1), pseudo columnar cells lining the tubule (2), lumen filled with sperm (3), the presence of smooth muscle fibers surrounding the tubule (4), the interstitial tissue (5; H&E stain, 400X).

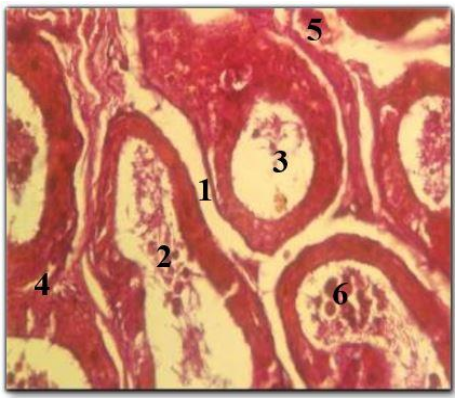

Fig. 11. histological section of the epididymis of mice of the fasting group in the second week, showing the presence of spaces between the tubules of the epididymis (1), a decrease in the diameter of the tubule lumen (2), a decrease of sperm in some of the tubules (3), detachment of the epithelial cells from the basement membrane (4), hyperemia (5), macrophage cells appearing in the lumen (6; H&E stain, 400X).

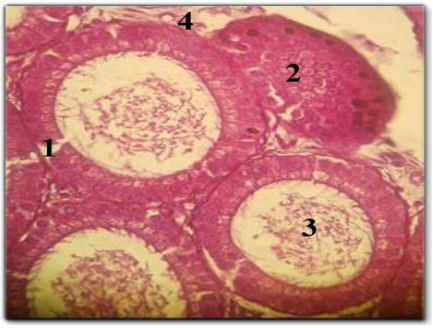

Fig. 12. Histological section of the epididymis of mice of the ketogenic diet group in the second week showing epithelial cell dissociation (1), atrophy of some epididymal tubules (2), lumen filling with sperm (3), decrease of smooth muscle fibers (4; H & E stain, 400 X).

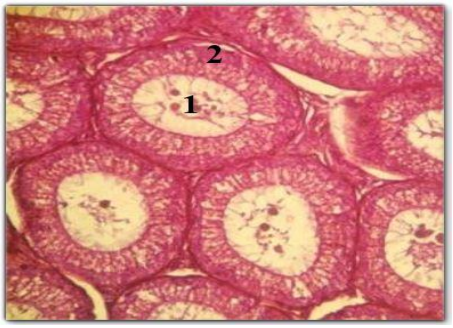

Fig. 13. Histological section of the epididymis of mice of the fasting group in the fourth week, showing the presence of some phagocytes in the lumen (1), detachment of epithelial cells from the basement membrane (2; H&E stain, 400 X).

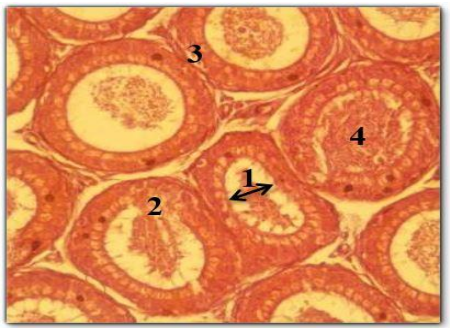

Fig. 14. Histological section of the epididymis of mice of the ketogenic diet group in the fourth week showing a decrease in the diameter of the tubular lumen (1), epithelial cell dissociation (2), detachment of epithelial cells from the basement membrane (3), and lumen filling with sperm (4; H&E stain, 400 X).

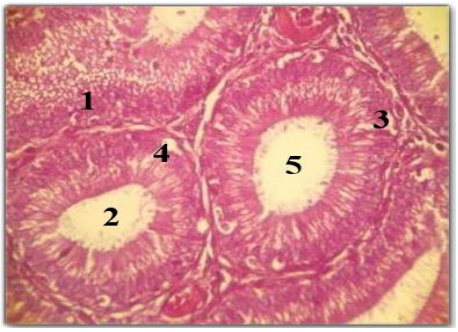

Fig. 15. Histological section of the epididymis of mice of the fasting group in the sixth week shows an increase in the thickness of the epithelium (1), decrease of tubular lumen (2), detachment of epithelial cells from the basement membrane (3), epithelial cell dissociation (4), decrease of sperm (5; H&E stain, 400 X).

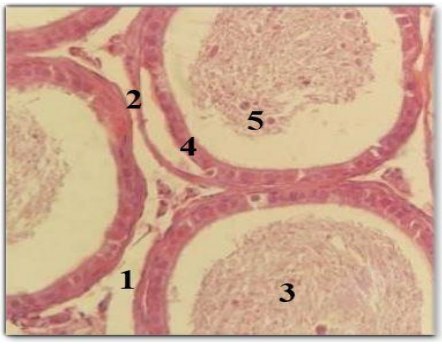

Fig. 16. Histological section of the epididymis of mice of the ketogenic diet group in the sixth week, showing the presence of space between the tubules (1), decrease of smooth muscle (2), tubule filling with sperm (3), decrease in epithelium thickness (4) appearance Macrophages in the lumen (5; H & E stain; 400 X).

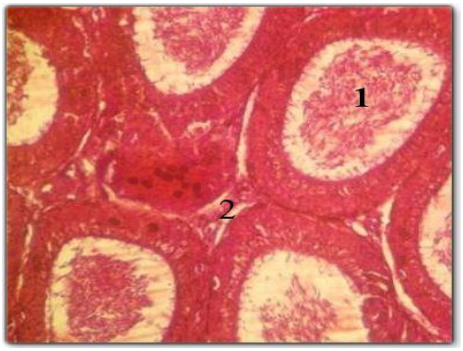

Fig. 17. A histological section of the epididymis of mice of the fasting group in the eighth week showing tubule filling In sperm (1), the presence of smooth muscle fibers around the tubules (2; H & E stain; 400 X).

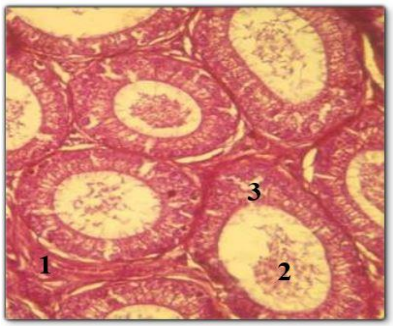

Fig. 18. Histological section of the epididymis of mice of the ketogenic diet group in the eighth week shows an increase in the smooth muscle surrounding the tubules (1), the presence of sperms inside the lumen (2), and the disintegration of the epithelial cells lining the tubule (3; H & E stain; 400 X).

Results of the histochemical study

Testis

The results of the histochemical study in the control group showed a strong interaction of the basement membrane of the seminiferous tubules with PAS (Fig. 19). In the 2nd week, it was weak with PAS (Fig. 20), while the in the ketogenic diet in the same period was strong (Fig. 21). In the 4th week, the interaction of the basement membrane in the fasting group was moderate with PAS (Fig. 22), while in ketogenic diet in the same period was strong (Fig. 23). In the 6th week, the interaction of the fasting group was also strong with PAS similar to the control group (Fig. 24), while the interaction in the ketogenic diet group in the same period was moderate (Fig. 25). In the 8th week, the interaction of both the fasting and ketogenic diet groups was strong with PAS (Figs. 26- 27).

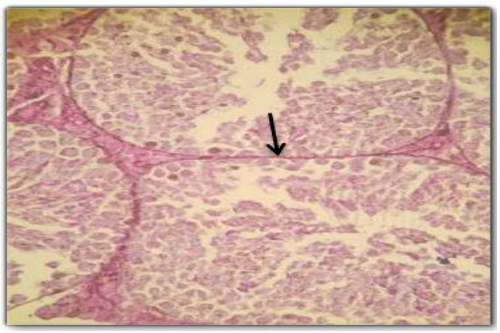

Fig. 19. testes of control group mice showed strong interaction of the basement membrane of the seminiferous tubules with PAS. 400X

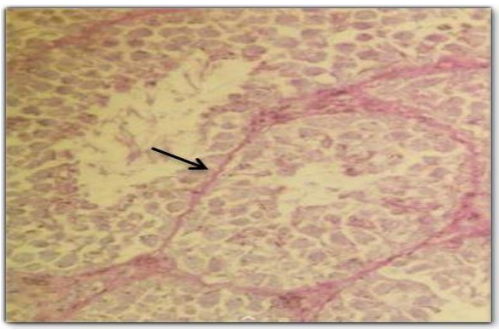

Fig. 20. testes of mice of the fasting group in the second week showed a weak interaction of the basement membrane of the seminiferous tubules with PAS. 400X

Epididymis

The results of the histochemical study showed a strong interaction of the basement membrane of the epididymal ducts in the control group with PAS (Fig. 28). The interaction was also strong in each of the two groups (fasting and ketogenic diet) in the 2nd week (Figs. 29-30). In the 4th week, the interaction was also strong in the fasting and ketogenic diet groups (Figs. 31- 32). In the 6th week, the interaction was strong with PAS in the two groups, similar to the previous two weeks (Figs. 33-34). Also, in the 8th week, the interaction was strong with PAS in the two groups, similar to the previous weeks (Figs. 35-36).

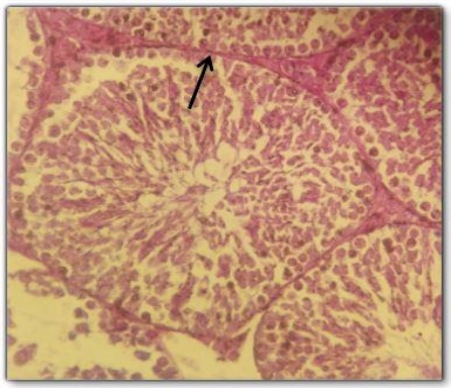

Fig. 21. Testes of mice of the ketogenic diet group in the second week showed strong interaction of the basement membrane with PAS. 400X

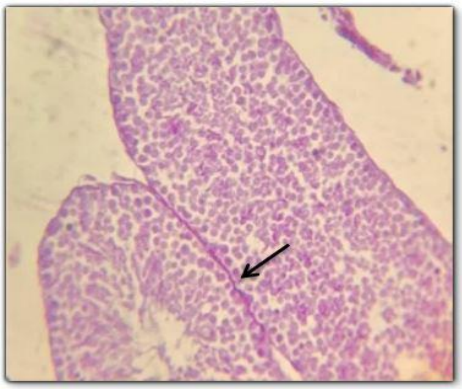

Fig. 22. Testes of mice of the fasting group in the fourth week showed moderate interaction the basement membrane with PAS. 400 X

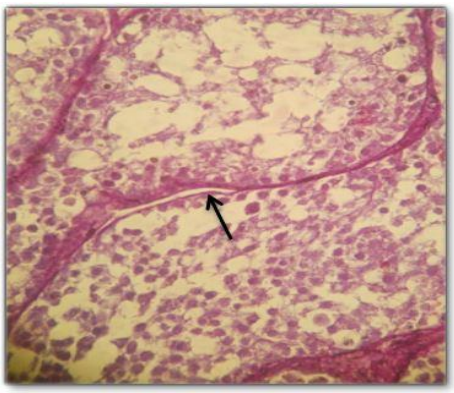

Fig. 23. Testes of mice of the ketogenic diet group in the fourth week showed strong interaction the basement membrane with PAS; 400X.

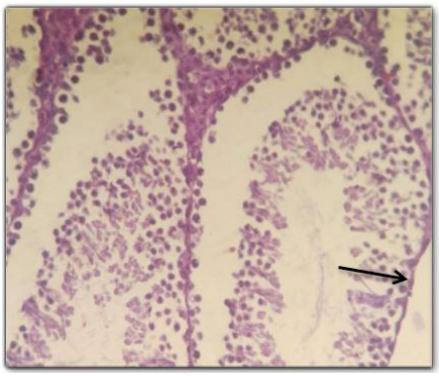

Fig. 24. Testes of mice of the fasting group in the sixth week showed strong interaction of the basement membrane with PAS (400 X).

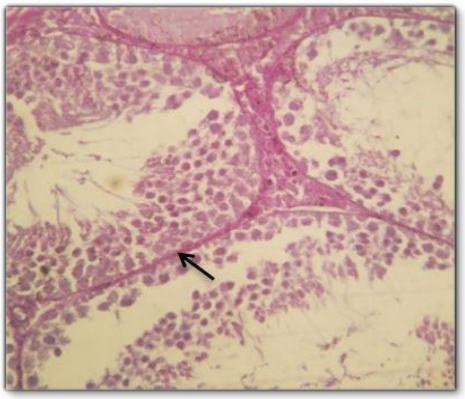

Fig. 25. Testes of mice of the ketogenic diet group in the sixth week showed a moderate reaction of the basement membrane with PAS (400 X

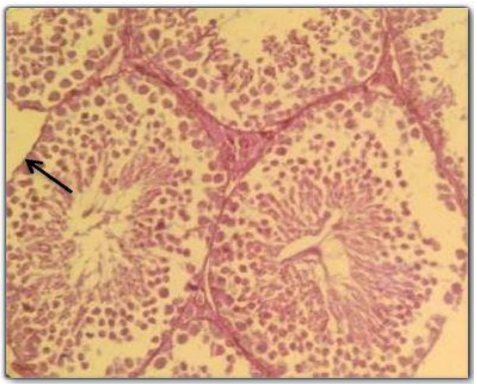

Fig. 26. Testes of mice of the fasting group in the eighth week showed strong interaction of the basement membrane with PAS (400 X)

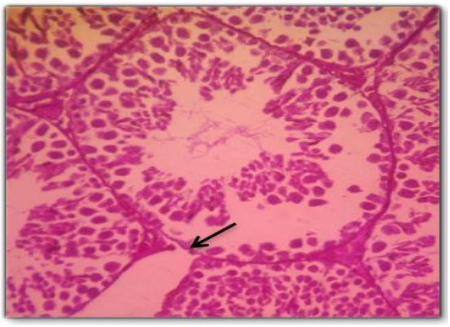

Fig. 27. Testes of mice of the ketogenic diet group in the eighth week showed strong interaction of the basement membrane with PAS (400 X).

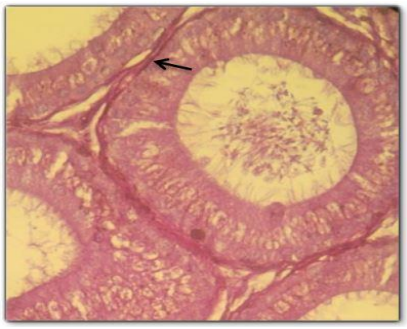

Fig. 28. The epididymis of control group mice showed strong interaction of the basement membrane with PAS (400 X).

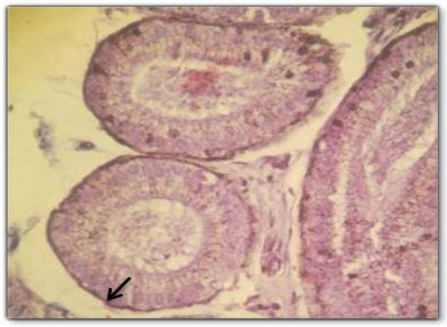

Fig. 29. The epididymis of the fasting group mice in the second strong showed a strong interaction of the basement membrane with PAS (400 X).

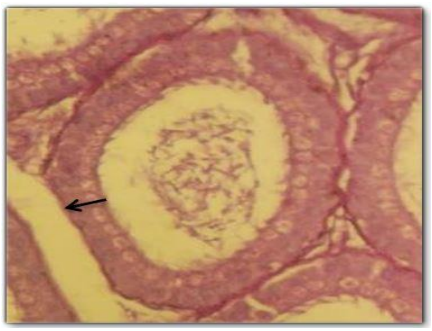

Fig. 30. the epididymis of mice of the ketogenic diet group in the second week showed a strong interaction of the basement membrane with PAS (400 X).

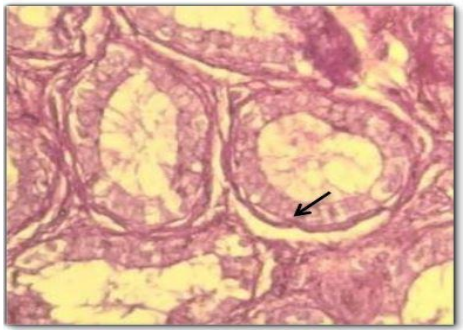

Fig. 31. The epididymis of the mice of the fasting group in the fourth strong showed a strong interaction of the basement membrane with PAS (400 X).

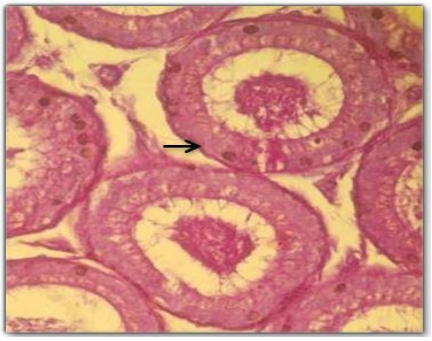

Fig. 32. The epididymis of mice of the ketogenic diet group in the fourth week showed strong interaction of the basement membrane with PAS (400 X).

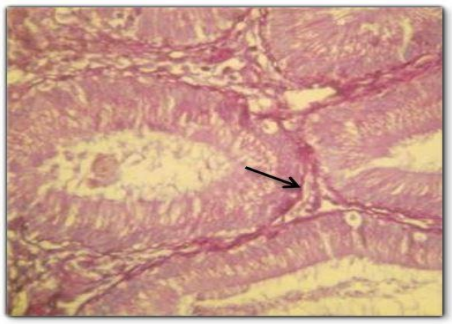

Fig. 33. The epididymis of the mice of the fasting group in the sixth strong showed a strong interaction of the basement membrane with PAS (400 X).

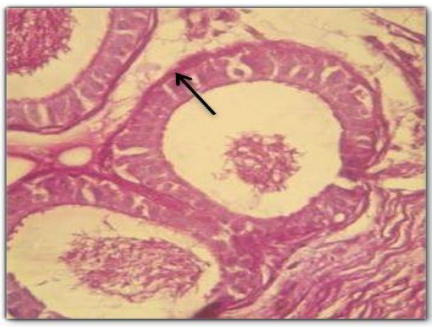

Fig. 34. The epididymis of mice of the ketogenic diet group in the sixth strong showed a strong interaction of the basement membrane with PAS (400 X).

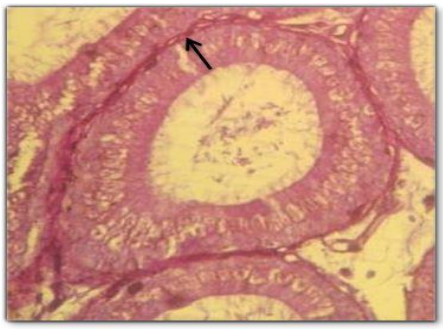

Fig. 35. The epididymis of the eighth week in the fasting group showed a strong interaction with PAS (400 X).

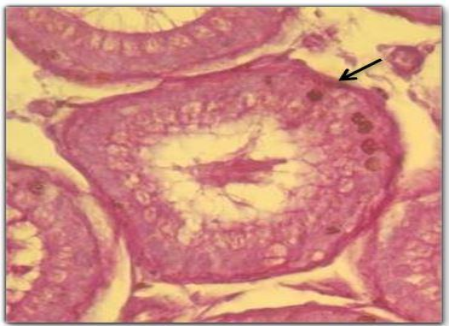

Fig. 36. the epididymis of the ketogenic diet group rat epididymis showed strong interaction with PAS (400 X).

DISCUSSION

The testes are the most important organ of the male reproductive system, as they have two main functions, including steroid production, and spermatogenesis (Carreau *et al.* 2002). The results of the current study showed the occurrence of histological alterations in the testes of the mice in the fasting group, exhibiting the presence of degeneration in the interstitial tissue, a decreased diameter of the lumen, an elevation in the number of layers of germ cells, the presence of spaces between spermatogonia and spermatid, and a drop in sperm number and Leydig cells. This study agrees with Samuel *et al.* (2015) who indicated that fasting causes histological alterations in the structure of the testis. Sertoli cells have an essential role in the development of germ cells through their formation of the blood-testis barrier that protects germ cells and transports nutrients as well as hormones to germ cells. It is believed that these alterations in testicular tissue are due to a defect in the structure and function of Sertoli cells (Reise *et al.* 2015). The decreasd sperm in some lumens of the seminiferous tubules, perhaps due to a drop in testosterone due to decline in the Leydig cells number (Manglang & Nager 2014). The results showed that the ketogenic diet caused the appearance of gaps among the germ epithelial cells, an elevation in the diameter of lumen, a decrease in Leydig cells, a drop in sperms, a thickening of parts of the basement membrane of the seminiferous tubules, and an upraise in the number of layers of germ cells. The thickness of the walls of the seminiferous tubules weakens the relationship between the wall and the interstitial tissue, when the wall thickness is elevated. Many alterations appear inside the testes, especially in the function of Sertoli cells, affecting the differentiation of germ cells and inhibiting spermatogenesis (Gulkesen *et al.* 2002). As for Winter & Huhtaniemi (2017), they concluded in their study that Sertoli cells secrete collagen fibers type IV, and these fibers cause an elevation in the thickness of the walls of the seminiferous tubules, thus lead to poor sperm formation. The results of the current study showed the occurrence of histological changes in the epididymis of the mice of the fasting group, as they showed the presence of spaces between the epididymal tubules, a drop in the diameter of the lumen, a decreased sperm, the removal of epithelial cells from the basement membrane, blood congestion, the appearance of some macrophages in the lumen, an upraise in the thickness of the epithelium, and the disintegration of epithelial cells. These alterations may be due to a drop in the level of testosterone produced by Leydig cells (Al-Gazali 2021). The epididymis is one of the androgen-dependent organs that plays important roles in the differentiation and proliferation of cells lining the epididymal tubules and these cells have androgen-

sensitive receptors (Boukenaoni *et al.* 2017). In the case of the presence of phagocytic cells in the lumen of some tubules, determining the origin of these cells is controversial, some of authors believed that they are real macrophages, while others attributed them to lymphocytes or suggest that they are Sertoli cells with phagocytic activity that devour the residual bodies of spermatid under normal conditions (Al-Aramy 2004). The results of the ketogenic diet group showed disintegration of epithelial cells, atrophy of some epididymal tubules, presence of sperm in the lumen, a decrease in the smooth muscle fibers, detachment the epithelial cells from the basement membrane, and the appearance of phagocytic cells in the lumen, which may be due to low levels of testosterone, produced by Leydig cells (Mori *et al.* 1998). The results of the histochemical study showed a strong interaction of the basement membrane of the seminiferous tubules with PAS in the control group, while in the fasting group the interaction ranged from weak to strong and in the ketogenic diet group it ranged from medium to strong. The changes in the basement membrane are associated with poor spermatogenesis (Schell *et al.* 2010; Moustafa *et al.* 2012). This can be explained by effect of ROS on the basement membrane (Mansour *et al.* 2018).

CONCLUSION

This study showed that both fasting and the ketogenic diets affect testicular and epididymal tissue. Fasting led to a decrease in Leydig cells and an elevation in the distances between the seminiferous tubules in the testis and in the epididymis, a reduction in the lumen of epididymal tubules and appearance of macrophage in the lumen, while the ketogenic diet led to a drop in the sperm number and a decrease in the smooth muscle fibers. The interaction of the basement membrane of seminiferous tubules with PAS ranged from weak to strong in the fasting group and from moderate to strong in the ketogenic diet group.

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