EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
ZOOLOGY

B

ISSN
2090-0759

WWW.EAJBS.EG.NET

Vol. 14 No. 2 (2022)

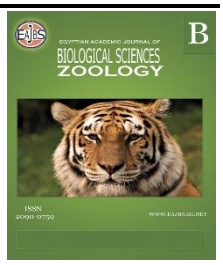
.

www.eajbs.eg.net

Citation: *Egypt. Acad. J. Biolog. Sci. (B. Zoology) Vol. 14(2) pp: 101-109(2022)*
DOI: 10.21608/EAJBSZ.2022.252142

Egypt. Acad. J. Biolog. Sci., 14(2):101-109 (2022)

Egyptian Academic Journal of Biological Sciences
B. Zoology
ISSN: 2090 – 0759
http://eajbsz.journals.ekb.eg/



Effect of Aging on Testis Structure, GLUT2, MTOR Expression and Testosterone Level in Spermatogenesis of The Rats

Maiada Moustafa* and Basma N. Hassan
Zoology and Entomology Dep., Faculty of Science, Helwan University
E-mail*: maiadamoustafa11@gmail.com

ARTICLE INFO
Article History
Received:30/5/2022
Accepted:1/8/2022
Available:5/8/2022

Keywords:
Testis, GLUT2, MTOR**,** testosterone hormone, spermatogenesis, Rats.

ABSTRACT

Background: Male ageing has been previously associated with declining sperm parameters, disrupted hormone secretion and increased time to pregnancy. Aging is a natural ongoing process characterized by morphological and structural degeneration and like other organs. Glucose is essential for testicular function; the uptake of carbohydrate-derived glucose by cells is mediated by glucose transporters (GLUTs). MTOR is also involved in the maintenance and restructuring of the blood-testis barrier (BTB), a key event in the seminiferous epithelium cycle **Aim of the work**: In the present study, we aimed to investigate the relationship between aging and the expression of GLUT2 and MTOR in the testis, the quality of the spermatogenesis process as well as the testosterone level.**Materials and methods:** Eighteen rats were classified into the following experimental age groups, premature, mature and aged the rats were weighed and sacrificed by decapitation under a mild dose of anesthetic ether testicular tissue was existed for histological, histochemical, immunohistochemical and molecular techniques.**Results:** In the aged group, Hematoxylin and Eosin stain, showed a decreased number of sperms that were observed in the lumen of seminiferous tubules, weak PAS reaction in basement membranes moderate immunohistochemical reaction for GLUT2 and MTOR antibodies, also decreased level of testosterone hormone was observed.**Conclusion**: spermatogenesis process is affected badly by aging that was clear histologically in testicular tissue and the decreased level of testosterone hormone.

INTRODUCTION

Aging is a normal process that has morphological and structural degenerative features in all organs including gonads (Eskenazi *et.al*, 2003). Testes play the role of producing male gametes as well as male hormones, testosterone. Infertile men have a high risk of hypogonadism because all the testicular functions are interrelated (O'Brien *et. al*., 2005). There are structural changes in senile male gonads like the reduction of the germ cell's numbers and size, and that affects negatively on fertility (Jiang *et. al.*, 2014). Lifestyle, diseases as well as the age have an impact on the production of gametes and fertility (Eva *et. al.*, 2021). The male has a point which is called andropause that is equivalent to menopause, in which the pattern of spermatogenesis and levels of testosterone are decreased (Juul & Skakkebaek, 2002; Perheentupa & Huhtaniemi, 2009 and Arnab *et*

al., 2014).

Normal reproductive functions need a proper amount of energy (Rato *et al.*, 2012). Metabolic regulators play crucial roles to control cellular senescence. Glucose is vital to most mammalian cells, especially testicular cells, and glucose passage across cell membranes is facilitated by the glucose transporters (GLUT-family) (Simpson *et al.*, 2008 and Arnab *et al.*, 2014).

The GLUT family has fourteen members of proteins and is divided into three classes depending on hexose affinity, structural homogeneity and tissue distribution (Bucci *et al*., 2010). Immunocytochemical analyses demonstrated that GLUT1, GLUT2, GLUT3 and GLUT8 are expressed in the rat testis (Kokk *et al.*, 2004, Carayannopoulos *et al.*, 2000; Doege *et al*., 2000). GLUTs are species-dependent, where GLUT2 is expressed in rats and mice, while GLUT3 in mice, rats, and humans (Kyu Ri Hahn *et al.*, 2017). All of the members of this family are mainly glucose transporter except GLUT2, which can also transport fructose (Bucci *et al*., 2010).

The process of the formation of the gametes needs metabolite transportation between blood and the germ cells. This blood-germ cell interaction is protected by junctions to control the exchanged substances that are called the blood-testis barrier (BTB), or Sertoli cell barrier (ScB) (Mruk & Cheng, 2010, Mruk & Cheng, 2012). The Sertoli cell barrier protects the testes from xenobiotics by exporting them back into the blood (Hogarth, 2015). The substances that are larger than 1KDa cannot pass from the basal to the adluminal side of the seminiferous tubules due to tight junctions which are formed between the adjacent Sertoli cells (Walker and Cheng, 2005). The BTB is important for normal spermatogenesis and germ cell maturation and it is formed in the mouse between 15 and 18dpp (Hogarth, 2015). The mitotic division in the multiplication phase of spermatogenesis is regulated by many proteins including MTOR (mechanistic Target of Rapamycin), which is also integrated into BTB. MTOR protein is strongly localized at BTB, suggesting that MTOR plays a crucial role in controlling its function, and also in the spermatogonia increase in number and death (Correia *et al*., 2020).

MATERIALS AND METHODS

A.Experimental Animals and Design:

Eighteen rats were classified into the following experimental age groups (n=6): (A) premature (one month); (B) mature (three months); (C) aged (22 months). The rats were weighed and sacrificed by decapitation under a mild dose of anesthetic ether. One testis from each animal was kept in -20^{o} C for the testosterone level evaluation in the tissue, and the other testis were fixed in neutral buffered formalin fluid for histology, histochemistry and immunohistochemistry.

B. Measurement of Total Testosterone Level in Testicular Tissue:

A 0.3 gram of frozen testicular tissue was excised into small pieces, approximately 1cm2, the tissue is washed with phosphate buffer saline (PBS) pH 7.4. Two mL of PBS was added to the washed tissue in a ratio of 3: 4 volume of buffer per volume of tissue. In a 5 mL test tube, the tissue was disrupted with tissue homogenizer for 1 minute until all solid tissue is disrupted, followed by centrifugation at 6000rpm for 10 minutes, then the supernatant was collected in 1.5 mL sterile Eppendorf and used for testing Testosterone concentration using Enzyme-linked immunosorbent assay, the remaining cell lysate was stored at -20^{o}c for further analysis. The Total Testosterone level was measured in the cell lysate of testicular tissue of rat species using the ELISA technique, the assay was conducted using Rat T(Testosterone) ELISA Kit, cat no: EH1642, Fine Biotech, Wuhan, Hubei, China.

C.Histological, Histochemical and Immunohistochemical Evaluation:

The excised organs had fixed in neutral buffered formalin solution for about 24 hours, cleared in xylene and impregnated in parablast for blocking, serial sections 5 u m thick were prepared and stained with the following techniques.

a-Hematoxylin and Eosin for histological examination of the general architecture of the organs **(**Drury and Wallington,1980).

b-Periodic acid Schiff's for demonstration of general carbohydrate (Bancroft and Gamble, 2002).

c-Bromophenol blue for demonstration of protein content (Pearse, 1980)

d- For immunostaining, testes of rat of different groups (premature, mature and aged) was paraffin-embedded, and 4mm sections were immunostained using anti- GLUT 2 and MTOR primary antibody (Invetrogen, Thermosintific, USA) for 90 minutes. This was followed by the secondary antibody application using the immunoperoxidase technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA).

Immunoreactive Score Calculation:

The immune-positive cells were counted in each region of interest (ROI) using a counting grid and their proportion among the total counterstained cell population. The stained areas of the ROI were digitally marked and the percentage of stained areas was determined using a computer program.

The protein expression intensity calculated using the was the immune-reactive score (IRS) for IHC-data interpretation. The immune-reactive score (IRS) gives a range of 0–12 as a product of multiplication between positive cells proportion score (0–4) and staining intensity score (0–3).

Statistical Analysis:

The data were presented as the mean±standard deviation (SD). Multiple variable comparisons were done using a one-way analysis of variance (ANOVA) in the statistical package program (SPSS version 20). Duncan's test was used to make statistical comparisons between the three groups. Statistical significance was defined when the p-value is equal to or lower than 0.05.

RESULTS

The level of testosterone in the testicular tissue in the mature group is significantly higher than in the immature and the aged groups and at the same time, its level in the immature group is significantly lower than its level in the aged group (Fig.1).

H&E stain for premature mouse showed normal arranged testicular tubules, incomplete spermatogenesis with few spermatozoa in the lumen also normal interstitial cells between the tubules was seen (Fig.2A). PAS reaction exhibited strong reaction for, tunica albuginea as well as in the inter-tubular connective tissue of testes while exhibit weak for spermatogenic cells (Fig.2B). The total proteins appeared in the testicular tissues of premature rats was deeply stained (Fig.2C).

Hematoxylin and Eosin examination of the testis of a mature rat revealed its normal structure. It showed different stages of spermatogenesis with well-developed spermatozoa and closely backed seminiferous tubules. The interstitial cells were presented clearly in between the tubules. Sertoli cells, also, were presented with their characteristic indentations (Fig.2D). PAS-positive materials appeared in tunica albuginea as well as in the inter-tubular connective tissue of testes of adult mice. The spermatogenic cells exhibited a strong reaction (Fig.2E).

The total proteins appeared in the testicular tissues of adult rats as deeply stained granules inside the nuclei and cytoplasm of all spermatogenic cells. The tunica albuginea,

inter-tubular connective tissue as well as the boundaries of seminiferous tubules showed a strong reaction, as well as the sperms, showed a strong reaction of total protein (Fig.2F).

Hematoxylin and Eosin examination of the testis of aged rats revealed many variable changes like loss of the arrangement of seminiferous tubules, with vacuolated and shrunken interstitial cells in between. In abnormal spermatogenesis with degenerated spermatids, a small number of spermatozoa emerged in Sertoli cells, not in the lumen (Fig.2G). PAS reaction exhibited weak reaction spermatogenic cells, tunica albuginea as well as in the inter-tubular connective tissue of testes (Fig.2H). The total proteins that appeared in the testicular tissues of adult rats were very weak (Fig.2I). Immunohistochemical results showed a mild reaction of both GLUT2 (Fig.3A&B) at Leydig cells and MTOR (Fig.4A&B) at Sertoli cells in premature rats, while a severe reaction was observed in adult rats in the same cells (Fig.3C&D) (Fig.4C&D) and the moderate reaction was noticed in testicular tissue of aged rats (Fig.3E&f) (Fig.4E&F).

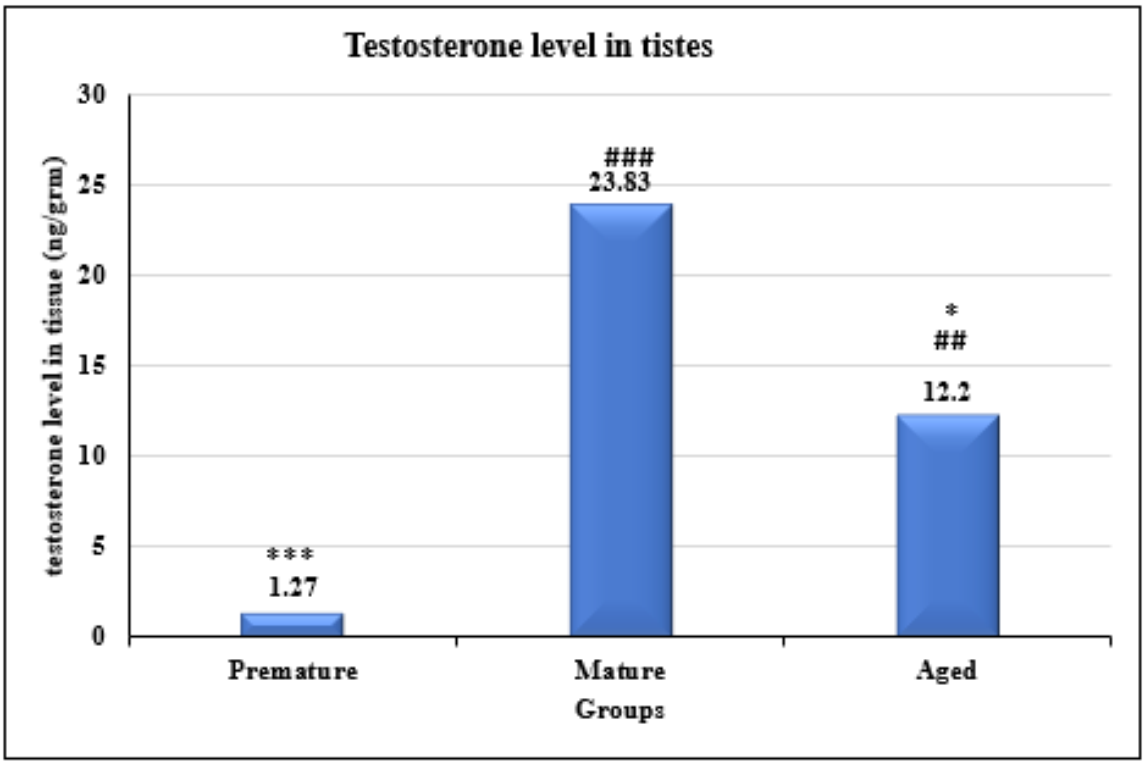


Fig. 1: Histogram illustrating the level of the testosterone (ng/grm) in the testicular tissue in the three studied groups. Values are mean ± SD at $p \leq 0.05$. *, significance against premature group. #, significance against aged group.

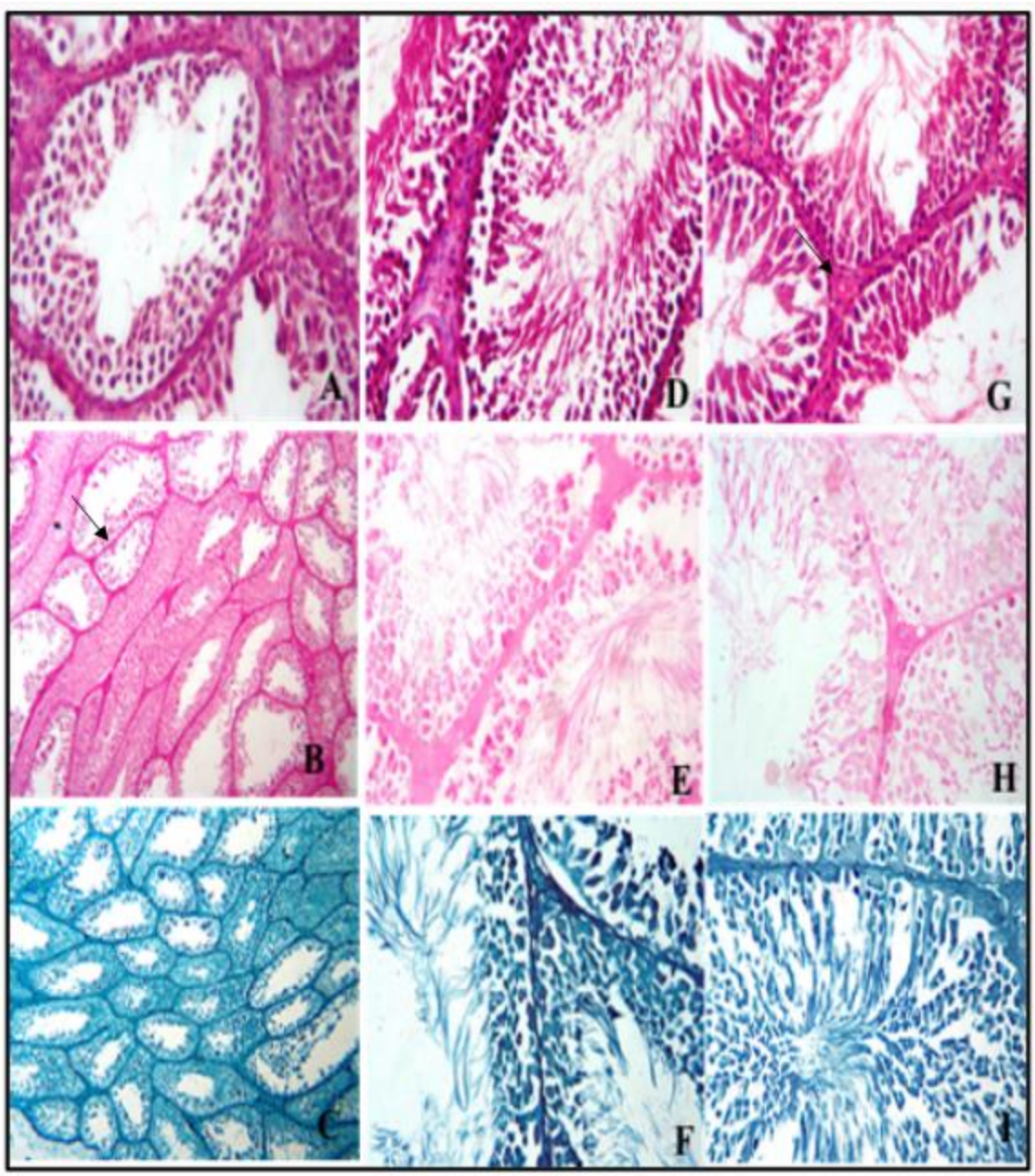


Fig. 2: Photomicrographs of cross section from testes showing: in premature group (A): normal Sertoli cells, spermatogenic cells and absence of spermatozoa., (B) PAS-positive reaction of the basement membrane (→) and in the inter-tubular connective tissue (×100). (C) showing decrease of total proteins content pf spermatogenic cells. Mature testes (D) increase of spermatogenic cells and normal spermatozoa well organized tubules and the Leydig cells in between, while aged testes. (E) showing PAS-positive reaction of the basement membrane and in boundaries and in the inter-tubular connective tissue. (F) showing strong protein contents in all layers of spermatogenic cells, Leydig cells, and sperms head. Aged testes a showing reduction of spermatogenic cells degenerated spermatids (G) small number of spermatozoa (→) shrunken and vacuolated interstitial cells (V). show abnormal spermatogenesis with sever defects in post-meiotic stages of spermatogenesis. (H) showing weak PAS- reaction of the basement membrane and in boundaries and in the inter-tubular connective tissue. (I) showing weak protein contents in all layers of spermatogenic cells, Leydig cells, and sperms head.

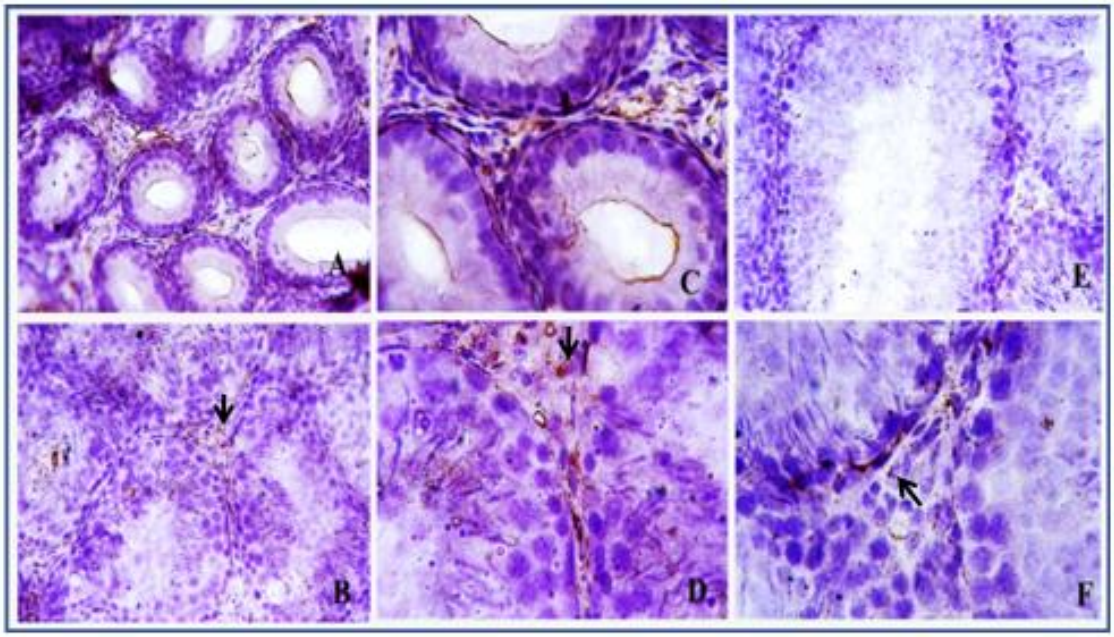


Fig. 3: A photomicrograph for immunohistochemical reaction for anti GLUT2 antibody (A) mild reaction in-between seminiferous tubules (B) also very clear mild reaction in Leydig cells (arrow). (C) severe reaction in-between seminiferous tubules (D) severe reaction in Leydig cells (arrow). (E) moderate reaction in-between seminiferous tubules (F) also a moderate reaction in Leydig cell (arrow).

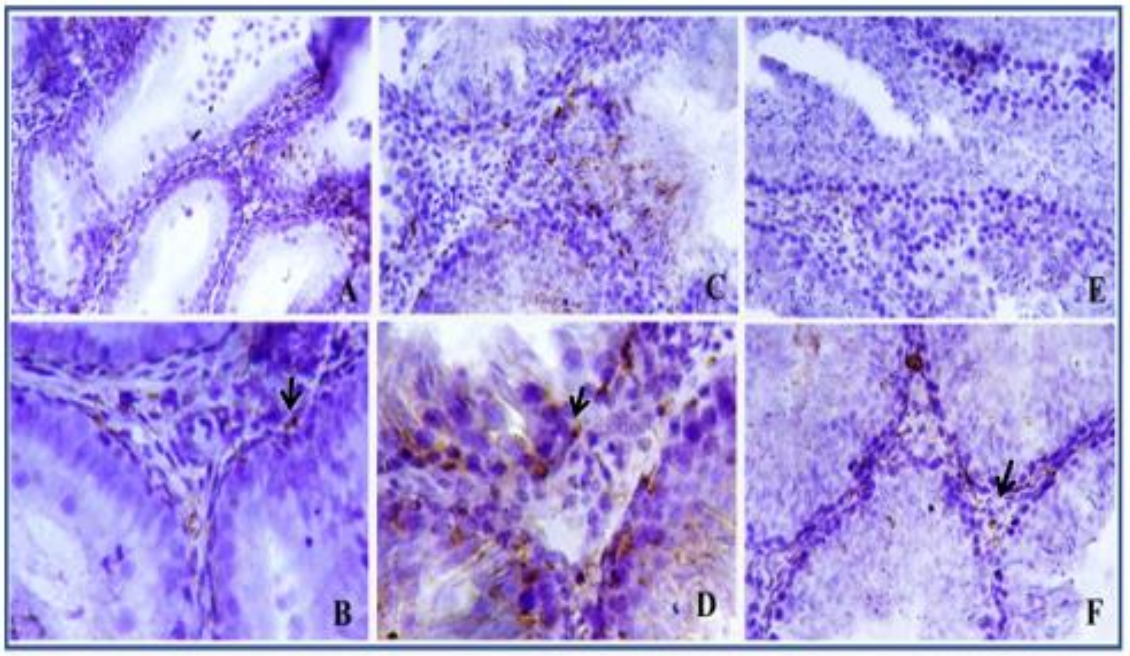


Fig. 4: A photomicrograph for an immunohistochemical reaction for anti MTOR antibody (A) mild reaction in tubular cells B) also a very clear mild reaction in Sertoli cells (arrow). (C) severe reaction in tubular cells D) severe reaction in Sertoli cells (arrow). (E) mild reaction in tubular cells (F) also a mild reaction in Sertoli cells (arrow).

Immunoreactivity score:

Table (1) showed the intensity and the IRS of the examined proteins in the premature, mature and aged groups of rats.

Table 1. Immunoreactivity Score of MTOR and GLUT2 in testis**.**

	Group	Positive cells		Fluorescence intensity		IRS (0-12)
		Percentage (%)	*Score*	*Intensity*	*Score*	
MTOR	premature	40	2	mild	1	2
	mature	70	3	intense	3	9
	aged	45	2	moderate	2	4
GLUT2	premature	25	2	mild	1	1
	mature	52	2	intense	3	6
	aged	42	2	moderate	2	4

IRS: immunoreactive score: 0-1 = **negative,** 2-3 = **mild,** 4-8 = **moderate** and 9-12 = i**ntense**

DISCUSSION

Our study revealed that premature rat testis stained with H&E showed normal arranged testicular tubules, the positivity of PAS reaction in the tubular basement membrane, and incomplete spermatogenesis with few spermatozoa. Also, GLUT2 and MTOR immunohistochemical markers showed mild reaction and decreased testosterone levels which indicated that the spermatogenesis process was in completed yet. Adult rats showed normal seminiferous tubules and spermatogenesis with a large number of spermatozoa. these histological results are in accordance with MTOR and GLUT2 immunohistochemical markers which showed severe reaction and increased level of testosterone hormone. Aged rats showed histological changes like loss in the arrangement of seminiferous tubules, as well as abnormal spermatogenesis and decreased spermatozoa. Degenerated spermatids were observed and there were few sperms in the lumen of seminiferous tubules. Also, vacuolated and shrunken Leydig cells were seen. Also, a moderate immunohistochemical reaction for GLUT2 and MTOR was observed, which explains the abnormality of spermatogenesis process. Vacuolated and shrunken Leydig cells were the main reason for the decreased level of testosterone in aged rat ants that's another factor that affects sperm production. These results are in match with the results of (Moustafa *et al*., 2014) who stated a decrease in testosterone levels associated with degenerated Leydig cells in the senile testes. This is explained by the gradual accumulation of molecular damage to cells as a response to the effect of the environmental xenobiotics and lifestyle (Gabry *et al*., 2014). Fructose and glucose are the main energy source for spermatozoa. Fructose seminal concentration is in a reverse relation with sperm concentration, which means that the production of the sperm resumes the seminal fructose which decreases its level in the semen (Nguyen *et al*., 2018). GLUT2 is the transporter of fructose and glucose into the testis (Bucci *et al*., 2010), which explains its elevated expression in mature testes than in the aged and premature testes. The development of BTB is related to the endocrine status of the organism, and also the postmeiotic germ cells and testosterone modulate the on/off of the BTB (González-Mariscal *et al.*, 2010). Also, the BTB is well developed and established in the mature testes and as the MTOR is integrated into its structure (Correia *et al*., 2020), it is normal to find a strong expression of GLUT2 in it than the premature and aged testes. These results revealed the results of Sahin *et al.*, (2018) who suggested that MTOR inhibition maintains the spermatogonia at the undifferentiated stage and subsequently a low number of sperms will be produced lumen. The low concentration of testosterone and also the low expression of the GLUT2 and MTOR together in the aged testes explain the affected structure of seminiferous tubules and also the affected spermatogenesis suggesting expected infertility in the aged rats.

REFERENCES

Alves MG, Martins AD, Cavaco JE, Socorro S, Oliveira PF (2015). Diabetes, insulin-mediated glucose metabolism and Sertoli/blood-testis barrier function. *Tissue Barriers*; 1: e23992

Arnab Banerjee, Anuradha, Kaustab Mukherjee, And Amitabh Krishna (2014). Testicular Glucose and Its Transporter GLUT 8 as a Marker of Age-Dependent Variation and Its Role in Steroidogenesis in Mice. *Journal of Experimental Zoology*. 321A:490–502.

Bancroft, J. D. & Gamble, M. (2002): Theory and practice of histological techniques.5th. Ed. Edinburgh. Churchill Livingstone Pub., pp 593-620.

Bucci D., Rodriguez-gil JE, Vallorani C, Spinaci M, Galeati G, and Tamanini C. (2010). GLUTs and Mammalian Sperm Metabolism. *Journal of Andrology*. 32(4):348-355.

Carayannopoulos MO, Chi MM, Cui Y. (2000). GLUT8 is a glucose transporter responsible for insulin-stimulated glucose uptake in the blastocyst. *Proceedings of the National Academy of Sciences.* 97:7313–7318.

Correia B, Sousa MI and Ramalho-Santos J. (2020). The mTOR pathway in reproduction: from gonadal function to developmental coordination. *Reproduction,* 159 R173–R188

De Miranda EP, Lorenzini F., Neves BVD., Melchioretto EF., Hota T. and De Fraga R. (2018). Stereological and morphological analysis of the e ects of aging on spermatogenesis in rat testis. *Acta Cirurgica Brasileira*, 33(10):904-913.

Doege H, Schurmann A, Bahrenberg G, Brauers A & Joost HG. 2000. GLUT8, a novel member of the sugar transport facilitator family with glucose transport activity. *Journal of Biological Education*, 275:16275–16280.

Drury R.A. and Wallington E.A. (1980) Carleton's Histological Technique. 5th Edition, Oxford University Press, New York.

Eskenazi B, Wyrobek AJ, Sloter E, Kidd SA, Moore L, Young S, Moore D. (2003). The associa on of age and semen quality in healthy men. *Human Reproduction*;18(2):447–54. PMID: 12571189.

Eva P, Jörg G, Joachim W and Sandra L. (2021). Healthy ageing and spermatogenesis. *Society for Reproduction and Fertility*. 161:89–101.

Gabry MS, Abdel Kader DM, Moustafa M and Elenany AA (2014): Effect of some antioxidants on the prostate of adult and aged albino rats: a histological and immunohistochemical study. *Journal of Applied Pharmaceutical Science,* 4 (02): 017-026.

González-Mariscal L., Garay E., Quirós M. (2010). Chapter 6 - Regulation of Claudins by Posttranslational Modifications and Cell-Signaling Cascades. *Current Topics in Membranes*, 65(C): 113-150.

Hellstrom WJ, Overstreet JW, Sikka SC, Denne J, Ahuja S, Hoover AM, Sides GD, Cordell WH, Harrison LM, Whitaker JS. (2006) Semen and sperm reference ranges for men 45 years of age and older. *Journal of Andrology*, 27(3):421–8.

Hogarth C. (2015). Retinoic acid metabolism, signaling, and function in the adult testis, Editor(s): Michael D. Griswold, Sertoli Cell Biology (Second Edition), Academic Press, pp247-272.

Juul A, Skakkebaek NE. (2002). Androgens and the ageing male. *Human Reproduction Update*, 8:423–433.

Kokk K, Verajankorva E, Wu XK, Tapfer H, Poldoja E, Pollanen P. (2004). Immunohistochemical detection of glucose transporters class

I subfamily in the mouse, rat and human testis. *Medicina (Kaunas)*, 40:156–160.

Kyu Ri Hahn, Jung H, Yoo DY, Kim JW, Kim YH, Jo YK, Kim GA, Chung JY, Choi JH, Hwang IK, Jang G and Yoon YS. (2017). Immunohistochemical localization of glucose transporter 1 and 3 in the scrotal and abdominal testes of a dog. *Laboratory Animal Research*, 33(2):114-118.

Moustafa M., Alazzouni AS and Gabri MS. (2014). Histological and Biochemical Evaluation of the Effects of Some Antioxidants on Aged Testes. *The Egyptian Journal of Hospital Medicine*, 57:598-611

Mruk DD, Cheng CY. (2010) Tight junctions in the testis: new perspectives. *Philosophical Transactions of the Royal Society B: Biological Sciences*; 365:1621-1635.

Mruk DD, Cheng CY. (2012). In search of suitable in vitro models to study germ cell movement across the blood-testis barrier. *Spermatogenesis*, 2:6- 10.

Nguyen T T, V T Huyen, Nguyen T T L, Trieu T S. (2018). Seminal Fructose Concentration in Man Infertility and the Fructose Test's Meaning in Diagnosis Reason of Azoospermia Man. *Biomedical Journal of Scientific and Technological Research*, 8(1):1-5.

O'Brien JH, Lazarou S, Deane L, Jarvi K, Zini A. (2005). Erectile dysfunction and andropause symptoms in infertile men. *Journal of Urology*;174(5):1932-1934.

Pearse, A. G. E. (1980). Histochemistry theoretical and applied. 4.ed. New York:Longman Group,. v.2.

Perheentupa A, Huhtaniemi I. (2009). Aging of the human ovary and testis. *Molecular Cellular Endocrinology*, 299:2–13.

Rato L, Alves MG, Socorro S. Socorro, S.; Carvalho, R.A.; Cavaco, J.E. and Oliveira, P.F. (2012). Metabolic regulation is important for spermatogenesis. *Nature*, 9:330–338.

Sahin P, Gungor-Ordueri NE & Celik-Ozenci C (2018). Inhibition of mTOR pathway decreases the expression of pre-meiotic and meiotic markers throughout postnatal development and in adult testes in mice. *Andrologia*, 50(e):12811 1–11.

Sharpe RM, McKinnell C, Kivlin C, Fisher JS. (2003). Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction*; 125:769- 84.

Simpson IA, Dwyer D, Malide D, Moley KH, Travis A, Vannucci SJ. (2008). The facilitative glucose transporter GLU T3: 20 years of distinction. *American Journal of Physiology and Endocrinology and Metabolism*, 295:242–253.

Walker WH, Cheng J. (2005). FSH and testosterone signaling in Sertoli cells. *Reproduction*; 130:15-28.