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Histological and Biochemical Evaluation of the Effects of Some Antioxidants on Aged Testes

Maiada Moustafa, Ahmed Said Alazzounui, Mohamed Said Gabri
Department of Zoology and Entomology, Faculty of Science, Helwan University.

Abstract

Background: Different studies have demonstrated that aging clearly affects male fertility which may be attributed to the androgen deficiency. Reactive oxygen species play a central role in the pathophysiology in the aged-related decrease in male fertility. Some antioxidants have ameliorative effects on different aged organs.

Material and Methods: The present study aimed to evaluate the effects of some antioxidants on aged testes. Ten adult and fifty aged male albino rats (*Rattus albus*) were divided into six groups. Group I (control adult), Group II (control aged), Group III (Vitamin E-treated aged), Group IV (Vitamin C-treated aged), Group V (Zinc sulphate-treated aged), Group VI (Vitamin E-, Vitamin C- and Zinc Sulphate-treated aged). Vitamin E, Vitamin C and Zinc were administrated in doses 2.52 mg, 3.15 mg and 0.693 mg, respectively. Histological and ultrastructural evaluation of the testes were examined as well as Follicle stimulating hormone (FSH), Luteinizing hormone (LH), total and free testosterone levels in the serum were measured. Counting the number of litters per animal and the teratogenic effects was noticed. **Results:** Giving zinc alone or combined with other antioxidants gave better ameliorative effects on the testicular structure and hormonal levels in the serum. No teratogenic effects of the aged animals`offspring were noticed.

Key words: aging, testis, zinc, vitamin E, vitamin C, combined antioxidants.

Introduction

Aging is an extremely complex and multifactorial process that proceeds with the gradual deterioration in functions. [(1)] It can be described as the gradual, lifelong accumulation of molecular damage to cells and tissues in response to exposure to stress associated with environment and lifestyle.[(2)] After maturity the signs of aging start to appear, when optimal health, strength and appearance are at the peak. After puberty, all the physiological functions gradually start to decline (e.g. the maximum lung, heart and kidney capacities are decreased, the secretion of sexual hormones is lowered, arthritic changes, skin wrinkling, etc).[(3)] The precise biological and cellular mechanisms responsible for the aging are not known, but according to **Fontana and Klein**[(3)] who are likely to involve a constellation of complex and interrelated factors, including oxidative stress-induced protein and DNA damage in conjunction with insufficient DNA damage repair, as well as genetic instability of mitochondrial and nuclear genomes. Age-related decline in testicular function could be overcome by the use of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) techniques. [(4)] It was found that semen concentration, sperm motility and morphology, were not decreased with advancing age, but there was age-dependent decrease in semen volume. [(5)] Embryo quality at the cleavage stage (days 2–3) was not affected by increasing males' age, while it was associated with a significant decrease in blastocyst embryo formation, probably reflecting male genomic activation within the embryo. [(4)] Zinc (Zn) plays an important role in the reproductive system. [(6)] It is the only metal found in almost all classes of enzymes. High concentrations of zinc in the testes and accessory sex glands show its pivotal role in the reproductive system. [(7)] Zn deficiency has been linked to hypogonadism and impaired sperm function.[(8,9)] Studies have showed that antioxidants have a far-reaching effect in andrology.[(10)] Vitamins A, E, D and C were reported to possess antioxidant functions.[(11)] Although efficient, the antioxidant enzymes and compounds do not prevent the oxidative damage completely. A series of damage

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removal and repairing the involved enzymes deal with this damage.[12] In this study, testicular structure, function and *in vivo* natural outcome of aged male albino rat, and the effect of some antioxidants against aging were evaluated.

Material and Methods

1- Material

A- Drugs:

Zinc sulphate capsule contained 110 mg zinc sulphate is equal to 25 mg zinc as white powder (October Pharma, 6 October City, Egypt). **Vitamin C** tablet contained 500 mg ascorbic acid (Kahira Pharma, Cairo, Egypt). **α-tocopherol** capsule contained 400 mg oily material (Pharcopharmaceutical , Alexandria, Egypt). The dose was calculated according to interspecies dosage conversion scheme of Goush.[13] Each dose was dissolved in 0.2 ml distilled water or sesame oil according to the drug.

B- Experimental animals and design:

Ten adult (6 months old) male albino rats (*Rattus albus*) ranging in weights from 120 to 150g and fifty aged (22 months old) male albino rats ranging in weights from 300 to 400g، were obtained from the farm of the Egyptian Organization of Biological Products and Vaccines in Helwan, Cairo. The animals were under hygienic conditions in the Medical Research Centre, Faculty of Medicine, Ain Shams University.

All the animals were divided into six groups with ten animals in each. **Group I** consisted of untreated adult rats and served as controls. **Group II** consisted of untreated aged rats. **Group III (Vitamin E-treated group)** consisted of aged rats, each received 2.52 mg therapeutic dose of α-tocopherol dissolved in sesame oil daily for 30 days. **Group IV (Vitamin C-treated group)** consisted of aged rats, each received 3.15 mg therapeutic dose of ascorbic acid dissolved in sesame oil daily for 30 days. **Group V (Zinc Sulphate-treated group)** consisted of aged rats, each received 0.693 mg therapeutic dose of Zn sulphate dissolved in sesame oil daily for 30 days. **Group VI (Vitamin E, Vitamin C and Zinc sulphate-group) (combined mixture-treated group)** consisted of aged rats, each received the three doses daily for 30 days.

Twenty-four hours after the last dose, each animal was bred with two female albino rats in one cage for three days. After pregnancy (appearance of a vaginal plug was considered as day one of pregnancy), the males were taken off the cages and the females were sacrificed on the 19th day of pregnancy. Teratogenic effect and number of animal per litter have been recorded.

2- Methods:

A- Histological and ultrastructural examination:

One testis from each sacrificed male was fixed in 10% formalin for 24 hours. They were then subjected to the normal procedures for paraffin blocks embedding, sectioning, and staining with haematoxylin and eosin stains [14].

The second testis of each sacrificed male was fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) at 4ºC for 24 hours. The specimens were post- fixed in 1% osmium tetroxide in 0.1M phosphate buffer at 4ºC for one hour. After fixation, dehydration in graded alcohol, and embedding in araldite cy 212 were done. Semithin sections were cut and stained for light microscopy examination. Ultrathin sections were cut, picked up on copper grids and then were double stained with uranyl acetate and lead citrate. The sections were examined and photographed using Zeiss 100s transmission electron microscope [15].

B- Morphometric measurements:

The number of the seminiferous tubules was counted using the "Leica Quin 500C" image analyzer computer system (Leica Imaging System Ltd., Cambridge, England). All the measurements were done within 10 non-overlapping fields/section for each animal, at the magnification 400in a standard frame.

C- Biochemical study:

CLIAgen Kits (ADALTIS,Italy), which are microplate chemiluminescence assay for quantitative determination of luteinizing hormone (LH), Follicle stimulating hormone (FSH), total and free testosterone levels in serum were used. The procedures were performed according to the kits' guidelines.

D- Statistical analysis:

The morphometric results were expressed as mean ±SD. Comparison between more than two different groups was carried out using the one-way analysis of variance (ANOVA) followed by Turkey-Kramer's Multiple Comparison Test [16], where $p<0.05$ was considered significant.

Results

A- Litter and teratogenic study:

Application of ANOVA test showed that the numbers of embryos per litter of aged rats were significantly decreased comparing to the control adult group (Fig. 1). The number of embryos per litter of zinc-treated group (Group V) showed the highest number comparing to the other treated aged groups (Fig. 1). No teratogenic morphology was recorded in the litters of the groups under study.

B- Histological observations:

The microscopical sections showed certain histological changes in the architecture of testes of rats under the oral administration of vitamin E or, vitamin C or, zinc or, combined mixture of these materials as antioxidants daily for one month in doses which are equivalent to that of human therapeutic doses.

B.1- The adult control group:

The testes of the control adult group have normal histological pattern. A number of seminiferous tubules (ST) are found separated by intact interstitial cells (ISC). The seminiferous tubules appeared as rounded or oval surrounded by peritubular myoid cells (MC). The tubules were lined with stratified germinal epithelium, which consists of two distinct populations of cells; the spermatogenic cells and the Sertoli cells. The spermatogenic cells represent the different stages of spermatogenesis, with the spermatogonia (Sg) resting on the basal lamina with small and dark nuclei and are arranged regularly in more than one layer. Primary spermatocytes (PS) appeared as large cells with large oval nuclei, followed by spermatids and well differentiated spermatozoa (dSp). There was narrow inter-lobular space with interstitial tissue that embodies clusters of Leydig cells (LC) with ovoid or polygonal shape and spherical nuclei; these are (androgen secreting cells) which is Leydig cells (LC). (Fig. 2a-c)

B.2- The aged group:

The structure of aged testes showed disorganization of the normal histological structure of the testes with overall different degrees of atrophy in the seminiferous tubules (ST) separated by wide inter-lobular space with degenerated interstitial cells. The number of spermatogonia (Sg) decreases, while the number of primary spermatocytes (PS) increase, spermatozoa were hardly seen, with degenerative changes of Sertoli cells. (Fig. 3a-c)

B.3- The vitamin E-treated group:

The structure of the testes of vitamin E-treated rats showed partial improvement in the structure of some seminiferous tubules (nST) still some disorganized seminiferous tubules were found (dST). Decreased number of spermatogonia (Sg) was observed. (Fig. 4a-c)

B.4- The vitamin C-treated group:

The structure of the testes of the vitamin C-treated rats showed degenerated interstitial C.T. between the seminiferous tubules with marked interstitial fluid (ISF). There was increase in number of spermatogonia (Sg) but with pyknotic nuclei and dense chromatin. Spermatogenesis was still disorganized at some parts of seminiferous tubules. In other parts of seminiferous tubules with organized spermatogenesis had differentiated spermatozoa (dSp) at their adlumenal compartment. (Fig. 5a-c)

B.5- The zinc sulphate-treated group:

The structure of the testes of the zinc sulphate-treated albino rats showed normal seminiferous tubules (ST) with normal epithelium and wide inter-lobular spaces. The spermatogonia (Sg) were normally distributed in more than one layer (mostly two layers). The primary spermatocytes (PS) appeared as large cells with large oval nuclei, followed by spermatids and well differentiated spermatozoa (dSp). (Fig. 6a-c)

B.6- The vitamin E, vitamin C and zinc sulphate-treated group:

The structure of the testes of the combined mixture-treated albino rats showed normal seminiferous tubules with wide inter-lobular

spaces filled with interstitial fluid (ISF). Congested blood vessel (c) was found. Hypertrophy in myoid cells (Mc) was also observed. Degenerated Sertoli cell and deformed spermatozoa with rounded head were obtained. (Fig. 7a-c)

C- Ultrastructural observation:

C.1- The control group:

The ultrastructural observation of the testes of the control rats showed normal testicular architecture. Well-developed Sertoli cells (SC) are found with oval shaped large nucleus, prominent nucleolus. Sertoli cells cytoplasm was extended from the basal lamina to the lumen of the seminiferous tubules and envelops the adjacent germinal elements. The primary spermatocytes (pSc) displayed round configurations with prominent nuclei; the nuclei have distinct chromatin networks and well defined nuclear membranes (Fig. 9 D). The spermatids (Sp) have characteristic well-defined nuclei with distinct nuclear membranes and chromatin networks, and normal peripheral arrangement of mitochondria (Mit) with abundant number and normal cell junctions (CJ) (Fig. 9 C, E&F). Clumps of normal interstitial cells (Leydig cell) are detected (LC) (Fig. 9A&B).

C.2- The aged group:

The ultrastructure of the aged group showed abundance of germ cells with many lysosomes (Ly) and residual particles in their cytoplasm resulting from organelle degeneration (Fig. 8A). Alterations in Sertoli cell nucleus and cytoplasm; nucleus was found dislocated from the basal portion and the cytoplasm was degenerated with several vacuoles and electron dense materials (Fig. 8B). There was abnormal structure of spermatozoa; many lysosomes and perinuclear vacuoles were observed (Fig. 8C&D).

C.3- The zinc sulphate-treated group:

The ultrastructure of the testes of zinc sulphate-treated group showed normal maturation of spermatids and spermatozoa (Fig. 9A&B).

C.4- The vitamin E, vitamin C and zinc sulphate-treated group:

The ultrastructure of the testes of combined mixture-treated aged albino rat showed marked interstitial fluid (ISF). (Fig. 11A) Developed spermatids had increasing number of lysosomes (Fig. 11B). The free spermatids and the well-developed detached spermatozoa had increased in number of mitochondria in the head region (Fig. 11C&D).

D- Seminiferous tubules morphometric analysis:

Statistical analysis of the counted numbers of seminiferous tubules in ten fields of each slide showed significant decrease in the aged testis comparing to the control adult testes. The testes of the antioxidant- treated aged animals of the different groups showed a significant increase of the counted numbers of seminiferous tubules almost to reach the normal number (Fig. 12).

E- Hormonal levels:

Statistical analysis of the FSH, LH, total and free testosterone levels indicated that: FSH level markedly increased in the aged group comparing to the control adult group. The treated groups showed lower levels of the FSH comparing to the aged group, but still higher than the level of the control adult group (Fig. 13).

LH level significantly increased in the aged group comparing to the control adult group. The treated groups showed lower levels of the LH comparing to the aged group except the vitamin C-treated group which showed marked increase if compared with the level of the control adult and aged groups (Fig. 13).

Total and free testosterone levels showed decrease in the aged group comparing to the control adult group. The treated groups showed decrease in the levels of the total and free testosterone comparing to the control adult group (Fig.13).

Discussion

The disorganized structure of testes of aged rats with degenerated Sertoli cells and Leydig cells was accompanied with increase in gonadotropins levels (FSH and LH), and also decrease in the number of seminiferous tubules, as well as, prominant decrease in the litter size. These results are in accordance with the studies of **Ramesh Babu *et al.*** [17] who stated that in infertile males with abnormal histopathology of testis (Sertoli cell only syndrome, hypospermatogenesis, and

spermatid arrest), the mean FSH levels were significantly elevated compared to the control group. **Yanam *et al.*** [18] also showed a significant increase in the mean FSH levels in infertile males with Sertoli cell only syndrome, hypospermatogenesis and maturation arrest. **Micic** [19], **Nistal *et al.*** [20] and **Turek *et al.*** [21] also showed significantly elevated mean FSH levels in infertile males with Sertoli-cell only syndrome. However, **Weiss *et al.*** [22] reported insignificant increase in the mean FSH levels in infertile men with Sertoli-cell only syndrome. Decrease in the level of testosterone is normally associated with the degeneration of the Leydig cells in the aged testes.

The absence of the secondary spermatocytes, which normally have short half life time, in the control adult testes revealed that the spermatogenesis is normal and regular, while the absence of differentiated spermatozoa and the increase in primary spermatocyte number revealed the spermatogenic arrest in the aged testes. This spermatogenic arrest was partially recovered by the antioxidants supplementation.

Improvement of the aged testis after zinc treatment, and combined mixture of antioxiadants; well-organized seminiferous tubules with interstitial cells in between and well-defined spermatogenesis were observed. **Tahmaze *et al.*** [23] stated that zinc sulphate has a possible role in testicular structure and function .Also, **Boran and Ozkan**[24] reported that zinc administration may prevent the progression of testicular injury which results from free radicals. **Rossman and Goncharova**[25] and **Gibbs *et al.***[26] suggested that the protective effect of zinc against reactive oxygen species is due to the direct binding of zinc to the sulphydryle-groups in proteins protecting it from being oxidized. This improvement was also accompanied with decrease in the gonadotropins (FSH, LH) levels, but still was higher than the control level. Although efficient, the antioxidant enzymes and compounds do not prevent the oxidative damage completely [12]; also the antioxidants did not ameliorate the abnormal structure of the aged testis up to the normal structure in the control group.

After the treatment of aged rat with vitamin E, the testes improved and the spermatogenesis process was better organized than the aged group. The same results were observed by **June *et al.*** [27] who stated the protective role of vitamin E against testicular damage. After treatment with vitamin C, in the present study, the aged testes tissue fairly improved; these results coincide with **Chang *et al.*** [28] who stated that vitamin C has a protective effect against the free radicals which cause testicular damage.

The ultrastructural evaluation of the different groups showed variable degrees of deterioration of spermatogenesis stages of the aged group; abnormal and non-nucleated primary spermatocytes up to ill-defined Sertoli cell and spermatogenic arrest were noticed. Mature spermatozoa showed the presence of perinuclear vacuoles in the aged testes, which explain the decrease in litter size of this group according to **Boitrelle *et al***. [29] who stated that the sperm-head vacuoles are nuclear in nature and are related to chromatin condensation failure and (in some cases) sperm DNA damage. All these abnormalities may be recovered in treated groups either with single antioxidants or as a collective mixture. The most recovered groups were zinc- and mixture-treated groups. The function of the blood-testis barrier is to protect germ cells from harmful influences. The barrier has three components: first, a physicochemical barrier consisting of continuous capillaries, Sertoli cells in the tubular wall, connected together with narrow tight junctions, and a myoid-cell layer around the seminiferous tubule. Second, an efflux-pump barrier that contains P-glycoprotein in the luminal capillary endothelium and on the myoid-cell layer; and multidrug-resistance associated protein 1 located basolaterally on Sertoli cells. Third, an immunological barrier consists of Fas ligand on Sertoli cells.[30] These mean that the destruction dysfunction in the either Sertoli cell or myoid cell cause physical dysfunction of testicular barrier and subsequently, cause spermatogenic arrest; and this may be one of the reasons of the spermatogenic arrest in the aged testes.

Aged testes showed a significant decrease in the litter size; this may be explained by the results of **Dain *et al.*** [5] that showed that there is age-dependent decrease in semen volume. The treated aged animals showed larger litter size, but not up to the control level; this reveals that the antioxidants have ameliorative effects against testes aging. And also there are no malformations in any of the aged groups; this confirms the study of **Wiener-Megnazi *et al*.** [4] who stated that although aging decreases the rate of blastula formation level in the IVF, no effect of aging on cleavage study.

All these results revealed that zinc alone or combined with other antioxidants has better ameliorative effects against the testes ageing.

Conclusion

Anti-oxidants have been used in a wide scale but may have some side effects, and some of them may be more effective than others. This study concluded that vitamins E and C, in addition to, zinc are anti-oxidants but some of them may be preferable and efficient than others. Zinc is the most effective and safer anti-oxidant than vitamin C and vitamin E. Antioxidants when taken separately or collectively in a mixture can give a recovery effect on physiological and histological damage of the aged testes.

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Figures

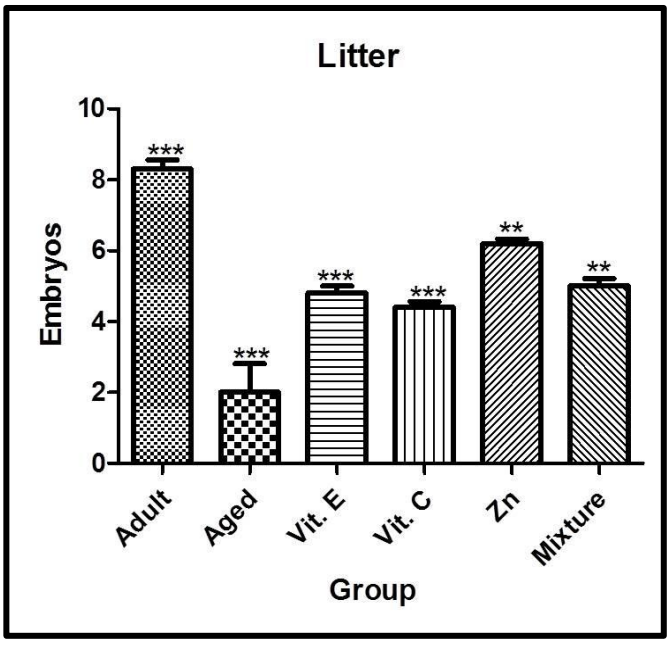

Figure 1: A histogram showing the number of embryos of each animal per litter. ** $p < 0.01$, *** $p < 0.001$

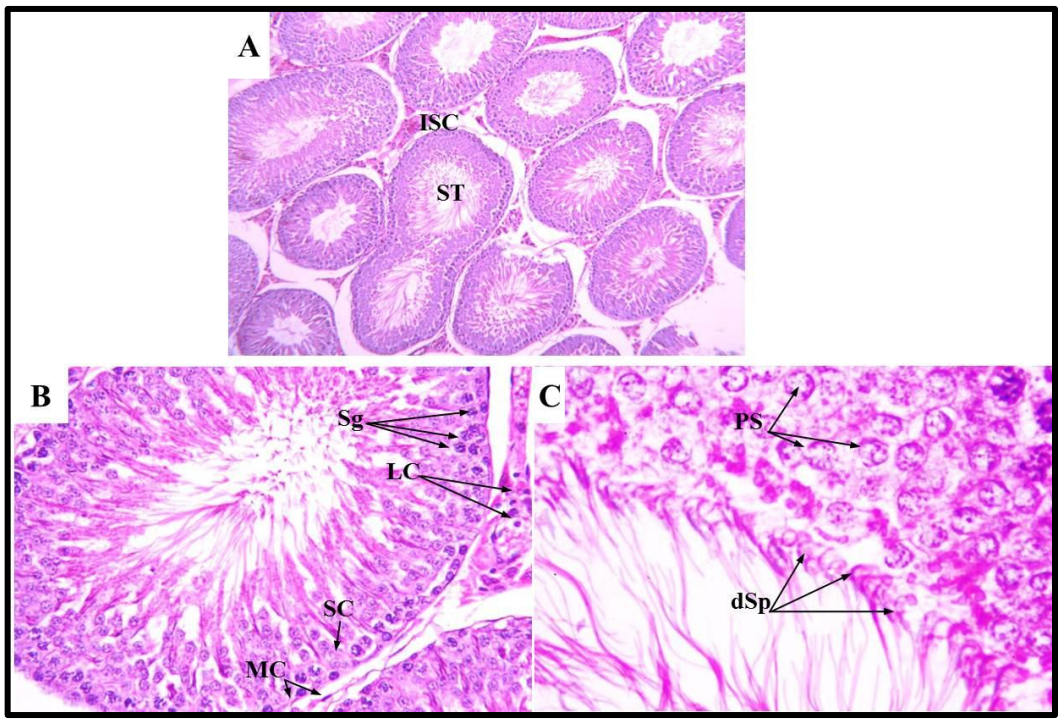

Figure 2: Photomicrographs showing the structure of a control testis with normal seminiferous tubules (ST) and interstitial cells (ISC) **(A)**. Arranged spermatogonia (Sg), Sertoli cell (SC), Myoid cells (MC) and Leydig cells (LC) are observed **(B)**. Primary spermatocytes (PS) and differentiated spermatozoa (dSp) are also found **(C)**. The magnifications are 10, 40, and 100, respectively.

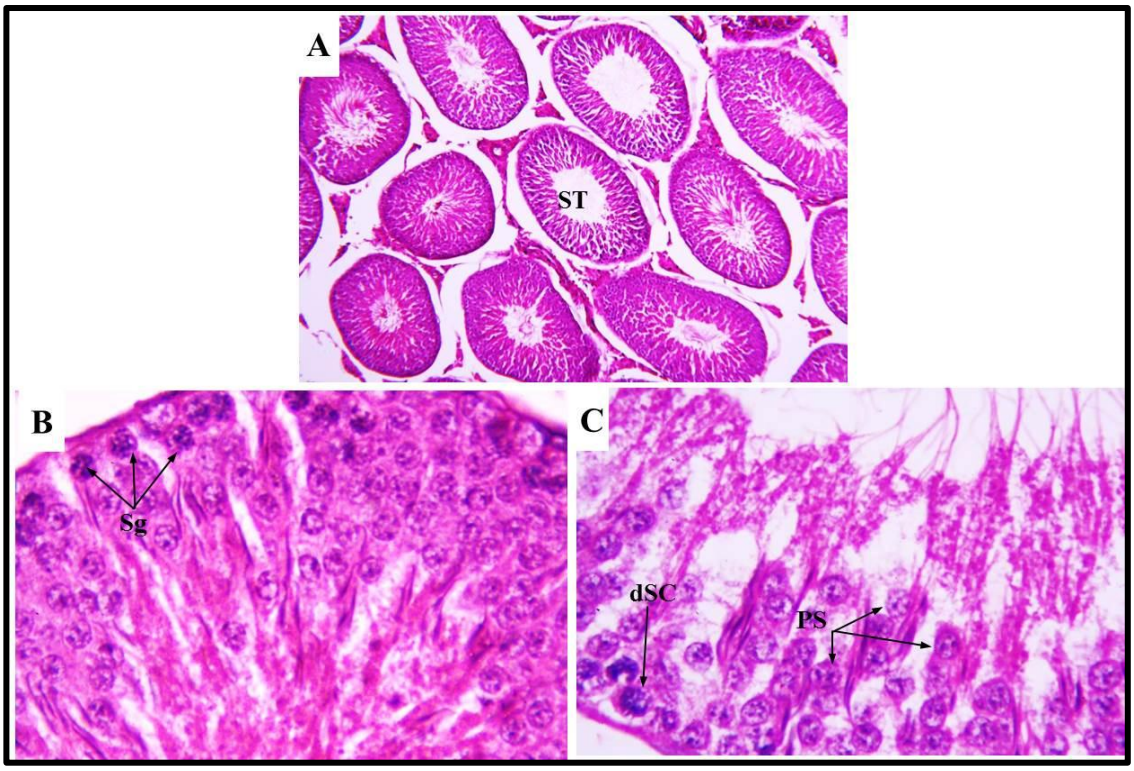

Figure 3: Photomicrographs showing the structure of the aged testis with atrophy in seminiferous tubules (ST) **(A)**, decreased number of spermatogonia (Sg) **(B)** and primary spermatocytes (PS) and degenerative Sertoli cells (dSC) are observed **(C)**. The magnifications are 10, 40, and 100, respectively.

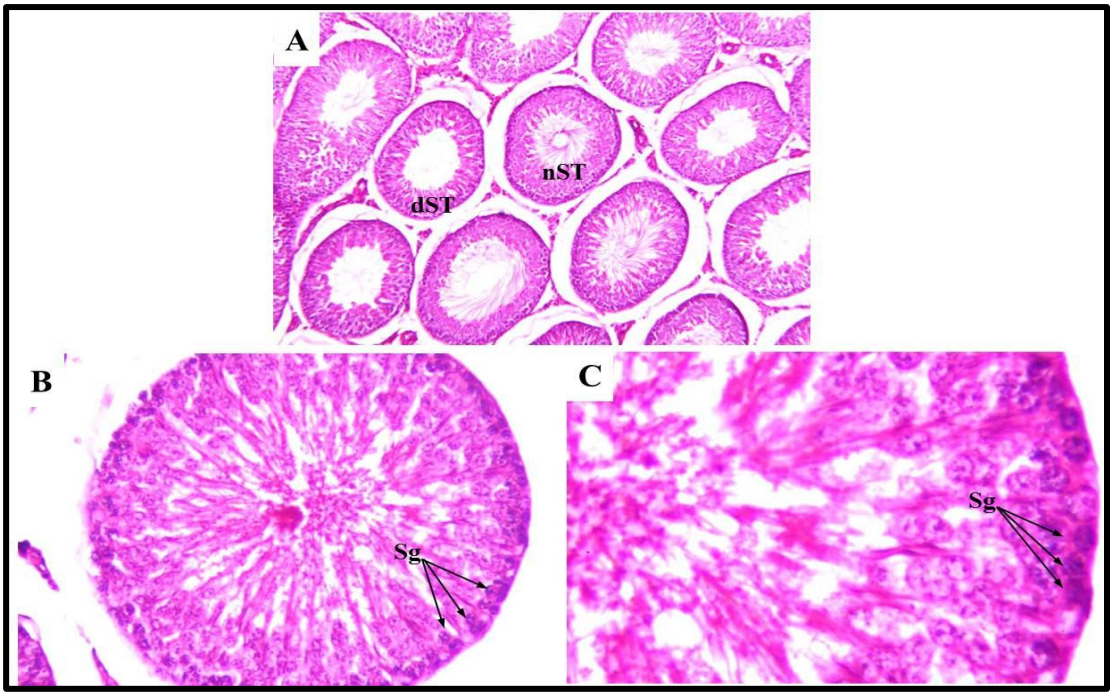

Figure 4: Photomicrographs showing the structure of testis of vitamin E-treated albino rat with some normal seminiferous tubules (nST) and some disorganized seminiferous tubules (dST) **(A)**. Regularly arranged spermatogonia (Sg) **(B&C)**. The magnifications are 10, 40, and 100, respectively.

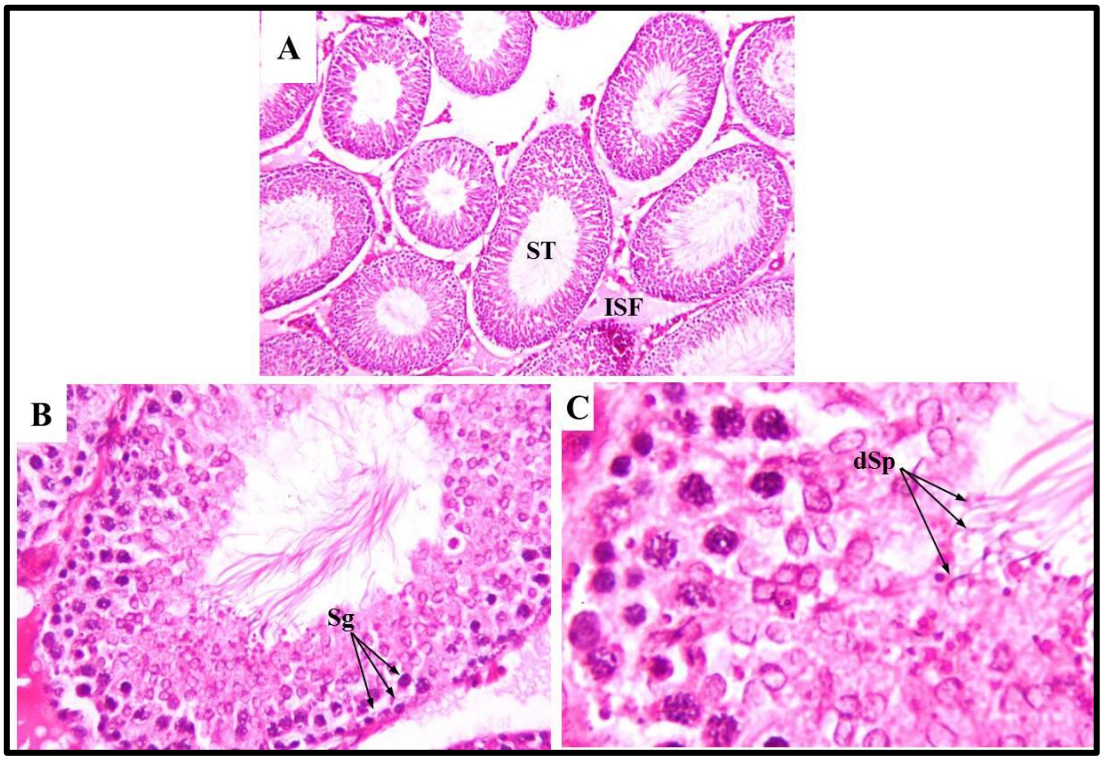

Figure 5: Photomicrographs showing the structure of testis of vitamin C-treated albino rat with normal seminiferous tubules (ST) but interstitial fluid (ISF) was observed **(A)**. Spermatogonia (Sg) **(B)** and differentiated spermatozoa (dSp) **(C)** were found. The magnifications are 10, 40, and 100, respectively.

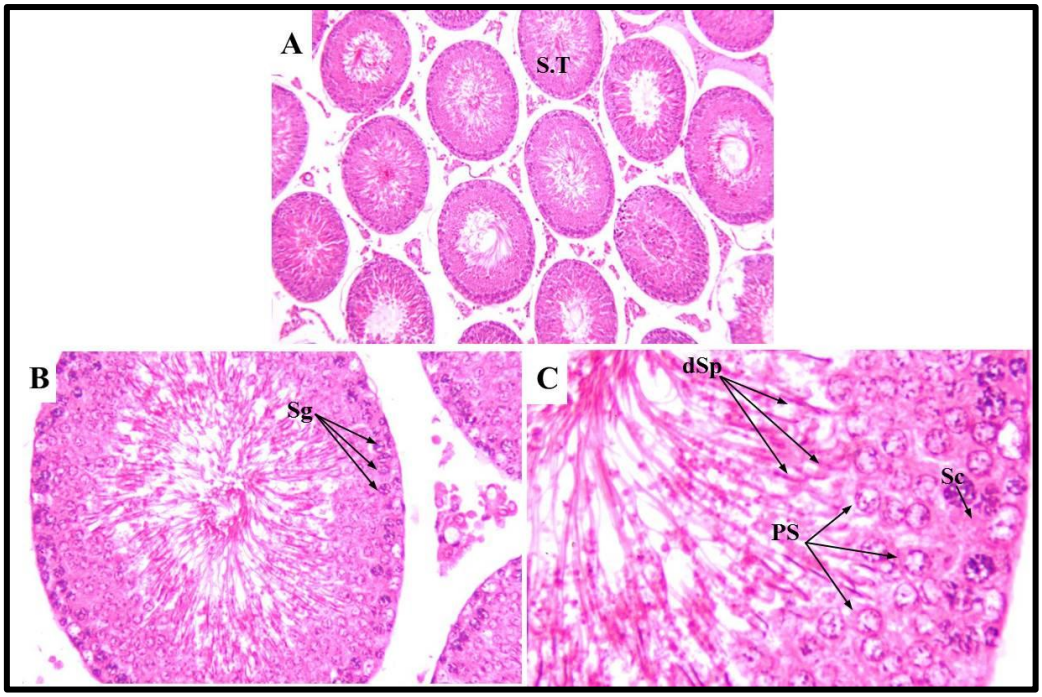

Figure 6: Photomicrographs showing the structure of testis of Zinc-treated albino rat with normal seminiferous tubules (S.T.) **(A)**. Normal spermatogonia (Sg) **(B)**, well-differentiated spermatozoa (dSp) and normal Sertoli cell (Sc) **(C)** were observed. The magnifications are 10, 40, and 100, respectively.

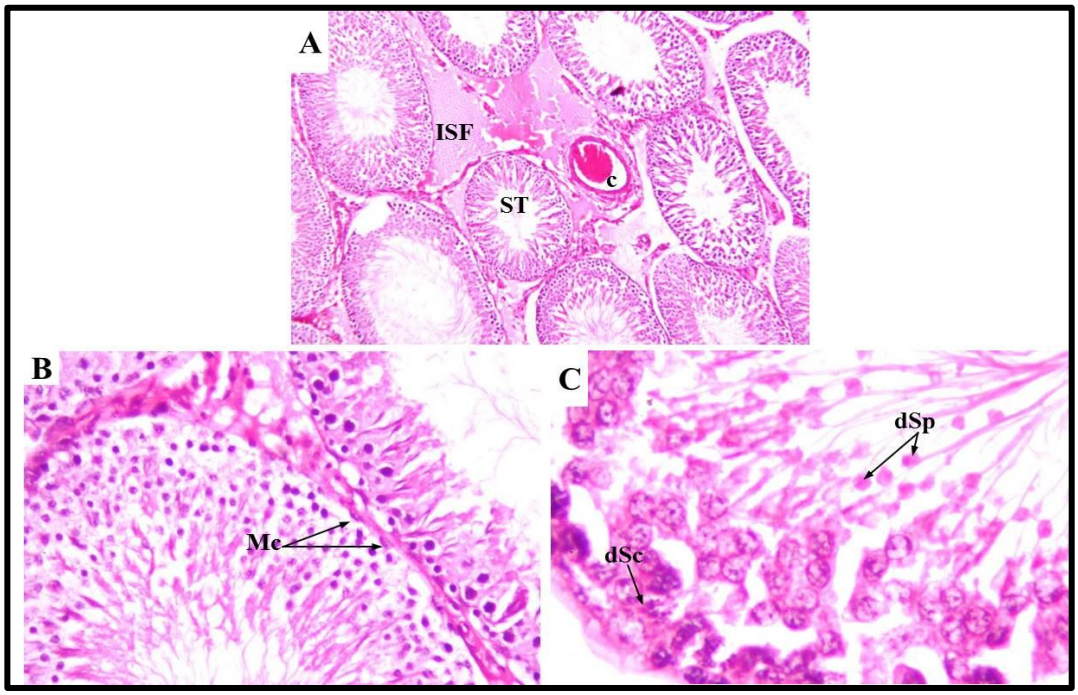

Figure 7: Photomicrographs showing the structure of testis of combined mixture-treated albino rat with seminiferous tubules (ST) but with congestion (c) and interstitial fluid (ISF) accumulation. **(A)** Distinct myoid cell (Mc) **(B)** and degenerated Sertoli cells (dSC) and deformed spermatozoa (dSC) **(C)**. The magnifications are 10, 40, and 100, respectively.

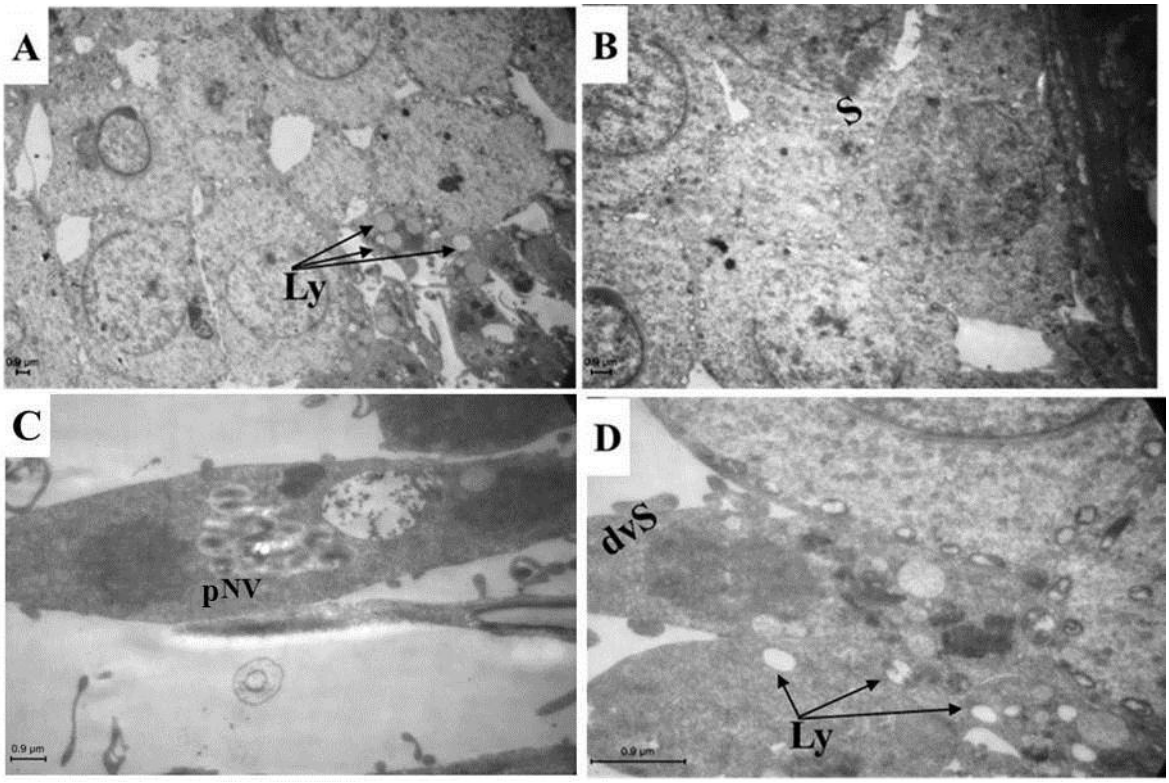

Figure 8: Electron micrographs of aged testis showing lysosomes (Ly) **(A)**, Sertoli cell (S) **(B)**, abnormal head with perinuclear vacuoles (pNV) **(C)**, developing sperms (dvS) **(D)**.

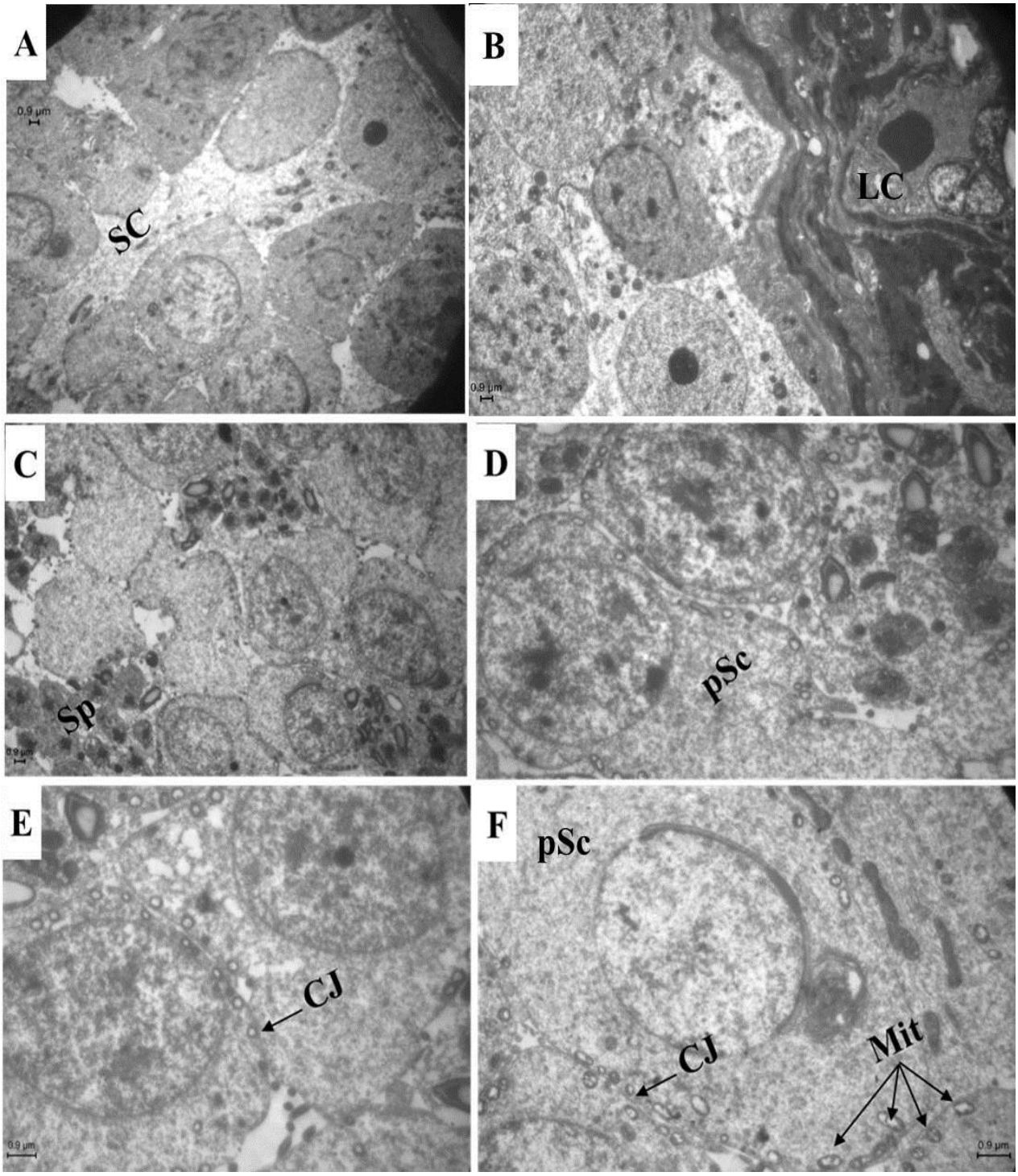

Figure 9: Electron micrographs of control adult testis showing normal Sertoli cell (Sc) **(A)**, Normal Leydig cell (LC) **(B)**, normal spermatid (Sp) **(C)**, normal primary spermatocyte (pSc) **(D)**, cell junction (CJ) **(E)**, mitochondria (Mit) **(F)**.

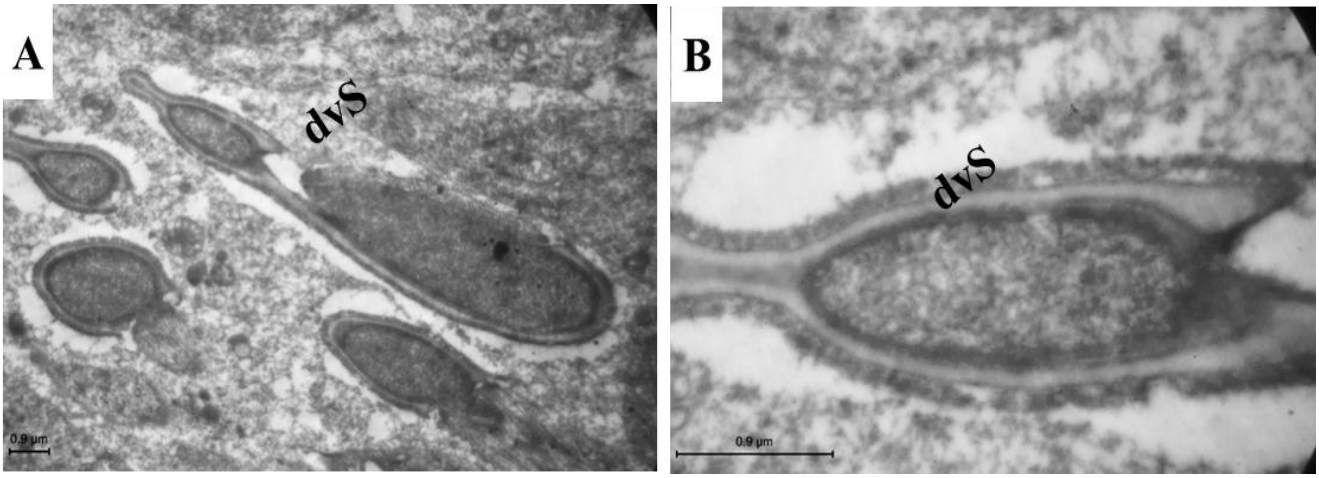

Figure 10: Electron micrographs of testes of zinc-treated aged albino rat showing (A&B) normal developing spermatozoa (dvS).

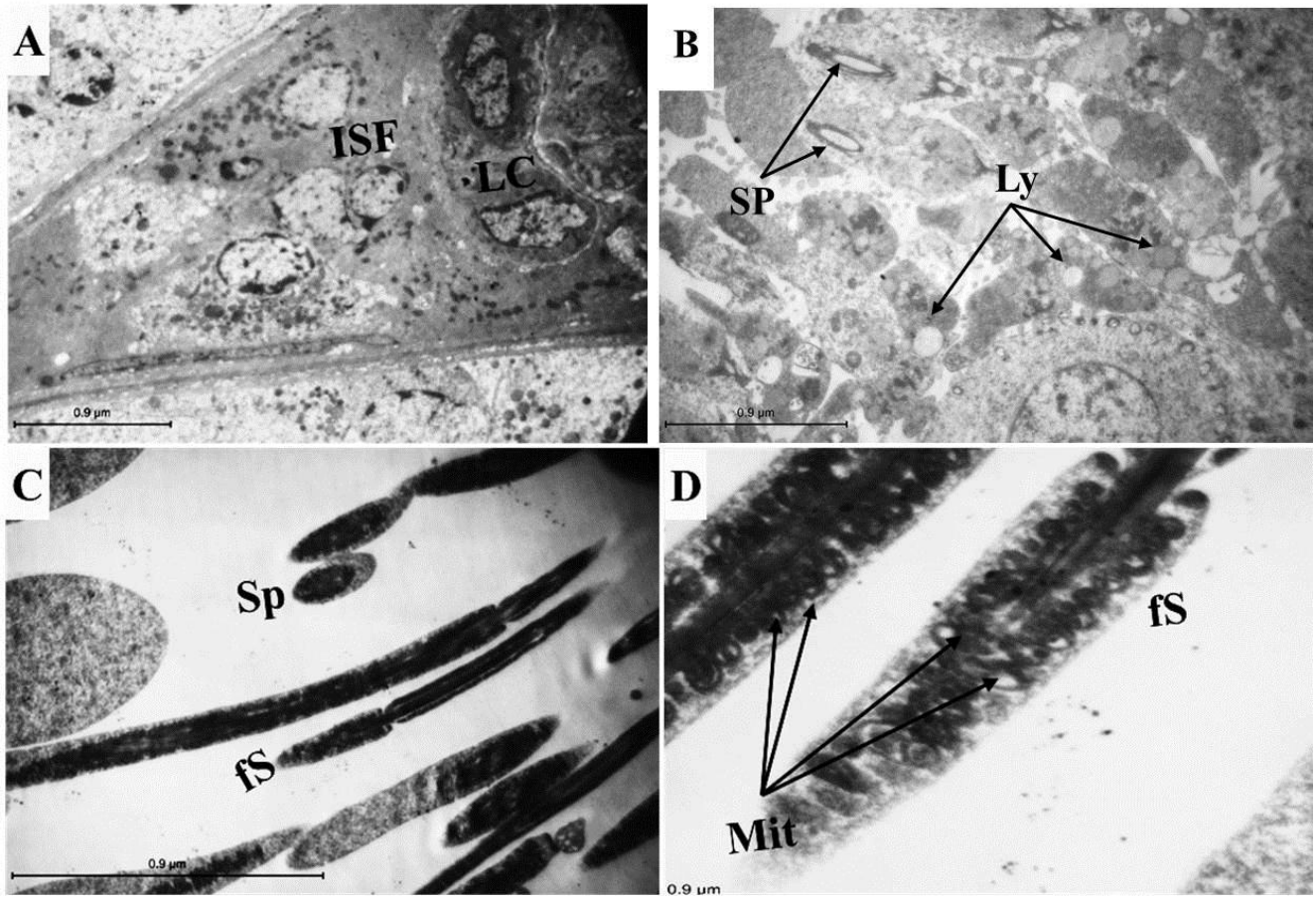

Figure 11: Electron micrograph of combined mixture-treated aged testis showing Leydig cell (LC) and interstitial fluid (ISF) **(A)**, and elongated spermatids (SP) mitochondreia (Mit) and lysosomes (Ly) **(B)**, developing spermatids (Sp) and free detached sperms (fS) **(C)**, head of free developed sperm (fS) with mitochondreia (Mit) **(D)**.

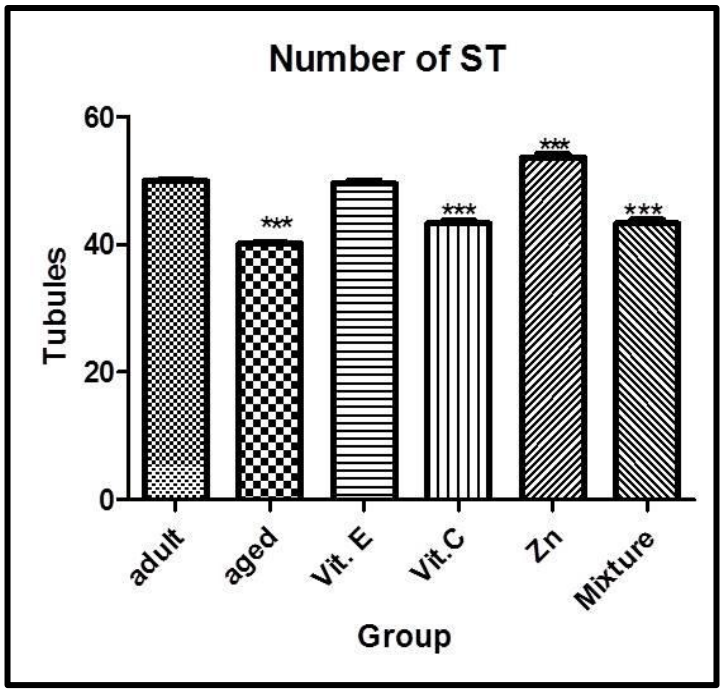

Figure 12: A histogram showing the mean number of seminiferous tubules (ST) in the different treated groups. ****p*<0.001

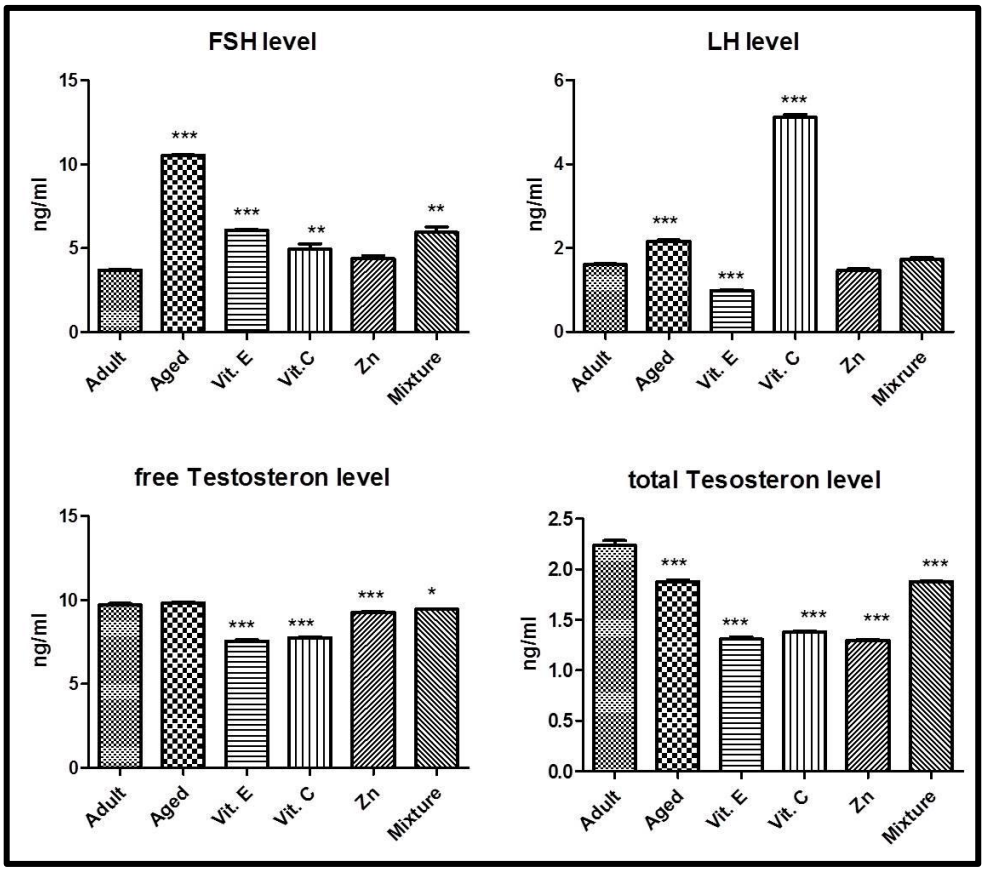

Figure 13: A histogram showing the level of FSH, LH, total and free testosterone in the sera of different animal groups.**p*<0.05, ***p*<0.01, ****p*<0.001.