

Effects of AL-Qutub (*Tribulus terrestris*) on the Spermatogenesis of the Mouse Testis: Histological, Histochemical and Morphometrical Studies

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Summary:

Backgrounds: *Tribulus terrestris* is one of the traditional herbs that have a revolutionary breakthrough in the management of erectile dysfunction and have become world –wide as an “instant treatment”

Materials & Methods: *Tribulus terrestris* was given daily to mature male mouse in a dose of 2 mg /kg body weight for 14 days .10% formalin fixed paraffin sections were performed for histological, histochemical and morphometrical studies.

Results: Histological and histochemical studies demonstrated a considerable increase in the number of spermatocytes, spermatids and sperms in parallel with an increase number of interstitial (Leydig) cells. Morphometrically, the thickness of seminiferous tubule is significantly increased together with a significant increase in the number of interstitial cells.

Conclusions: *Tribulus terrestris* increases the number of Leydig cells, and the androgens produced by these cells are directly responsible for enhanced spermatogenesis.

Key Words: Mouse Testis, *Tribulus terrestris*, Periodic Acid Schiff's reaction, Alcian blue.

Fac Med Baghdad
2008; Vol.50, No.2
Received July 2007
Accepted Oct.2007

Introduction:

Tribulus terrestris which is called Al-Qutub (in Iraqi dialect) or Qutiba (in Classical Arabic Medicine) has been commonly used in folk medicine to energize, vitalize and improve sexual function and physical performance in men. Although different effects of *Tribulus terrestris* on animals (1,2,3) and men (4,5,6,7) have been evaluated and many active compounds from *Tribulus terrestris* extract have been established (8,9,10,11) , the mode of its action and efficacy remains uncertain and controversial .

Tribulus terrestris owes its health and medicinal effects to a number of active phytochemicals :1) Steroidal saponins ; dioscin , protodioscin and diosgenin ; these substances stimulate sexual performance by increasing the percentage of free available testosterone levels for men and they even affect pregnenolone ,progesterone and estrogen 2)Sterols such as β -sitosterols and stigmasterols.

Several studies of questionable worth support the pre- erectile effects of *Tribulus terrestris*. These studies found that oral *Tribulus terrestris* increases serum dehydroepiandrosterone (DHEA) (12) , increases sexual function in men (13) , exhibits a proerectile effect in isolated rabbit corpus

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cavernosum (14) and improves sexual behaviors and intracavernous pressure in rats

(2). Parameters like sexual behavior, intracavernous pressure and androgen levels were used in these studies to scientifically validate the claim of *Tribulus terrestris* as an aphrodisiac but no research until now (midline 1965 – 2007) has been done to correlate these functional changes with structural changes in the testis. Therefore, the aim of this study is to determine the effect of *Tribulus terrestris* on mouse testis making use of the currently available histological, histochemical and morphometrical means

Thirty apparently normal mature male mice (*Mus musculus*), aged between 6-8 weeks, were used in this study. They were housed in the Animal Breeding Centre / College of Medicine / University of Baghdad under normal diurnal lighting conditions, kept at constant temperature (about 25 °C) and given free access to tap water and food.

Animals were divided into twenty experimental and ten controls. Each experimental mouse was treated with 2 mg / kg body weight of *Tribulus terrestris* powder (2) which was mixed with 3 ml distilled water and was administered as a single oral daily dose for two weeks. All mice were weighed just before the experiment and before their sacrifice.

From each deeply ether anesthetized animal, testis was removed and immediately fixed in a 10% formalin solution. Fixed tissues were processed for routine paraffin-wax embedding and sectioned serially at 5 micrometers thickness using electric microtome. From each specimen, thirty-two sections were obtained and sub-grouped into four sets. The 1st set for routine haematoxylin & eosin stain , the 2nd for Periodic Acid Schiff (PAS) method (15) , the 3rd set for staining with alcian blue – PAS (15 & 16) method and the 4th set for alcian blue pH 2.6 (15 & 17) . PAS with diastase was also used for the demonstration of glycogen that usually stains strongly positive with PAS stain and disappears after the use of diastase enzyme to differentiate it from neutral mucins (15).

Morphometry was done using an eye piece micrometer fitted to a light microscope at 10 x 40 magnifications. It was used to study the following parameters: (a) the diameter of seminiferous tubule, (b) the number of

interstitial cells of Leydig per field. The data collected were analyzed using the computer facility with the available software statistical packages SPSS 10.0 (Statistical Packages for Social Sciences, Version 10.0). Results were presented in simple measures of mean +/- standard deviation.

Results:

All experimental mice maintained good general health and showed rather hyperactivity and better appetite. Additionally, experimental mice revealed a slight, however, non – significant rise in body weight when compared with their controls.

Histological examination using haematoxylin & eosin stain revealed that the testis of control mouse exhibited seminiferous tubules with spermatogonia resting on the basement membrane, next to the latter cells; there were primary spermatocytes, spermatids and sperms located near their lumen (Fig.1). The seminiferous tubules of experimental group elicited the same spermatogenic series but with an increase in the number of spermatids and sperms which completely filled their lumen (Fig.2).

In all d-PAS stained testicular sections for the experimental animals and their controls , the basal lamina and fibrous structures in the connective tissue between the lobules and underneath the wall of the testis are PAS positive (Fig.7 A) . The spermatogenic stages up to spermatids show light PAS staining which remain visible in diastase– treated sections (Fig. 7 B). Spermatids in the later stages of development as well as mature sperms show more diffuse reactions after PAS staining especially in the sperm heads. This reaction also remains visible in control section (Fig.3). The lumen of seminiferous tubules contains PAS positive globular structures. These structures were markedly increased in experimental sections (Fig.4). The interstitial tissue of experimental group demonstrated an increase in the number of interstitial cells of Leydig when compared with control group (Fig.5&6). The cytoplasm of these cells stained darkly with PAS (Fig. 6).

The cell membranes in spermatogenic stages from secondary spermatocytes to mature sperms showed a crescent – shaped structures after d-PAS- alcian blue 2.8 staining. These were located

at one pole of the cells, and are markedly increased in experimental group (Fig. 8C)

After alcian blue staining (pH 2.8) , the interstitial cells of Leydig developed a relatively dark purplish – blue diffuse cytoplasmic reaction (Fig.8 A & B) and a diffuse greenish reaction is also observed in these cells and in spermatids , spermatozoa and in Sertoli cells . This reaction was markedly increased in experimental group (Fig.8 A & B).

Morphometrical measurements listed in table I illustrated that the diameter of seminiferous tubule was significantly increased ($P < 0.004$) in experimental group and the number of interstitial (Leydig) cells were significantly increased ($P < 0.000.0$) in that group.

Discussion:

Oral *Tribulus terrestris* greatly increases the number of spermatids and sperms that nearly fill the lumen of the seminiferous tubules in parallel with an increase in number of interstitial (Leydig) cells. It is concluded that *Tribulus terrestris* appears to enhance spermatogenesis and increase Leydig cells probably due to its constituent "steroid saponin " which may be responsible for the androgen increasing property of this herb . The saponin components of *Tribulus terrestris* (9, 18, and 19) have a steroidal action which explains the increment of both seminiferous tubule size and sperm contents. It was concluded by Tyler *et. al.* (20) that the presence of sterols especially cholesterol are more readily incorporated in sex steroid synthesis in animal tissue .

The production of testosterone by the Leydig cells is apparently under the influence of the pituitary gonadotropin luteinizing hormone (LH) which not only increases the production of testosterone by individual cells but also increases the number of Leydig cells by differentiation possibly from peritubular myoid cells or undifferentiated stem cells (21). And since the number of Leydig cells increased markedly in the present experimental group , it may be concluded that *Tribulus terrestris* itself increases the level of LH either directly by its effect on the anterior pituitary cells or indirectly by its effect on the hypothalamus and the increased LH level responsible for the formation of more Leydig cells by differentiation from undifferentiated stem cells .

Histochemically, testes of control and experimental groups, exhibited a positive d-PAS on the basal lamina and in the germ cells, primary spermatocytes , spermatids and mature sperm . It may therefore be attributed to reactive aldehydes which might be similar in both groups. Such a PAS positive picture was also observed in the testis of various mammals (17, 22, 23, and 24). The lumen of seminiferous tubules of experimental group showed a remarkable increase in the number of d-PAS – positive globular structures. These globules are carbohydrate rich material released in the lumen of the seminiferous tubules. These carbohydrate materials are not glycogen since diastase fail to remove the staining. Sasso-Cerri *et.al.* (17) found that PAS positive globular structures contained acid glycoconjugates which appear to be Sertoli cell apical portions which are accumulated in the lumen of the seminiferous tubules mainly during spermiation . Thus providing further evidence that spermiation markedly increased in *Tribulus terrestris*–treated group.

The distinct crescent–shaped deposits, observed in the cell membranes from secondary spermatocytes to mature sperms after d- PAS – AB 2.8 staining, was distinguished from other membranes and organelles in these cells. They may have a function in sperm development or maintenance perhaps by delivering special components to plasma and acrosomal membranes in spermatocytes and spermatids. By electron microscopy Mollenhauser & Morre (25) in guinea pig and Schindelmeiser (24) in amphibian found a number of vesicles , parts of plasmalemma and of the acrosome that showed enhanced electron density in the testis of these species and these structures seem to play a role in development and maintenance of germ cell structures and since these d-PAS-AB 2.8 positive crescent – shaped structures were markedly increased in the present experimental group , it may be suggested that *Tribulus terrestris* enhanced spermatogenesis .

Morphometrically, the significant increase in the diameter of seminiferous tubules in the experimental group may indicate that *Tribulus terrestris* induces growing of the seminiferous tubules as well as increases production of sperms which may occur secondary to increased level of testosterone. This fact is supported by the observation of Meachem *et.*

al. (26) where they considered high local levels of testosterone accelerate seminiferous tubule development in the rat and it was suggested that testosterone exerts this effect through its action on Sertoli cells (27). On the other hand, the significant increase in the number of Leydig cells in experimental group may indicate that *Tribulus terrestris* increases the level of LH since it has been found by Kelly *et. al.* (21) that LH is responsible for the proliferation of Leydig cells.

On the basis of histology, histochemistry and morphometrical findings, *Tribulus terrestris* may exert a direct action on the hypothalamus and / or other brain structures such as anterior pituitary. On the other hand, a peripheral effect is assumed to be present as well.

Table 1: Measurements of the seminiferous tubules in μm and Leydig cells count per high power field (HPF), n=6.

Groups	Thickness of the seminiferous tubules in μm		Number of interstitial Leydig cells	
	Control	Experimental	Control	Experimental
Mean	12.83	20.50	5.83	20.83
SD	2.23	1.64	0.75	2.48
P value	0.004<		0.000 <	

Fig.1 Testis of control mouse showing seminiferous tubules with the usual spermatogenic series (H&E) (X320).

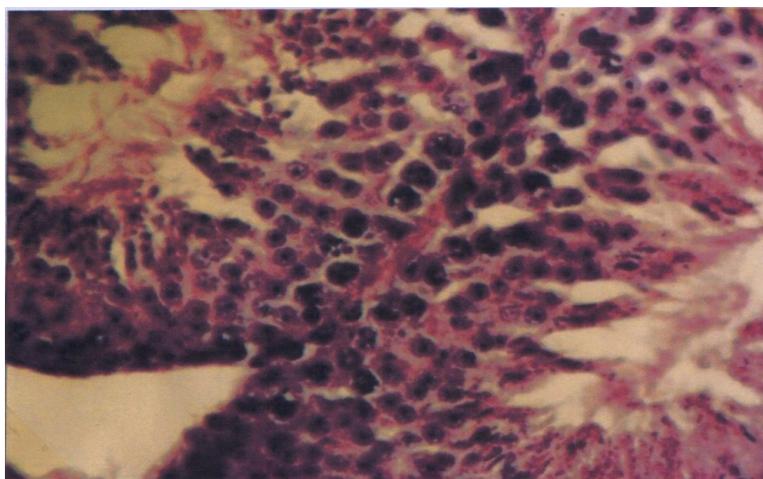


Fig.2 Testis of experimental mouse exhibited an increase in the thickness of seminiferous tubules with sperms nearly filled the lumen (H&E) (X320).

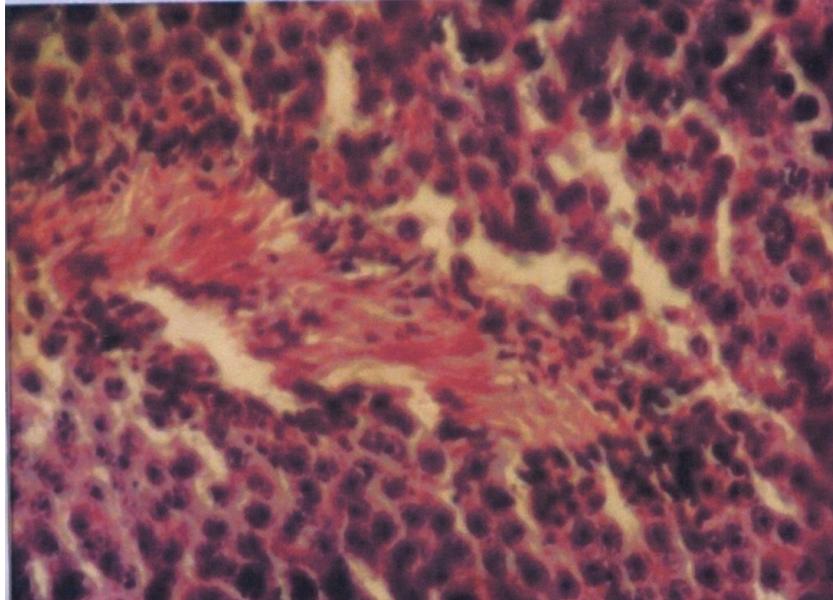


Fig.3 Testis of control mouse manifested tubules with the usual spermatogenic series (PAS- Haematoxylin Stain) (320).

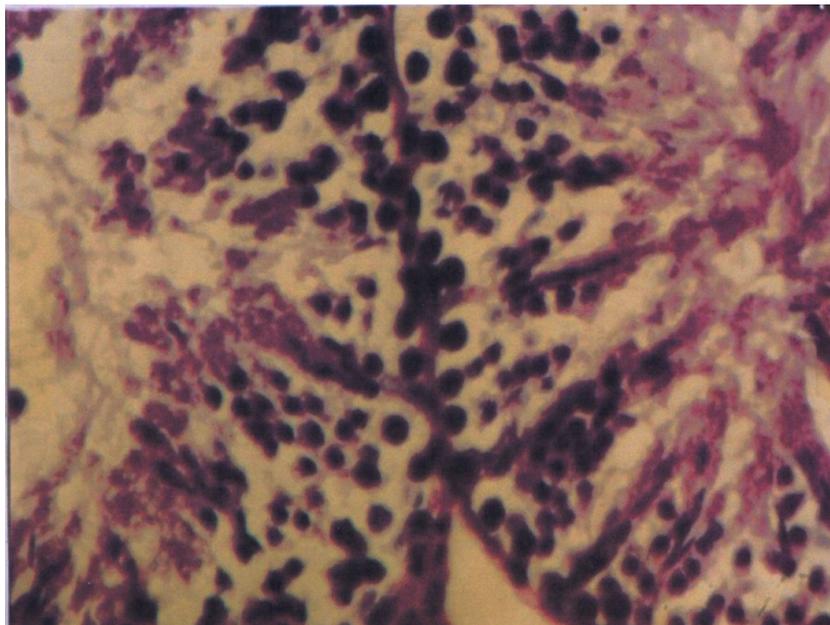


Fig.4 Testis of experimental mouse exhibited seminiferous tubules with sperms filling its lumen in addition there are PAS positive globular structures which are markedly increased in this group (blue arrows) (PAS-Haematoxylin Stain) (320)

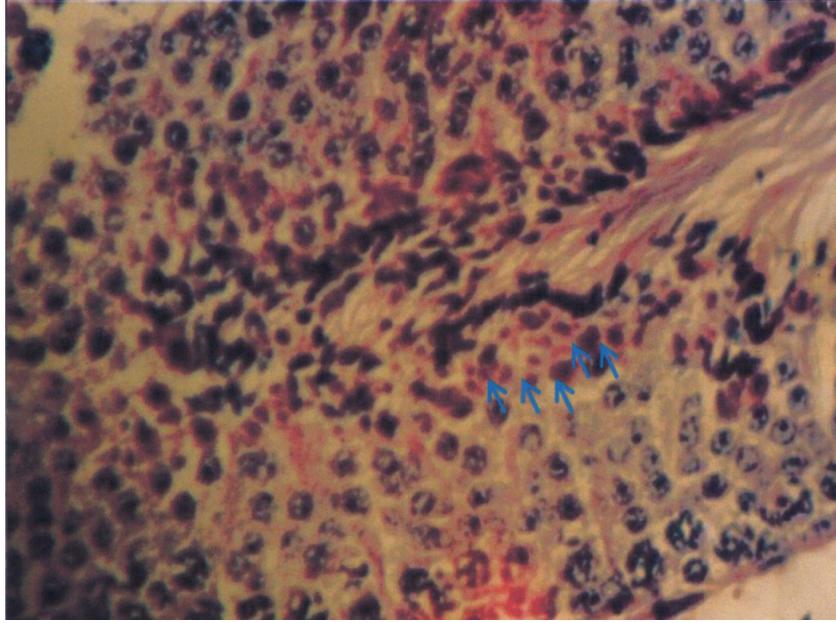


Fig.5 Testis of control mouse showing interstitial cells within the interstitial tissues (arrows) (PAS-Haematoxylin Stain) (X400).

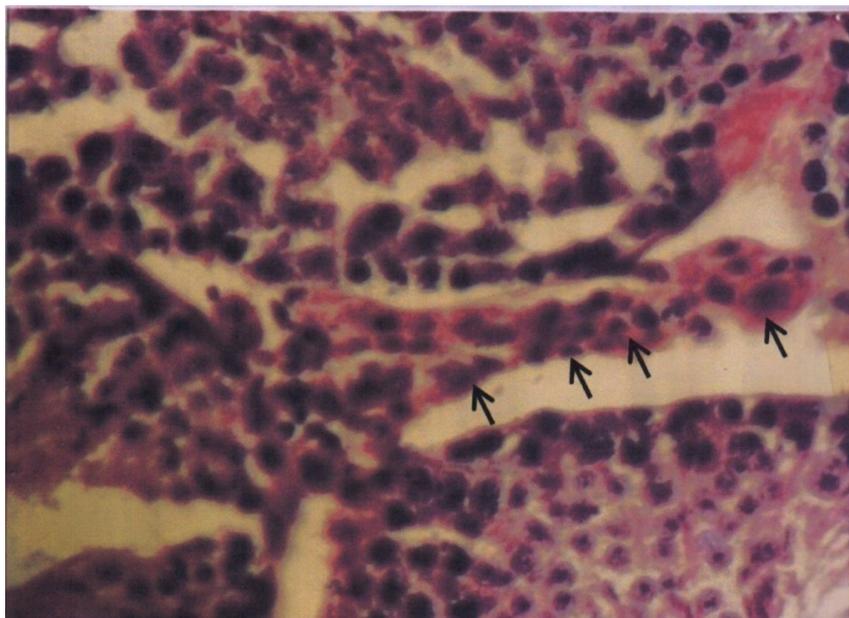


Fig.6 Testis of experimental group showing a remarkable increase in the number of interstitial cells (PAS-Haematoxylin Stain) (X400).

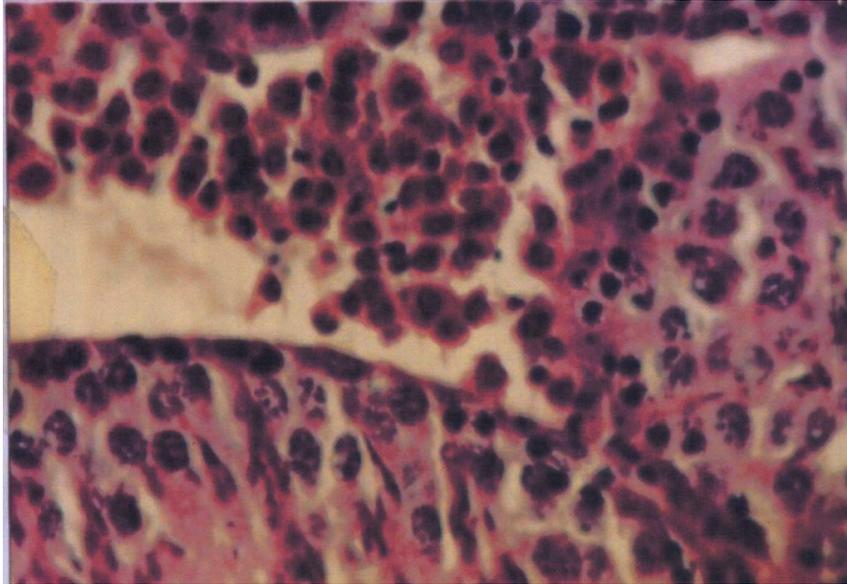


Fig.7 PAS stained testicular sections for the experimental animals, A: Non-Diastase treated sections; note the positive PAS staining reaction in the basal lamina and fibrous structures in the connective tissue between the lobules (X400). B: Diastase treated sections; note the light staining reaction for the cells of the spermatogenic stages up to spermatids (small arrows), and the relative darker staining of the interstitial cells of Leydig (large open arrows) (X400).

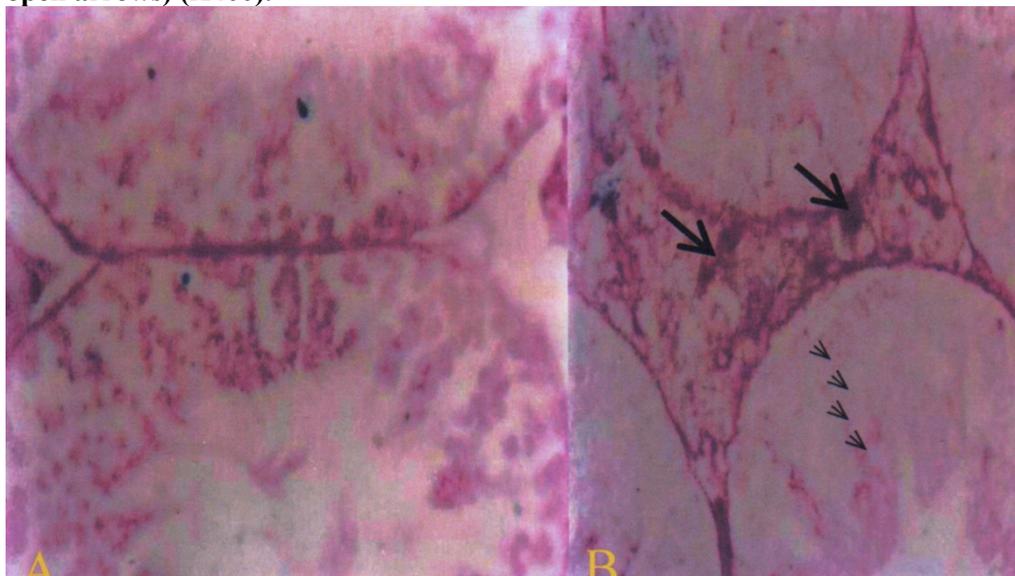
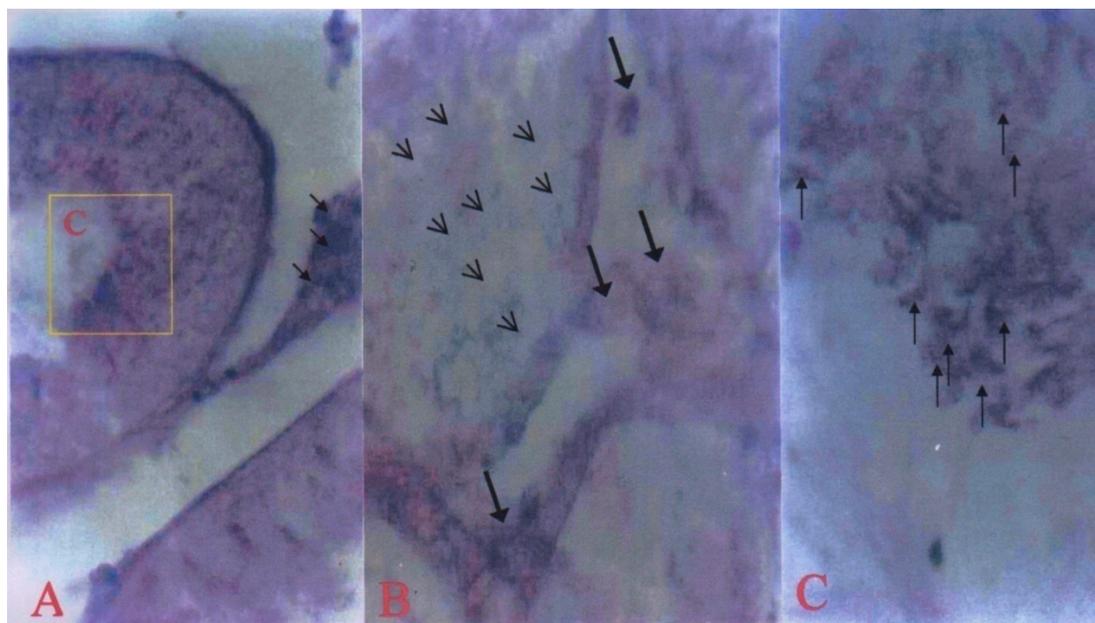


Fig.8 Alcain Blue 2.8-PAS stained experimental sections. **A:** Note the relatively dark bluish staining of the interstitial cells of Leydig (arrows)(X300). **B:** High magnification of the interstitium; note the relatively dark purplish-blue diffuse cytoplasmic reaction of the interstitial cells (long arrows), and the diffuse greenish cytoplasmic reaction in spermatids, spermatozoa and in Sertoli cells (short open-end arrows) (X400). **C:** High magnification of the boxed area in A; Note the Alcainophilic nature of the crescent-shaped structures at one pole of the cells of the spermatogenic stages (arrows) (X400).



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