Changes in the Cytoskeletal Intermediate Filaments of Testicular Tissues of Rabbits Related with Age and the Prophylactic Role of Vitamin E

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ABSTRACT

The present study is planned to investigate the changeable of molecular structure of testes vimentin and cytokeratin of different ages of rabbits (young, adult and elder) by using immunohistochemical method and the prophylactic role of vitamin E on senescent animals. Male New Zeland rabbits were divided into four groups according to age. Group I: represented the young rabbits (one month age, weighing 1 ± 0.4 kg), group II: adult rabbits (6 months age, weighing 4 ± 0.5 kg), group III: elder rabbits (24 month age, weighing 7.5 ± 0.5 kg and group (IV) aged rabbits administered orally with the therapeutic dose of vitamin E daily at a dose of 10 mg/kg b.w / day for 60 days. The intensity of either vimentin or cytokeratin filaments immunoreactivity to the testes was gradually increased with age; from moderate in young to strong in adult and intense in elderly rabbit testes. Vimentin was expressed in the interstitial connective tissue, boundary of the seminiferous tubules, around blood vessels periphery to Sertoli cells and in tunica albuginea, but not present in Leydig cells and spermatogonia. Cytokeratin was expressed in the endothelia of blood vessels either present inbetween the seminiferous tubules or those present in the interstitial areas. It was expressed as scany in young, moderate in adult and intense in aged rabbit testes. After administration of senescent animals with vitamin E at a therapeutic dose of 10 mg/kg b.w/day orally for 60 days, the testicular tissues showed an obvious improvement and a reduction of either vimentin or cytokeratin immunoreactivity and they demonstrated almost similar to the adult ones. The results indicated that vitamin E should be used to aged New Zeland rabbits to improve the testicular disturbances of the cytoskeletal intermediate vimentin & cytokeratin filaments and to improve the fertility of aged-animals.

Key Words:Age,Testes,Cytoskeleton,intermediate filaments,Immunohistochemistry,Mammals,Vitamin E

INTRODUCTION

Aging is a complex biological process leads to gradual loss of ability of an individual to maintain homeostasis **(1).** It is known that ageing of the human seminiferous epithelium or the condition of spermatogenetic maturation arrest involves alterations of structural and functional features of the spermatogenetic & Sertoli cells **(2&3),** including a general or local lowering of the efficiency of the Sertoli cell barrier. This phenomenon appears to coincide with significant alterations of the Sertoli cells cytoskeleton **(4).**

The cytoskeleton, which comprises actin, microtubules and intermediate filaments, is believed to function in the spermatogenic cells events to cytoskeletal dynamics in the testis **(5).** Modulation of the cytoskeletal network changes the mechanical properties of the cells that are essential for functions such as in cell motility, movement of organelles, maintenance cell-shape, participate in cell-cell and cell-matrix junctions **(6).**

Intermediate filaments (IFs) are the most abundant cytoskeletal proteins and include; vimentin, cytokeratin, desmin, neurofilament proteins, glial fibrillary acidic proteins, nestin and nuclear lamin. Vimentin is widely distributed in the cells of mesenchymal nature and in stroma **(7)** and plays a role in anchoring germ cells to the

seminiferous epithelium and these filaments are important component of the Sertoli cytoskeleton. Vimentin mediates tight junction contact between neighboring Sertoli cells, as well as the desmosome-like junctions located between Sertoli cells and germ cells **(8)**. Vimentin is proposed to conistitute a regulatory structure at the receptor enabling efficient signal transmission **(9).**

Seminiferous tubules of elderly men with spermatogenetic arrest at different levels display an increase in vimentin levels expression pattern **(10).** Increased vimentin expression has also been reported in various epithelial cancers including prostate cancer, CNS tumors, breast cancer, malignant melanoma, lung cancer and other types of cancers **(11)** as well as in colon and upper gastrointestinal tract cancer **(6).**

Cytokeratins are found in the epithelial cells and participate in epithelial cell protection from mechanical and non-mechanical stressors. Cytokeratin filamentous proteins used as diagnostic markers in tumor pathology, particularly for the differential diagnosis of carcinomas at the histologic level **(12).** The rete testis epithelium was positive to cytokeratin; while the Sertoli cells, stromal cells and Leydig cells were positive for vimentin. Cytokeratin-positive cells were also found lining atrophic seminiferous tubules, and were occasionally seen within nonatrophic seminiferous tubules. Both the immature and the mature epithelial structures showed cytokeratin positivit **(13)**.

The distribution patterns of cytokeratin and vimentin by means of immunohistochemical techniques in testicular biopsies of infertile men were explained by **Bergmann and Kliesch (14)**. They found that the Sertoli cells were regularly found to show vimentin expression in seminiferous tubules with normal spermatogenesis as well as in tubules with any kind of spermatogenic impairment. The authors demonstrated that cytokeratin expression was common in Sertoli cells of tubules with arrest of spermatogenesis at the level of spermatogonia, and was occasionally associated with arrest at the level of primary spermatocytes.

Moreover, the cytokeratin and vimentin can take place in fetal, postnatal and adult rat testes and only vimentin is in the adult rat testes **(15)**. Vimentin and keratin are also expressed in the Sertoli cells of young and elderly men by means of quantitative immunohistochemical methods **(10)**. They recorded that Sertoli cells from young men showed moderate immunogold labelling to vimentin throughout the entire cytoplasm between the cell organelles in tubules showing complete spermatogenesis. Immunogold particles were more numerous in the perinuclear cytoplasm and beneath the plasma membrane. The Sertoli cells of severely damaged tubules of elderly expressed more intense to vimentin throughout the whole cytoplasm. Also, the authors found that keratin expressed neither in young men nor in tubules of ageing men while vimentin increased in Sertoli cells.

These events are also associated with extensive changes in cell shape and size and germ cell movement. **Pop *et al*. (16)** found that the seminiferous tubules of testes of men between 50 and 80 years were designed which underwent sclerosis showed an intense positive reaction for actin and desmin but only focal positive reaction for vimentin.

Vitamin E is the most important lipophilic antioxidant and resides mainly in the mitochondria thus helping to maintain membrane stability and decreased the cell death which is due to the oxidants and free radicals in different cells. The antioxidants play an important role in preventing free radicals damage assiociated with age by

interfering in the generation of radicals or by scavenging **(17).** The effect of antioxidant supplementation was studied on sperm quality, lipid peroxidation and testosterone levels of male rats **(18)**. They recorded the improvement of reproductive traits of male rats that are associated with high fertility. The level of lipid peroxidation is significantly decreased in the testis. Epididymal sperm concentration and plasma testosterone levels are significantly increased in the ascorbic acid treated animals when compared to the control animals.

The aging is associated with mitochondrial changes & damages that may be enhanced by deficiencies in antioxidants**.** Vitamin E treatment to aging mice could minimize the age-related mitochondrial changes in the mouse liver **(19)**, improve sperm quality and mobility in the testis **(20)**, and improved the histological and intermediate filaments, vimentin and cytokeratin, of thyrocytes of aged – rabbits (**21),** restored the histological structures of the testes with an obvious improvement of Leydig and well organized spermatogenic cells of the seminiferous tubules (**22).**

The present study is planned to study the age- related changes in the cytoskeletal intermediate filaments vimentin & cytokeratin of rabbit testes of different ages (young, adult and elder) immunohistochemically, and after administration of antioxidant vitamin E to aged- animals.

Materials and Methods

Animals:

Twenty eight different ages of male New Zeland rabbits from one to 24 months weighing from one kg to 8 kg were collected during autumn and housed in environmentally controlled optimal conditions for one week. Diet and water were allowed *ad-libitium.* All experiments were conducted in accordance with guidelines approved by the Institutional animal Care and Use Committee.

Experimental design:

The animals were divided into four groups according to age; Group I: young rabbits (one month age, weighing 1 ± 0.4 kg), Group II: adult rabbits (6 months age, weighing 4 ± 0.5 kg). Group III: senescent animals (24 month age, weighing 7.5 ± 0.5 kg) and Group IV: aged rabbits administered daily with a therapeutic dose of vitamin E orally, (E-Viton,Kahra Pharm &Chem. Ind.Co) at a dose of 10 mg/kg b.w/day for 60 days . Vitamin E (Alpha tocopheryl acetate) dosage was estimated according to **Baydas *et al.* (23).**

The animal groups:.I, II & III were sacrificed after one week, while the animals group (IV) that given with vitamin E was sacrificed after 60 days. The testes were removed carefully from all groups and cut into small pieces, then fixed in 10% neutral buffered formalin for 24 hrs. The fixed specimens were washed and transferred to 70% ethanol, dehydrated in ascending ethanol, cleared in xylene, embedded in paraffin wax and sectiond at 5µ thickness. Paraffin sections were used for immunohistochemical (IHC) studies. Monoclonal antibodies either anti- pan cytokeratin (anti-CK AE1/AE3) or anti-vimentin (V9) (received from Dako Carpinteria, CA 93013 USA) were used and obtained from Thermo Fisher Scientific Industries.Avidin-biotin immunoperoxidase technique was applied in which a biotinylated secondary antibody reacts with peroxidase conjugated streptavidin molecules. Colour reaction of cytokeratin or vimentin immunoreactivity was developed by using diamino-benzidine (DAB) that

gave brown colour and haematoxylin was used for counter staining **(24).**

Results

Immunohistochemical(IHC) observations:

1-Vimentin

By IHC study, vimentin is expressed in the interstitial connective tissue, at the basal part of boundary of the connective tissue of seminiferous tubules and around blood vessels, in Sertoli cells and also seen in tunica albuginea but not present in Leydig cells and spermatogonia as: a moderate immunoreactivity in young rabbits (Fig. 1a&b), a moderate to strong immunoreactivity in adult animals (Fig.2) and intensive immunoreaction in aged rabbits (Fig. 3a&b).

After administration of aged animals with vitamin E at a dose of 10 mg/kg b.w/day orally for 60 days, the testicular tissue showed an obvious improvement and a reduction of vimentin immunoreactivity in the connective tissue boundary of the seminiferous tubules, and disappearance of it from the majority of Sertoli cells (Fig. 4a&b).The vimentin filaments demonstrated almost similar to the adult ones.

2- Cytokeratin.

In young studied animals, IHC study demonstrated a scanty of immunoreactivity to cytokeratin in the endothelia of blood vessels inbetween the seminiferous tubules and in the endothelia of blood vessels in interstitial area (Fig. 5a&b). In adult rabbits, a moderate expression of cytokeratin immunoreaction was elucidated in the endothelia of blood vessels either present inbetween the seminiferous tubules, or those present in the interstitial areas, and no expression of immunostain to cytokeratin at the germinal epithelial cells of the tubules and Sertoli cells (Fig. 6a&b). In aged animals, cytokeratin is characteristically shown as intense expression of immunoreactivity in the endothelia of blood vessels boundary the seminiferous tubules and in the interstitial area (Fig. 7). No cytokeratin immunoreactivity could be expressed in Sertoli cells in all aged.

The testis of aged rabbit treated with vitamin E at a dose of 10 mg/kg b.w/day for 60 days, showed a decline in the cytokeratin immunoreactivity in the endothelia of blood vessels (Fig. 8a&b).

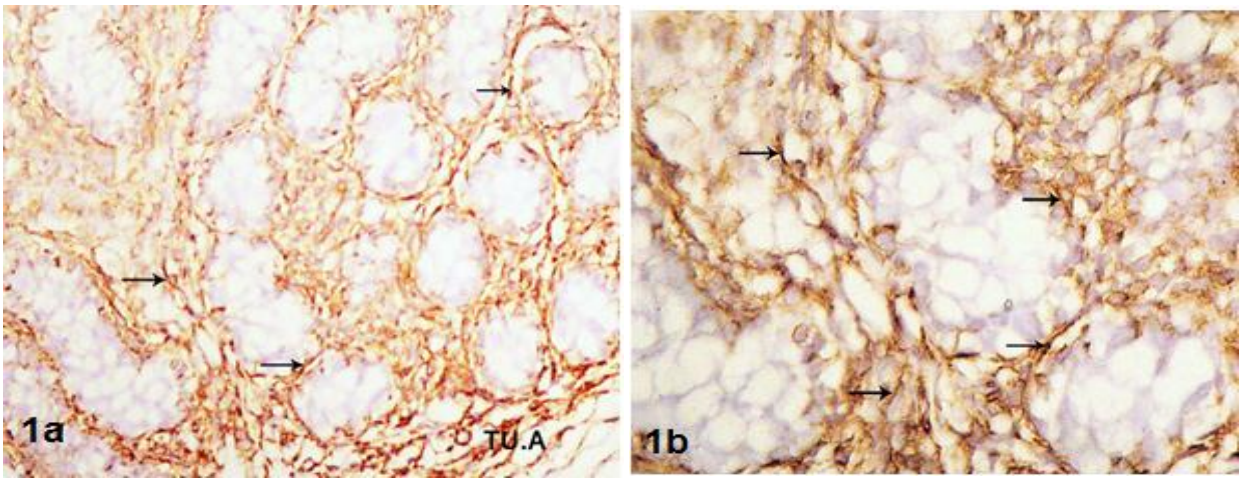


Fig.(1a&b): Sections in the testes of a young rabbits expressing moderate vimentin immunoreactivity in the interstitial tissue, boundary of the seminiferous tubules and around blood vessels (arrows), and tunica albuginea (TU.A). Vimentin immunostain, X200 & X400

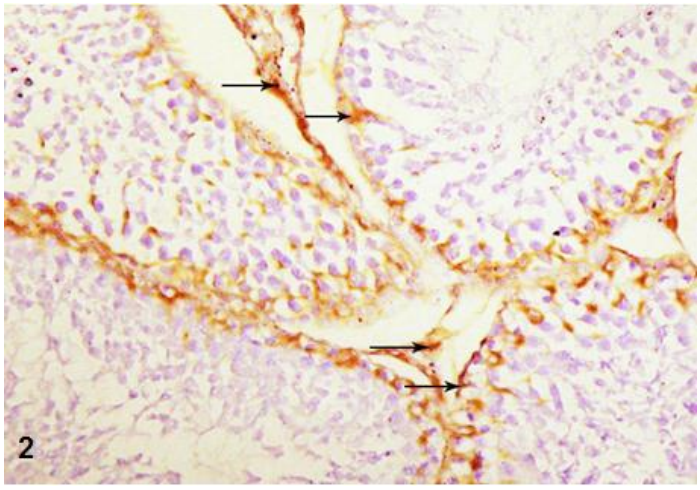


Fig.(2): Section in the testis of an adult rabbit showing moderate to strong vimentin immunoreactivity in the connective tissue of the interstitium, at the basal part of boundary connective tissue of seminiferous tubules and in Sertoli cells (arrows). Vimentin immunostain, X200

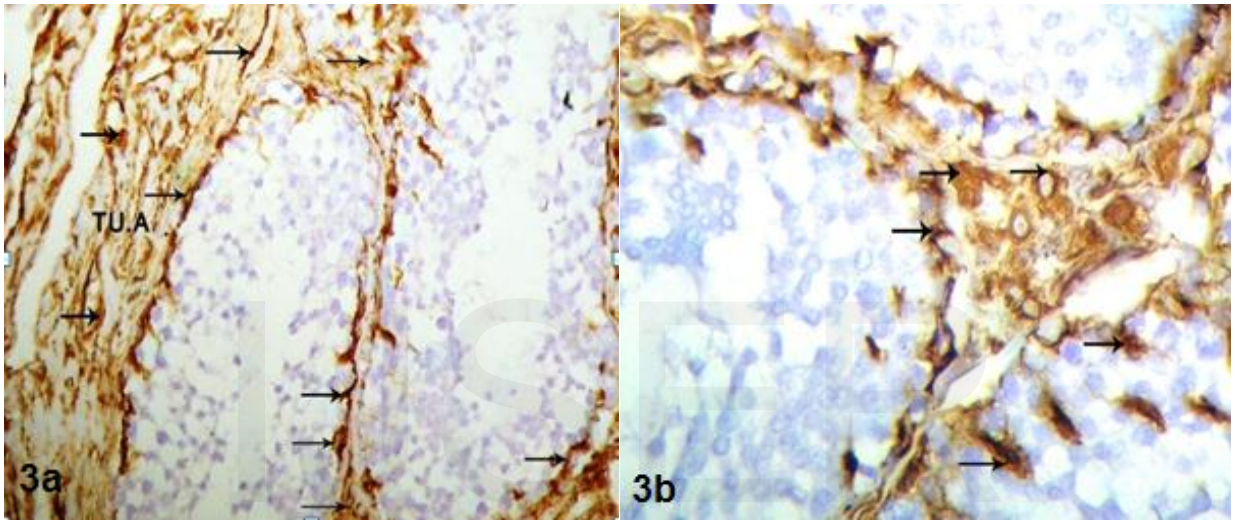


Fig.(3a&b): Sections in the testes of aged rabbits showing an obvious intensive vimentin immunoreactivity in tunica albuginea (TU.A), in the basal part of spermatogonia, in Sertoli cells and in connective tissue boundary of seminiferous tubules , in the interstitial tissue and boundary of the blood vessels (arrows). Vimentin immunostain, X200& X400

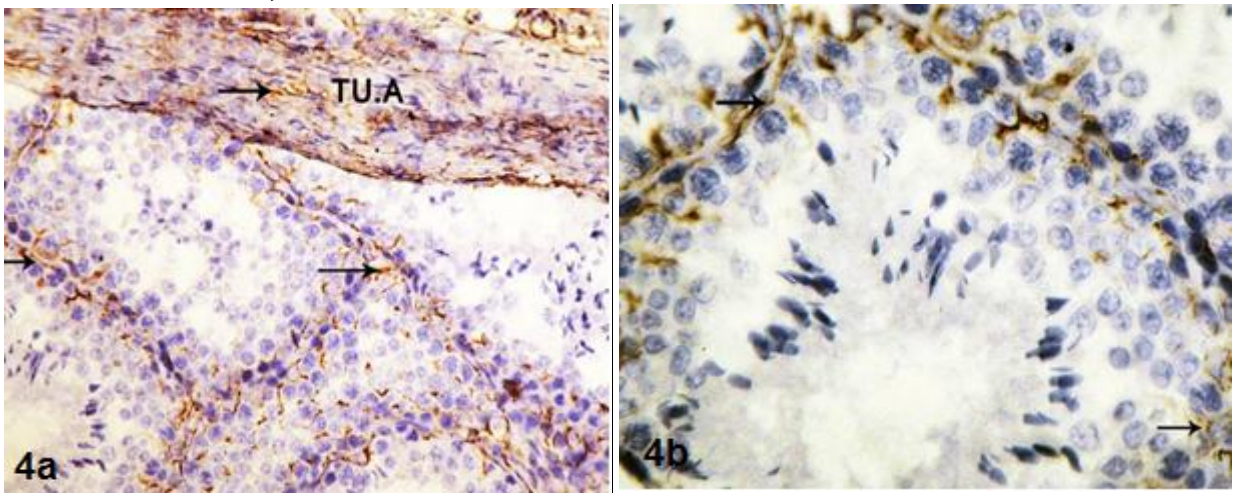
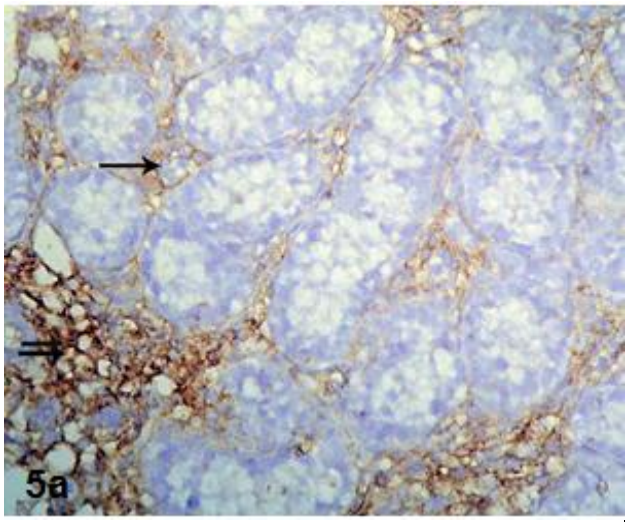


Fig.(4a&b): Sections in the testes of aged rabbits treated with vitamin E showing an obvious reduction of vimentin immunoreactivity in tunica albuginea (TU.A), in the connective tissue boundary of the seminiferous tubules and disappearance of it from the majority of Sertoli cells (arrows). Vimentin immunostain, X 200 & X1000.



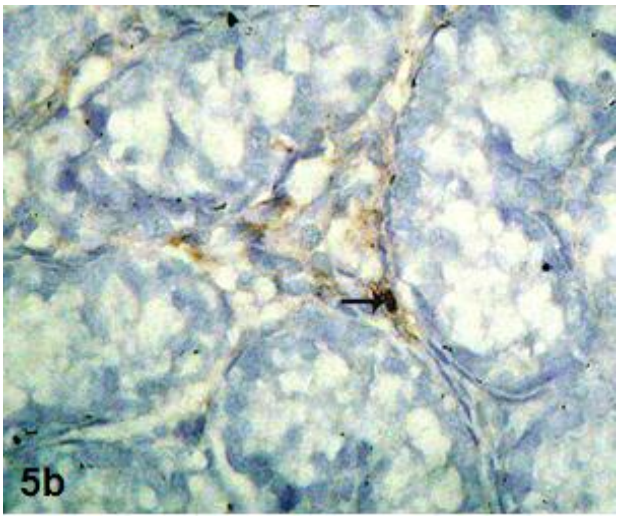
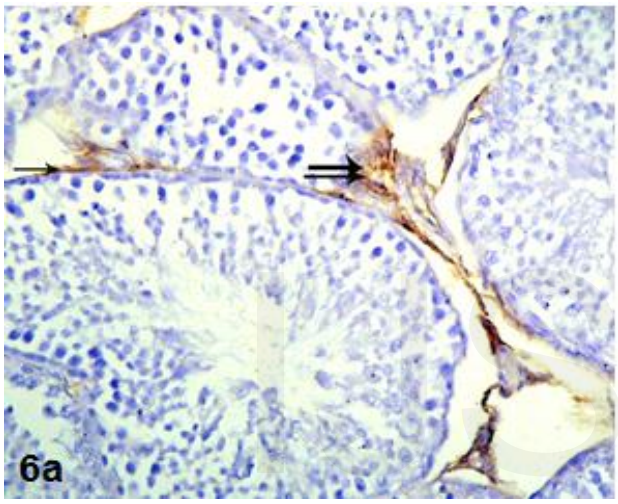


Fig. (5 a&b): Section in the testis of young rabbit showing a scanty immunoreactivity to cytokeratin in the endothelia of blood vessels, inbetween the seminiferous tubules (arrow) and in the endothelia of blood vessels of the interstitial area (double arrow). Cytokeratin immunostain, X 200 & X400



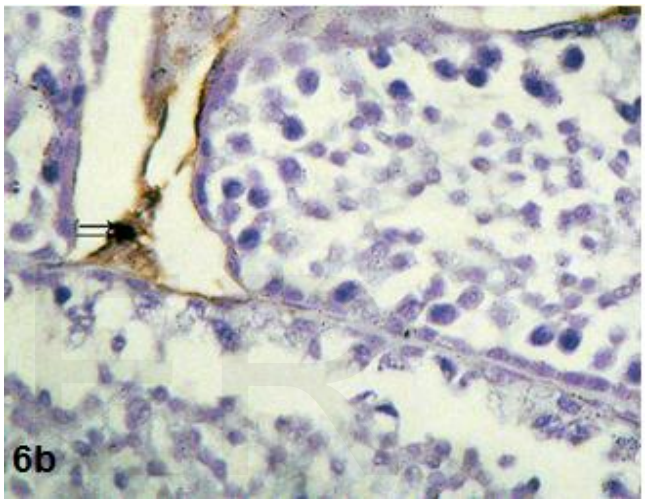


Fig. (6 a&b): Sections in the testes of adult rabbits showing a moderate expression of immunostain to cytokeratin in the endothelia of blood vessels inbetween the seminiferous tubules (arrow) and in the endothelia of blood vessels of the interstitial area (double arrow).Noticed no expression of immunostain to cytokeratin at the germinal epithelial and Sertoli cells. Cytokeratin immunostain, X 200 & X400.

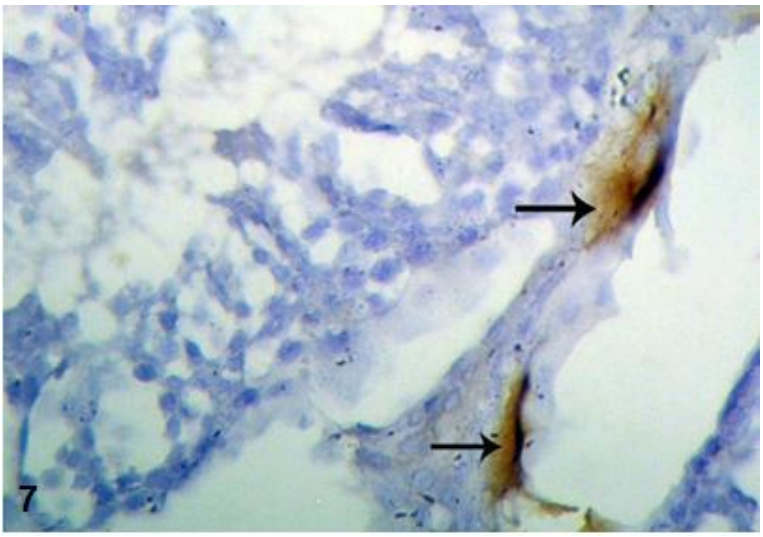
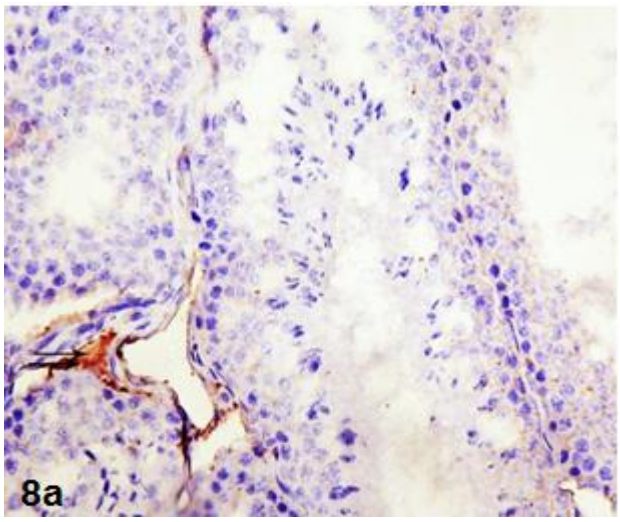


Fig. (7): Section of the testis of aged rabbit showing the seminiferous tubules with intense cytokeratin expression in the endothelia of blood vessels in the interstitial area (arrows).Noticed no expression of immunostain to cytokeratin at the germinal epithelial and Sertoli cells. Cytokeratin immunostain, X 400



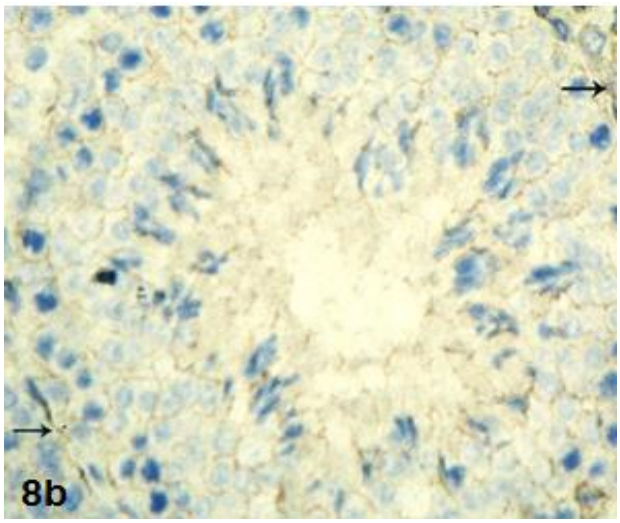


Fig. (8a&b): Sections in the testes of aged rabbits treated with vitamin E showing a marked reduction in the cytokeratin immunostain of the endothelia of blood vessels in the area of interstitial cells (double arrow). No cytokeratin expression in the germinal and Sertoli cells of the seminiferous tubules almost similar to cytokeratin of adult testis. Cytokeratin immunostain, X200 & X400

Discussion

Vimentin in the present work is found in the interstitial connective tissue and boundary of the seminiferous tubules at the basal part of spermatogonia, around blood vessels either in tunica albuginea or in interstital areas and Sertoli cells in a moderate expression in young, moderate to strong in adult and more intense in aged studied animals. Additionally, cytokeratin immunoreactivity in the current work is clearly expressed in the endothelia of blood vessels inbetween the seminiferous tubules and in the endothelia of blood vessels in the interstitial area; in a scanty expression in young, moderate in adult and intense expression in aged rabbits. No expression of immunoreactivity to cytokeratin in Sertoli cells and at the germinal epithelial cells of the seminiferous tubules.

Vimentin is widly distributed in cells of mesenchymal nature **(7)**. Normal seminiferous tubules showed vimentin positivity in the Sertoli cells, which confirmed previous reports. Vimentin is an important-Sertoli cell cytoskeleton component and provide mechanical resiliency and strength to cells **(25)**. **Lie *et al.* (5)** illustrated that the cytoskeleton are associated with the germ cells movement and their changeable.

Cytokeratin is a family of water insoluble, intracellular fibrous protein present in almost all epithelia **(26).** Keratin antibodies also failed to react with any intratubular cells in the normal testes. The cytokeratin-positive cells could belong to the rete testis system **(27). Dinges *et al*. (28)** found that the vimentin and cytokeratin filaments in rete testis of children up to 2 years of age were more homogeneous. Predominance of the basal cell portions for vimentin, and the apical regions for cytokeratin staining were less pronounced than in adult testes.

The Sertoli cells of severely damaged tubules of elderly men expressed more intense to vimentin, and keratin expressed neither in young men nor in tubules of ageing men while vimentin increased in Sertoli cells (**10**). They suggested that the changes in IFs are related to the local factors associated with completion of spermatogenesis causing functional changes in Sertoli cells.

Zhang *et al*. (29) showed disrupted expression of intermediate filaments in the testis of rhesus monkey after experimental

cryptorchidism and they found that vimentin was localized in the perinuclear region of Sertoli cells of the normal testis. An intense increase in vimentin immunoreactivity was observed with appearance of disorganized staining in the Sertoli cells of the cryptorchid testes. Cytokeratin 18, a marker of immature Sertoli cells, re-expressed in the cells of the adult cryptorchid testes. Desmin was also observed in the Sertoli cells in addition to the peritubular myoid cells after 30 days the cryptorchid operation. The authors concluded that the altered changes in intermediate filaments could be possible to induce the Sertoli cell functional changes that would partially contribute to the germ cell apoptosis leading to azoospermia or oligozoospermia.

Cruzana *et al.* (30) found that the vimentin was present in the modified Sertoli cells of the terminal segmenting of the seminiferous tubules suggested that these proteins are involved in maintaining the cytoskeletal framework to be able to carry out their function as a valve-like device preventing the reflux of spermatozoa and tubular fluid and aid in regulating the secretory activity of the modified Sertoli cells.

Cytokeratins (CK8 and CK18) were found in varying amounts in Sertoli cells of fetal, prepubertal and senile human testes and in all cases of pathological alteration. Cytokeratins were completely absent only in normal mature seminiferous tubules. Therefore, the immunohistochemical detection of cytokeratins in Sertoli cells seems to provide a sensitive marker for immature or damaged testes **(31)**. The detection of cytokeratin in the extremely thin seminiferous epithelia is one of the most characteristic phenomena of age-related testicular changes in Sertoli cells of older mice **(32).**

During embryonal development prominent cytokeratin expression disappeared after 20th week of gestation**.** The desmin intermediate filament was prominently present in Sertoli cells in the vast majority of the cases investigated as well as vimentin and cytokeratin coexpression **(33). Sharpe *et al*. (34)** found that cytokeratin expression as a marker showing lack of differentiation was common in Sertoli cells of tubules with arrest of spermatogenesis at the level of spermatogonia, and was occasionally associated with arrest at the level of primary spermatocytes.

The presence of prominent immunoreactions with all cytoskeletal proteins in the testis of swamp-type water buffalo (*Bubalus bubalis*) was studied **(30)**, they occurred in the peritubular cells, Sertoli cells, modified Sertoli cells of the terminal segment of the seminiferous tubule, intratesticular excurrent ducts and blood vessels. Leydig cells and spermatogenic cells were negative to all the cytoskeletal proteins. They showed that the pattern of distribution of some cytoskeletal proteins changes from immature to mature testis. Moreover, the vimentin is present in Sertoli cells of the adult testis. In addition, the appearance of cytokeratin may be related to ageing rather than having an undifferentiated immature feature. They demonstrated the appearance of cytokeratin may be related to ageing rather than having an undifferentiated immature feature. **Dawei *et al.* (35)** reported that the dysfunction of spermatogenesis may be due to the disruption of the intermediate filament cytoskeleton in Sertoli cells.

In the present study aged- rabbits treated with antioxidant vitamin E a dose for 60 days demonstrated improvement of the intermediate filament and reduced the changeable of cytokeratin and vimentin immunoreactivity in the testes. In accordance, **Sahoo *et al.* (36)** found that antioxidant defense profile of rat testicular

mitochondria exhibited age related alterations which might play a critical role in regulating physiological functions of the testis such as protein carbonylation, steroidogenesis and spermatogenesis. The free radicals are formed with aging and caused disturbance damage of many tissues **(37& 38)**.

Zamoner *et al*. (39) supported the idea that an increase in mitochondrial reactive oxygen species generation, underlying cellular oxidative damage, is a side effect of hyperthyroid-induced biochemical changes by which rat testis increase their metabolic capacity. Biomarkers of oxidative stress demonstrated an increased basal metabolic rate measured by tissue oxygen consumption. They found that maturation of testis showed an intense in protein synthesis and processing, supporting the enhancement in vimentin synthesis in hyperthyroid testis. The enzymatic and non-enzymatic antioxidant defenses appeared to respond according to the augmented oxygen consumption by decreasing total glutathione levels, with enhancement of reduced glutathione, whereas most of the antioxidant enzyme activities were induced.

Hong *et al.* (40) found that the supplementation of vitamin E can increase activity of glutathione peroxidase (GSH-PX) and can protect testis from damage by preoxidation. Vitamin E could up- also regulate genes expression responsible for sperm mobility in the testis of the aging mouse **(20).** In the previous study, aged rabbits (2 years age) exhibited a numerous number of irregular follicles lined with flattened thyrocytes, and after supplementation with vitamin E at a dose 10 mg/kg b.w / day for 60 days, an obvious improvement of the thyroid structure and the recovery of cytoskeletal vimentin and cytokeratin immunoreactivity to normal form **(21).** Moreover, vimentin and cytokeratin alterations of rat colon and liver exposured to immobilization stress were recerovery after diazepam treatment **(41& 42).**

In brief, an obvious remodulation of intermediate vimentin and cytokeratin filaments after the treatment of vitamin E to senescent testes New Zeland rabbits was demonstrated. The results indicated that the elder animals should be used vitamin E to improve the fertility of the testis of aged-animals to increase offspring of these animals.

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