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Involution of seminiferous tubules in aged hamsters: an ultrastructural, immunohistochemical and quantitative morphological study

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Summary. In this study, we examined the age-related changes on morphometric parameters and ultrastructure of seminiferous tubules, and on the expression of extracellular matrix proteins in lamina propria of Syrian hamsters. A significant decrease in the percentage of normal tubules and an increase in the percentage of hypospermatogenic and arrested maturation tubules was observed with aging. Aged animals showed a decrease in tubular diameter, tubular lumen, seminiferous epithelium volume and total tubular volume. However, the total length of seminiferous tubules was significantly increased with aging. The most important ultrastructural changes with aging were the thickening of the lamina propria, the presence of diverse abnormalities in the spermiogenesis process, degeneration of germ cells, and vacuolization and flattening of Sertoli cells showing abundant lipofucsin droplets and residual bodies. Laminin immunoreactivity was found along the lamina propria of seminiferous tubules both in young and aged animals. Fibronectin immunoreactivity was found along the lamina propria and blood vessels. Both laminin and fibronectin total volume of immunostaining per testis was increased in aged hamsters. In conclusion, the age-related changes in seminiferous tubules of hamster include: a decrease in tubular width and an increase in tubular length; widening of the lamina propria caused by a more extensive connective matrix between the peritubular cells and the basal membrane; and a strong disarrangement of the seminiferous epithelium, including germ cell degeneration and important alterations in both spermiogenesis and Sertoli cell structure.

Key words: Aging, Testes, Immunohistochemistry, Hamster, Morphometry, Ultrastructure

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Introduction

Specific age-related changes both in function and structure of the seminiferous tubules have been reported in most mammals including man (Paniagua et al., 1986, 1987, 1991; Johnson et al., 1986, 1988), the donkey (Nipken and Wrobel, 1997), the rat (Wright et al., 1993; Levy et al., 1999) and the hamster (Horn et al., 1996; Morales et al., 2003). Tubular involution in aged men includes the occurrence of unspecific morphological alterations, the loss of seminiferous epithelium, and ultrastructural alterations including the abundance of lysosomes and lipids in the Sertoli cells and the thickening of the lamina propria (Paniagua et al., 1987, 1991). Also, quantitative morphological studies have shown a diminution in the volume, diameter and length of seminiferous tubules with advancing age in men (Johnson et al., 1986, 1988; Paniagua et al., 1987). However, studies focused on ultrastructural and morphological quantitative changes with age in experimental animals such as mouse and rat are limited (Wright et al., 1993; Richardson et al., 1995) and non-existent in the case of the hamster.

The seminiferous tubules of vertebrates are surrounded by a "lamina propria", a permeability barrier for substances penetrating the seminiferous tubule from the interstitium (Fawcett et al., 1970), consisting of myofibroblast, fibroblast, collagen fibers, ground substance and a basal lamina on which spermatogenic and Sertoli cells rest (Burgos et al., 1970).

In many testicular disorders associated with germ-cell depletion, the tubular diameter decreases while the tubular lamina propria undergoes a progressive enlargement that ends in a complete tubular sclerosis (Prijono and Schirren, 1985; Söderstrom, 1986). Germ cell depletion and tubular sclerosis have also been associated with aging in men (Jonhson et al., 1986; Paniagua et al., 1987). In some mammals, the tubular lamina propria thickens with advancing age and displays long irregular projections into the seminiferous epithelium (Paniagua et al., 1991; Nipken and Wrobel,

1997). However, there is no clear evidence if this thickening is accompained by an increase in extracellular matrix or if it is just the result of tissue shrinkage.

In addition, it is doubtful whether the lamina propria enlargement is either the result or the cause of germ cell depletion in testes. It has been postulated that the diminution of seminiferous tubule volume caused by germ cell loss results in the increase in collagen fibers around the tubules in an attempt to fill the empty space and replace the lost volume (Söderstrom, 1986). However, other authors assume that enlargement of the tunica propria hinders the metabolic exchange between the seminiferous epithelium and testicular interstitium, thus leading to germ cell loss and tubular atrophy (Bustos-Obregón, 1976).

The hamster has been widely used as an animal model for testicular aging (Swanson et al., 1982; Horn et al., 1996; Calvo et al., 1995, 1997, 1999; Morales et al., 2003), but the ultrastructural and quantitative morphological alterations of seminiferous tubules, and the co-related changes in lamina propria due to aging have not been studied. Thus, the objectives of the present report are: a) to analyze the morphological quantitative changes in seminiferous tubules with advancing age; b) to describe the ultrastructure of regressed seminiferous tubules in aging; and c) to determine the possible modifications in lamina propria with aging using ultrastructural and immunohistochemical techniques for detection of its components: laminin and fibronectin.

Materials and methods

Animals, tissue preparation and conventional histology

Thirteen male Syrian hamsters (*Mesocricetus auratus*) were obtained from the animal facility of Murcia University: 6 were aged 6 months (young) and 7 were aged 24 months (aged). All animals were maintained at a constant temperature between 25-28 ºC and received food and water ad libitum. All the animals were housed in a 14/10 light/dark cycle. The hamsters were all sacrificed by an intraperitoneal overdose of pentobarbital and weighed. Immediately after removal the testes were weighed and measured. Subsequently, they were fixed in methacarn (methanol:chloroform: acetic acid, 6:3:1) for quantitative morphological and immunohistochemical study. Representative samples were chosen, dehydrated, immersed in toluene and embedded in Paraplast Plus. For histological examination 4 mm-thick sections were stained with hematoxylin-eosin and PAS.

Immunohistochemistry

4 μm-thick sections were used for immunohistochemical demonstration of fibronectin and laminin, following the streptavidin-biotin-peroxidase (ABP) method. Sections were deparaffinized and rehydrated with PBS. Endogenous peroxidase was inactivated by 0.5% H_2O_2. Before immunostaining, the sections were treated for 3 min with 0.1% pepsin in 0.01 M HCl at 37 ºC for laminin and fibronectin staining. Subsequently, the samples were incubated with the primary antibodies rabbit anti-fibronectin (Biogenex, Dublin, USA) and rabbit anti-laminin (Eurodiagnostic, Amsterdam, Nederlands), diluted 1:400 and 1:40, respectively, in PBS containing 1% BSA. Primary antibody incubation was performed in a humidified chamber overnight at 4 ºC for all antibodies. After extensive washing steps in PBS, samples were incubated for 30 min in a secondary biotinylated anti-rabbit (Dako, Glostrup, Denmark) diluted 1:400 in PBS containing 1% BSA, in a humidified chamber. The ABP reaction was performed according to the manufacturer´s protocol. To visualize the immunostainings, sections were washed in PBS and incubated for 10 min in TBS including 3,3'-diaminobenzidine (DAB) to which 0.3% H_2O_2 was added. Sections were counterstained with hematoxylin.

Electron microscopy

A central slice of each testis was cut into pieces of 1 mm^3. These were fixed for 4 h in 3.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. They were then washed in 0.855 mg/100 ml saccharose-cacodylate, postfixed in 1% osmium tetroxide (for 2 h), dehydrated in a graded acetone series and embedded in Epon 812 (Serva). Semithin sections were stained with toluidine blue. Ultrathin sections were cut using a Reichert-Imy Ultracut Ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined with a Zeiss EM/10CR electron microscope.

Morphometrical study

Testicular volume and weight. The testes were weighed immediately after removal by using a Mettler PS 360 balance. The testicular volumes (V_T) were estimated by considering the testis as a triaxial ellipsoid (Paniagua et al., 1990; Santamaría et al., 1995). The three testicular transverse axes (2a, 2b and 2c) were measured by a Vernier calliper immediately after removing the testes from animals, and the testicular volume in fresh was calculated by the equation for triaxial ellipsoid, $V_T=4/3(\pi abc)$.

Semiquantitative histological study. For semiquantitative analysis, the degree of tubular degeneration was classified into 5 categories: 1) normal tubules; 2) hypospermatogenic tubules characterized by a decrease in the thickness of the seminiferous epithelium which contained all cellular types of the germ-line and occasional slough off of germ cells; 3) arrested maturation, where the seminiferous epithelium showed hypospermatogenic areas joined to others with arrested maturation of germ-line, predominantly at the level of spermatocytes; 4) tubules containing spermatogonia and Sertoli cells; and 5) only Sertoli cell tubules. For this purpose two 4 μm-thick sections of

each testis, chosen randomly, were used and 100 tubular cross-sections were examined from each section.

Morphological parameters of the testis. For the morphometrical study of histological parameters and immunoreactivity quantification, the microscopic images were recorded using an image analysis program (Micron, Barcelona, Spain). The images were analyzed by a computerized program of analysis of area designed for the study. For morphometric analysis corrected measures in fresh were used. To transform the parameters, which were measured in histological sections, to their actual measurements in fresh testes, the change in volume during tissue preparation was calculated. To obtain the correction factor for tissue shrinkage, the maximal slice of fixed testes was embedded in paraffin. Five 4 μm cross-sections were obtained and stained with hematoxylin-eosin. In each cross-section, the transverse axes were measured using an image analysis program (Micron). To obtain the correction factor for shrinkage, the difference between the transverse fresh axes and the transverse axes measured in maximal cross-sections was performed and the correction factor was calculated. A correction factor of 1.2 was applied to correct means of the morphological parameters of testes for shrinkage.

The epithelial area, tubular perimeter, mean tubular diameter (MTD) and mean lumen diameter (MLD) were calculated using an image analyzer (Micron) with a x10 objective. For this purpose five 4 μm-thick sections of each testis, which were chosen randomly, were used, and five exactly cross-sectioned tubules were measured from each section (25 exactly cross-sectioned tubules per testis).

Subsequently the following variables were calculated: (1) The volume density of seminiferous tubules (VD_{ST}), i. e. the ratio of "seminiferous tubule surface/reference area (8947 μm^2). (2) The total volume of seminiferous tubule per testis V_{ST}, calculated by multiplying $VD_{ST} \times V_T$. (3) The total length of seminiferous tubules (L_{ST}). All tubules were assumed to form a single cylinder with a length L, a radius R (MTD/2), and a volume V_{ST}. The length of seminiferous tubule per volume unit (L_v) was calculated employing the established formula: $L_v = VD_{ST} / \pi R^2$ (Wing and Christensen, 1982; Santamaria et al., 1995). From $L_{ST} = L_v \times V_T$. (4) The mean cylinder area of seminiferous tubules = $2\pi R \times L_{ST}$. (5) The total volume of seminiferous epithelium (V_{SE}), calculated by multiplying the area of seminiferous epithelium x L_{ST}.

The average thickness of the lamina propria was calculated in at least five different sites of each type of tubule (n=7, per tubular type) by measuring the distance from the basal membrane to the inner basal membrane of the peritubular cells using representative electron microphotographs of seminiferous tubules.

Quantitative evaluation of the immunohistochemical study

For immunoreaction area measurement, four 4 μm-thick cross-sections per testis were used. In each section five randomly chosen fields were examined. The volume density of immunoreactivities (VD_I) was calculated as mean imunoreactive area / reference area (8947 μm^2) using the image analyzer system with a x40 objective, and the total volumes of the immunoreactivities (V_I) were calculated by multiplying $VD_I \times V_T$.

Statistical analysis

Statistical evaluation of mean differences between the two groups was performed by a Student *t* test. The test was performed using ln-transformed data. Data were back-transformed following analyses, and are reported as mean ± SEM. The difference among means corresponding to lamina thickness was analyzed by the one-way ANOVA test and completed by a Student *t* test for independent data. Mean differences were considered statistically significant when $P < 0.05$.

Results

Histology

At 6 months of age, the seminiferous tubules did not show any relevant morphological alterations. The seminiferous epithelium showed all the typical associations of germ cell types and Sertoli cells configuring the different stages of the seminiferous cycle. The majority of tubular cross-sections showed a normal seminiferous epithelium. However, some hypospermatogenic zones were also observed. Normal myoid cells were observed surrounding the seminiferous tubule. Leydig cells, located near to interstitial blood vessels, were morphologically normal (Fig. 1A).

At 24 months of age, histologically normal seminiferous tubules were detected alternating with tubules showing different degrees of histological degeneration, including hypospermatogenesis (Fig. 1B), maturation arrest at the level of spermatocytes (Fig. 1C) and spermatids, tubules with spermatogonia and Sertoli cells, and even Sertoli cell-only tubules.

Immunohistochemical results

Laminin immunoreactivity was found along the lamina propria of seminiferous tubules, principally in the inner zone. Also, laminin immunoreactivity was observed surrounding the endothelium of interstitial capillaries (Fig. 1D). In 24-month-old animals laminin immunoreactivity showed an undulating profile, corresponding with the lamina propria folds (Fig. 1E,F).

In seminiferous tubules fibronectin immunoreactivity was found along the lamina propria. In the interstitium the immunoreactivity to fibronectin was observed basically in the blood vessels. In 24-month-old hamsters an increased immunoreactivity was observed. The strongly positive lamina propria of aged animals was enlarged and showed several folds (Fig. 1G). Myoid cells were strongly positive to fibronectin fundamentally in aged animals (Fig. 1G), although positive

immunoreactivity to fibronectin was also observed in myoid cells of young animals.

Ultrastructure

6 months

Young animals showed a normal spermatogenesis in the different stages of the seminiferous epithelium cycle. The Sertoli cells extended from the base of the tubule to enrichen its lumen surrounding the neighboring germ cells. Sertoli cell nuclei were located in the basal zone of the seminiferous epithelium directly adjacent to the basal membrane and they showed a normal ultrastructural pattern. Typically, prominent nucleoli were observed in the indented and irregular nuclei. The cytoplasmic organelles showed their habitual morphology (Fig. 2A).

The basal compartment of the seminiferous epithelium included spermatogonia and early spermatocytes disposed close to the Sertoli cell nucleus

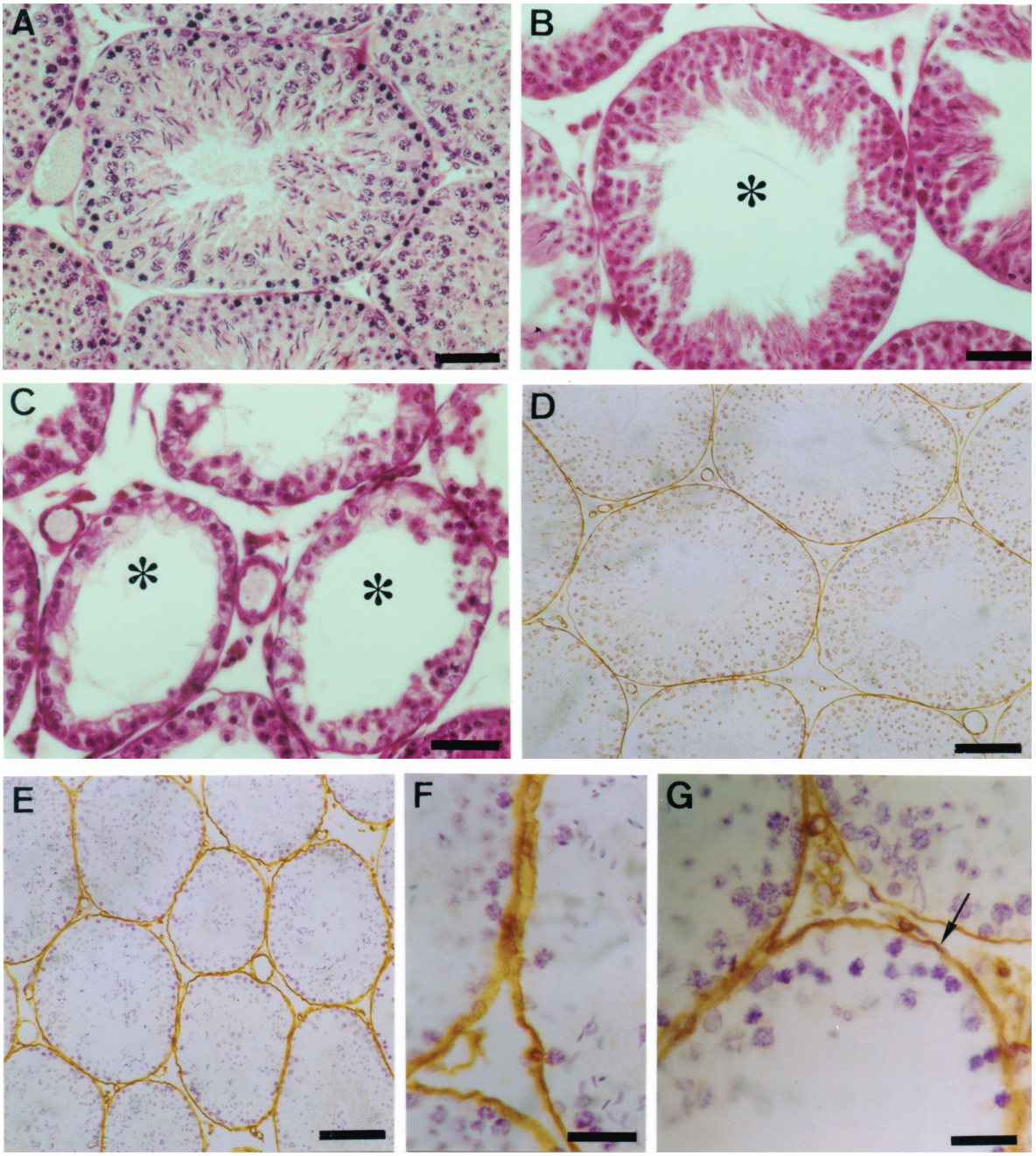

Fig. 1. A. Testis cross section from 6-month-old hamsters stained with hematoxylin and eosin. **(B, C)** Testis cross sections from 24-month-old hamsters showing degenerated tubules (asterisks). Hypospermatogenic tubule **(B)** and arrested maturation tubules **(C)** stained with hematoxylin and eosin. **(D, E and F)** Immunohistochemical demonstration of laminin immunoreactivity in a 6-month-old hamster **(D)** and a 24-month-old hamster **(E, F)**. **G.** Immunohistochemical demonstration of fibronectin in 24-month-old hamsters. Myoid cell (arrow). Scale bars: A, 94 μm: B, 97 μm; C, 71 μm; D, 315 μm; E, 267 μm; F, 100 μm; G, 75 μm.

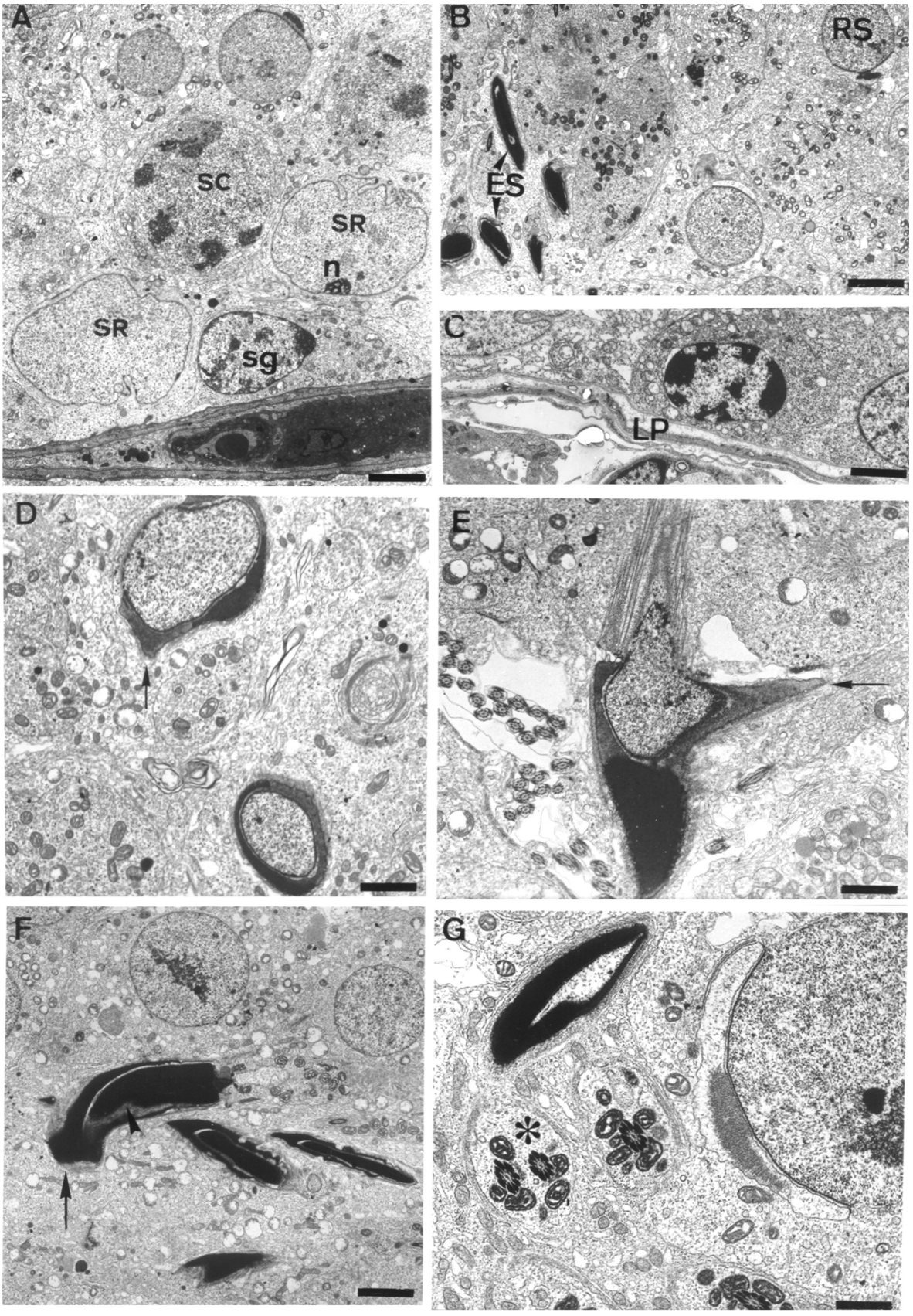

Fig. 2. A, B and C. Representative electron microscopic photographs of seminiferous epithelium from 6-month-old hamsters. **A.** The basal compartment is composed of a Sertoli cell (SR) nucleus showing prominent nucleoli (n), spermatogonia (sg) and spermatocytes (SC). **B.** The adluminal compartment is composed fundamentally of round (RS) and elongated (ES) spermatids. **C.** A typical aspect of lamina propria (LP) in young animals. **D, E, F and G.** Alterations in the spermiogenesis process in 24-month-old hamsters. **D.** Deformation of acrosome (arrow) in an early phase of formation. **E.** Abnormal extension (arrow) of the acrosome of spermatozoa in formation. **F.** Spermatozoa head showing a "mallet" shape (arrow) and an indented nucleus (arrowhead). **G.** Double flagellum surrounded by a single membrane (asterisk). Scale bars: A, B, 4 μm; C, 3.3 μm; D, 2.64 μm; E, 1.8 μm; F, G, 3.33 μm

(Fig. 2A). The adluminal compartment contained the other types of germ cells: late spermatocytes, round and elongated spermatids and spermatozoa (Fig. 2B). The characteristic germ cell associations or stages of seminiferous epithelium cycle were also observed.

The lamina propria of young animals showed a smooth basal membrane along the seminiferous tubule. The single layer of myoid cells was separated from the basal membrane by an electron-lucent layer of extracellular matrix containing multiple collagen fibers of varying orientation. Myoid cells showed an elongated and spindle-shaped nucleus with abundant perinuclear ribosomes and mitochondria. Outside this was another clear layer with some collagen fibers, which separated the myoid cells from the lymphatic endothelium (Fig. 2C).

24 months

Hypospermatogenic tubules. In aged animals the different phases of transformation of the spermatid into spermatozoa were identified both in normal and hypospermatogenic tubules as being similar to control hamsters. Some abnormalities were frequently observed in the ultrastructure of the acrosome and the nucleus of spermatids and subsequently in the spermatozoa head in formation (Fig. 2D-F). Also, both late spermatids and luminal spermatozoa showed multiple flagella surrounded by a single membrane. Ultrastructural alterations were frequently observed in these flagella (Fig. 2G). Hypospermatogenic tubules showed complete spermatogenesis with a decrease in seminiferous epithelium thickness and a relative increase in tubular lumen. In addition, sloughing off of germ cells was observed in some tubules. Sertoli cells showed numerous electron-dense granules of lipofucsin, residual bodies, electron-lucent lipid droplets (Fig. 3A) and intracellular vacuoles. In the lamina propria, the basal membrane showed an undulating profile with many irregular projections towards the seminiferous epithelium (Fig. 3A). The non-cellular inner layer of the extracellular matrix was thickened and displayed abundant fascicles of collagen fibers. Germ cell degeneration was a common process in hypospermatogenic tubules. Degenerated cells, principally spermatocytes and spermatids (Fig. 3B), were characterized by condensation of nuclear chromatin along the perimeter of the nucleus.

Tubules with arrested maturation at spermatocyte and spermatid level. Tubules showing arrested maturation of spermatogenesis were characterized by a seminiferous epithelium containing spermatogonia, spermatocytes, Sertoli cells and a few round spermatids. The Sertoli cell nucleus was reduced in height and sometimes it was displaced from the basal position toward the luminal compartment (Fig. 3C). The Sertoli cell cytoplasm exhibited abundant lipids and vacuoles situated principally at the basal compartment. Degenerating germ cells were also commonly seen throughout the seminiferous tubules which showed arrested maturation. The lamina propria was markedly thickened. The basal membrane displayed large, deep infoldings towards the seminiferous epithelium and the inner non-cellular layer of the connective tissue was enlarged and contained abundant fascicles of collagen fibers.

Tubules with only spermatogonia and Sertoli cells and tubules with only Sertoli cells. Tubules with advanced histological degeneration were composed of spermatogonia and Sertoli cells, and tubules with only Sertoli cells were also observed. Some Sertoli cells lacked the nuclear indentations and displayed a regular outlined nucleus. However, other Sertoli cells conserved the characteristic indented nucleus and the prominent nucleolus. Sertoli cells showed numerous large cytoplasmatic vacuoles (Fig. 3D). The vacuoles showed an irregular profile and were located at different levels of the Sertoli cytoplasm from the basal to the luminal zone. They contained amorphous material similar to that observed in the tubule lumen and were surrounded by smooth endoplasmic reticulum cisternae. Also, abundant lipid droplets were observed (Fig. 3E). The lamina propria was markedly thickened. The nuclei of myoid cells lost the spindle-shaped aspect and were thickened, showing an irregular outline with profound invaginations (Fig. 3E). The basal membrane presented numerous, irregular folds and the fascicles of collagen fibers were conspicuous (Fig. 3F).

Morphometrical results (summarized in Table 1)

From 6 to 24 months, the percentage of normal tubules was reduced and the percentage of hypospermatogenic and arrested maturation tubules was significantly increased with aging. No significant differences were found in the percentage of tubules containing Sertoli cells and spermatogonia or only Sertoli cell tubules between 6 months and 24 months.

No significant differences were found in animal weight, testis weight and testicular volume between young and aged hamsters. The total volume of seminiferous tubules, the tubular diameter, the lumen diameter and the total volume of the seminiferous epithelium was significantly decreased in aged animals with respect to the young group ($P < 0.05$). However, the total length of the seminiferous tubule in aged animals was significantly higher in the aged group compared with the control group ($P < 0.05$) (Table 1).

Both the area and the total volume per testis of immunoreactivity for laminin and fibronectin were significantly higher in aged animals compared with the control group ($P< 0.05$) (Table 1). Significant differences in thickness of the lamina propria were found between controls and each group of histological degeneration of the aged hamsters. Also, in the aged group a significant increase in lamina propria thickness was observed between normal-hypospermatogenic tubules and arrested

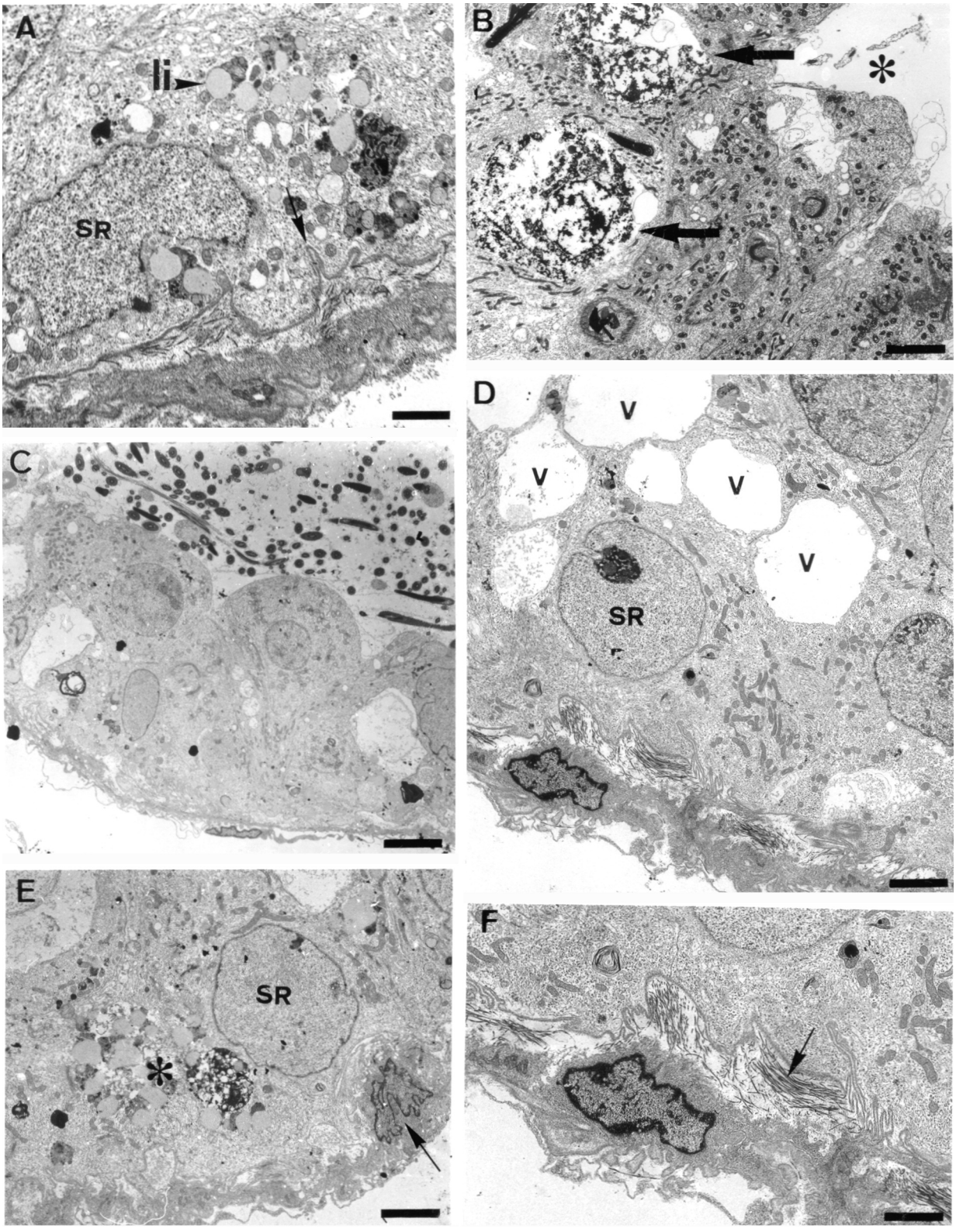

Fig. 3. Representative electron microscopic view of seminiferous tubules from 24-month-old hamsters. **A.** The basal compartment of seminiferous epithelium shows basal lamina infoldings (arrow) and lipid droplets (li) in Sertoli cell (SR) cytoplasm. **B.** Hypospermatogenic zone of seminiferous tubules (asterisk) showing round spermatids in degeneration (arrows). **C.** Tubular cross-section of arrested maturation at spermatocyte level. **(D)** Tubules with only Sertoli cells (SR) are characterized by the presence of numerous, large vacuoles (V). **E.** Sertoli cell cytoplasm contains numerous lipid droplets and residual bodies (asterisk). Also, the nucleus of myoid cells presents an irregular outline (arrow). **F.** In the advanced phase of tubular degeneration the lamina propria is markedly thickened. The basal membrane shows irregular folds and the fascicles of collagen fibers are conspicuous (arrow). Scale bars: A, 2.36 μm; B, 5 μm; C, 6.25 μm; D, 1.93 μm; E, 3.63 μm; F, 1.2 μm.

maturation, but no significant differences were found between arrested maturation and tubules with spermatogonia and Sertoli cells (Table 1).

Discussion

In this study we examined the age-related changes on morphometric parameters of seminiferous tubules, ultrastructural changes in seminiferous epithelium, and the expression of extracellular matrix proteins in the lamina propria of Syrian hamsters.

Our study expands previous observations of aging of the hamster testis by demonstrating that regression of the seminiferous epithelium occurs in a progressive manner (Horn et al., 1996). This process begins with hypospermatogenesis, continues with arrested maturation and finally results in only Sertoli cell tubules. In contrast to other animals, such as the Brown Norway rat, in which the majority of tubules are fully regressed at 24 months (Wright et al., 1993; Levy et al., 1999), 24-month-old hamsters showed a high percentage of normal tubules with an increase in tubules with hypospermatogenesis and arrested maturation.

The quantitative morphological results indicate that the hamster seminiferous tubules suffer a decrease in tubular diameter, lumen diameter and total volume with aging, and that these alterations are accompanied by an increase in the total length of the tubules. The aging testicular regression is also characterized by a decrease in total volume of seminiferous epithelium. All these alterations are in agreement with previous data reported in men (Johnson et al., 1986; Paniagua et al., 1987, 1991) and donkeys (Nipken and Wrobel, 1997) except for the increase in the tubule length observed in aged hamsters. At the present no studies are available on the modifications of seminiferous tubule length with aging in rodents, such as the rat or the mouse. This increase in tubule length with aging in the hamster may be a characteristic of this species or may be a phenomenon which occurs in early phases of testicular aging, since the aged testes of hamsters did not present a complete and generalized regression of seminiferous tubules. The results suggest that aging of seminiferous tubules in the hamster is characterized by a shrinkage of the tubules in the transversal plane but not in the longitudinal plane.

The present study showed that lamina propria thickness was significantly increased in comparison between normal tubules and the different degrees of tubular degeneration observed in aged hamsters. The expansion of the intercellular layers of the matrix

Table 1. Testicular parameters in control (6 months) and aged testes (24 months) of Syrian hamsters.

	CONTROLS TESTES	AGED TESTES
Nº of males	6	7
Animal weight (g)	148.33±15.21	150.28±4.43
Testicular weight (g)	1.84±0.13	1.50±0.09
VT (mm^3) ($x10^3$)	2.21±0.17	1.85±0.08
Seminiferous tubules		
VD_{ST} (%)	87±2	81±5*
V_{ST} (mm3) (10^3)	1.92±0.15	1.50±0.07*
L_{ST} (mm) (10^7)	2.38±0.14	2.87±0.11*
MTD(μm)	320±8.31	257±5.03*
MLD (μm)	157±3.86	141±3.63*
Mean cylinder area (μm^2) ($x10^{10}$)	2.40±0.15	2.31±0.09
VSE (mm^3) ($x10^3$)	1.48±0.10	1.03±0.04*
Percentage of tubular types		
Normal tubules	68.87±4.10	48.25±6.80*
Hypospermatogenic tubules	29.52±3.49	44.55±5.35*
Arrested maturation tubules	1.77±0.77	6.31±2.53*
Tubules with SG and Sertoli cells	0	0.32±0.27
Only Sertoli-cell tubules	0	0.44±0.44
Lamina propria		
VI of laminin (ml)	0.1002±0.0101	0.1715±0.0134*
VI of fibronectin (ml)	0.0983±0.0106	0.1557±0.0089*
Average thickness (μm)		
Normal-hypospermatogenic tubules	0.376±0.013*	0.811±0.032*[a]
Arrested maturation tubules	-	1.777±0.113*[a]
Tubules with SG and Sertoli cells and only Sertoli-cell tubules	-	1.614±0.114*

Values are representing means ± SEM. Abbreviations: VT, testicular volume; VDST, volume density of seminiferous tubules; VST, volume of seminiferous tubules; LST, total length of seminiferous tubules; MTD: mean tubular diameter; MLD, mean lumen diameter; VSE, volume of the seminiferous epithelium; VI, total volume of immunoreactivity; SG: Spermatogonia. *: Significant differences between control group and aged hamsters ($P< 0.05$). [a]: Significant differences ($P<0.0001$).

components of the connective tissue was responsible for the thickness of the lamina propria. In addition, the tubular wall of aged hamsters showed ultrastructural changes.

Previous reports have suggested that the age-related thickening of the boundary tissue results from a reduction both in the length and in the diameter of the seminiferous tubules (Johnson et al., 1986, 1988). However, the thickening of the lamina propria has been observed in tubules prior to the degeneration of seminiferous tubules and the shrinkage of the tubule in aged Norway rats (Richardson et al., 1995) and it has also been associated to functional decline with aging of the blood-testis barrier (Fukuda et al., 2001). In the present study the thickening of the lamina propria was associated to an increase in tubular length. A significant increase in lamina propria thickening was also observed in seminiferous tubules prior to aging degeneration in aged hamsters in normal and hypospermatogenic tubules. Thus, our data suggest that the thickening of the lamina propria is probably not a result of tubular shrinkage but may be due to an increase in synthesis or deposition of extracellular matrix. The lamina propria thickness and the projections of the basal lamina are not specific to the aging process of seminiferous tubules since these alterations have been found in other testicular situations such as varicocele (Santamaria et al., 1992), cryptorchidism (Paniagua et al., 1990) and azoospermia (Gulkesen et al., 2002).

Also, both laminin and fibronectin volume of immunoreactivity per testis were significantly increased in aged testes compared with young testes. Similar results have been reported in atrophied testes after epinephrine treatment (Santamaria et al., 1995) and in azoospermia (Gulkensen et al., 2002). In rats (Hadley and Dym, 1987; Yazama et al., 1997) and men (Davidoff et al., 1990; Gulkesen et al., 2002), the laminin staining has been principally described in the basal membrane of the seminiferous tubules. With aging, laminin immunoreactivity showed an undulating profile, probably corresponding with the basal membrane projections towards the seminiferous epithelium. Laminin is principally produced by Sertoli cells (Skinner et al., 1985; Borland et al., 1986). The peritubular cells in normal testis do not have active laminin synthesis, but through the direct or indirect effect of stimuli deteriorating the testicular structure, the peritubular cells start laminin synthesis (Gulkesen et al., 2002). Therefore, the increase in laminin content in aging may be due to a disregulation in Sertoli cell production or to an active laminin synthesis in the peritubular cells. It has been suggested that peritubular myoid cells can change their immunophenotype and increase the secretion of extracellular matrix components which provokes tubular fibrosis (Santamaria et al., 1995).

Fibronectin has been observed in the outer non-cellular layer of the lamina propria of rats (Hadley and Dym, 1987) and men (Davidoff et al., 1990). Fibronectin has also been detected in peritubular cells (Tung and Fritz, 1984; Gulkesen et al., 2002) and it is well known that fibronectin is secreted by peritubular myoid cells (Tung and Fritz, 1984; Skinner et al., 1985; Hadley and Dym, 1987). Under pathological situations the cells of the lamina propria produce increased levels of extracellular matrix components (Davidoff et al., 1990). The higher volume of immunoreactivity of fibronectin in aged testes may be caused not only by a generally increased production of extracellular connective-tissue material, but also by transformation of the myofibroblasts of the inner layers to fibroblasts during the aging process, as has been proposed by other authors (Arenas et al., 1997; Fukuda et al., 2001). This hypothesis is in concordance with the strong immunoreactivity to fibronectin found in myoid peritubular cells of aged hamsters in comparison with the control group. These cells establish their myoid characteristics only when they continuously interact with extracellular matrix components and with neighboring Sertoli cells, otherwise they appear as fibroblasts (Tung and Fritz, 1986). In addition, the thickening and hyalinization of the lamina propria might hamper the relationship between the Sertoli cells and myofibroblast and contribute to the alterations observed in these cells (Heindel and Treinen, 1989). The present results suggest that the increase in fibronectin and laminin amounts is due to an increased deposition or synthesis during aging of seminiferous tubules that may be related to the transformation of myoid cells into myofibroblast.

In the present study ultrastructural changes with aging have been observed in the seminiferous epithelium, affecting principally the spermiogenic process, especially in the acrosome and flagellum, and Sertoli cells. Previous studies have documented an increase in sperm abnormalities in aged hamsters in the epididymal tract, which include the absence of acrosome, headless spermatozoa and coiled spermatozoa (Calvo et al., 1997). Other abnormalities have also been observed with aging: double-headed spermatozoa; tail thickness; sperm with head alterations; and double axoneme enclosed in the same plasma membrane (Calvo et al., 1997). No modifications of the intermediate piece, neck or head of the spermatozoa in relation with the absence of the acrosome, coiling or loss of the head were observed in the tubular lumen or seminiferous epithelium of aged hamsters, suggesting that these abnormalities are a secondary defect originating in the epididymis (Calvo et al., 1997; Syntin and Robaire, 2001). However, the alterations in the flagellum and acrosome observed in aged hamsters could be a primary defect originating during the spermiogenesis process. The presence of two flagella surrounded by a single membrane have been described in rats (Syntin and Robaire, 2001), and this can reflect on some occasions the so-called " Dag defect" (Blom and Birch-Andersen, 1996) which is related to the "bending or coiling" of the spermatozoa, as has previously been indicated (Calvo et al., 1995). This alteration could also be due to a primary defect which occurs specially in aged hamsters and

which is associated to mitochondrial modifications. This defect corresponds to the multi-tailed spermatozoa described by Nistal et al. (1977). In summary, aging principally affects the process of spermiogenesis in the formation of the acrosome and the tail structure.

Ultrastructural alterations in Sertoli cells in aging include cytoplasm vacuolation and an increase in lipid droplets, lipofucsin and residual bodies. Sertoli cell vacuolation, a non-specific lesion, is a phenomenon often occurring in several different pathological conditions such as Sertoli-cell-only tubules, post-radioactive testicular atrophy, and atrophies caused by drug administration in tumor treatments (Nistal and Paniagua, 1984). It appears before extensive germ cell degeneration, and it is believed to be an early indicator of damage to the Sertoli cell (Russell et al., 1990). In addition, germ cell loss probably determines the development of vacuoles in the Sertoli cells (Paniagua et al., 1991). The progressive accumulation of lipid droplets in aged Sertoli cells has also been described in man (Lynch and Scott, 1950; Paniagua et al., 1987, 1990). It is suggested that lipid accumulation is due to degenerating germ cells which have been phagocytized by Sertoli cells (Paniagua 1987; Sinha Hikim et al., 1988). The numerous residual bodies observed in aged Sertoli cell cytoplasm support this hypothesis. An accumulation of lipofucsin has also been seen in many aged tissues (Sohal and Brunk, 1989; Serre and Robaire, 1998; Calvo et al., 1999). This accumulation may be caused by a decline in the degradation pathways or by a buildup of oxidative stress due to the aging process.

Our study has demonstrated that modifications with aging of the lamina propria of the seminiferous tubule are accompanied by severe changes in the epithelium. As indicated by other authors, the lamina propria plays a part in the maintenance (Davidoff et al., 1990) and differentiation of epithelia (Lustig et al., 2000). An intact lamina propria is necessary for the normal structural and functional integrity of the seminiferous epithelium and its modifications with aging might contribute to epithelium degeneration. However, additional evidence has revealed that the lamina propria is not the sole factor involved, and Sertoli cells may also be involved in the atrophy of the seminiferous epithelium with aging because they are implicated in the maintenance and differentiation of germ cells (Tindall et al., 1985). The loss of pachytene spermatocytes (Horn et al., 1996) and the decrease in proliferative activity together with the increase in apoptosis of germ cells observed in aged degenerative seminiferous tubules of hamsters (Morales et al., 2003) suggests the appearance of modifications to the Sertoli cell-germ cell interactions.

The aging mechanisms of seminiferous tubule damage are unknown, but the present results suggest the participation of the lamina propria. Various hypotheses can be deduced from these results. For example, a primary alteration of the Sertoli cell as responsible for the alterations in the epithelium and lamina propria. Another possibility is that the thickening of the lamina propria may contribute to the observed alterations in Sertoli cells in aged testes and subsequently lead to impairment of spermatogenesis; or perhaps both Sertoli cell damage and the thickening of the lamina propria are independent processes that contribute to the degeneration of the seminiferous epithelium at the same time.

In conclusion, in aged hamster testes, the seminiferous tubules show important structural changes both in the lamina propria and the epithelium. The lamina propria has an increase in extracellular matrix and proteins, and the seminiferous epithelium shows important modifications in Sertoli cells and spermiogenesis.

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References

Arenas M.I., Bethencourt F.R., De Miguel M.P., Fraile B., Romo E. and Paniagua R. (1997). Immunocytochemical and quantitative study of actin, desmin and vimentin in the peritubular cells of the testes from elderly men. J. Reprod. Fertil. 110, 183-93.

Blom E. and Birch-Andersen A. (1996). The ultrastructure of the new hereditary sterlizing defect (the Dag defect) in the bull sperm tail. Proc. Vth. Congr. Fertil. Excerpt. Med. Fdn. Int. Congr. Ser. 133, 602-605.

Borland K., Muffly K.E. and Hall P.F. (1986). Production of components of extracellular matrix by cultured rat Sertoli cells. Biol. Reprod. 35, 997-1008.

Bustos-Obregón E. (1976). Ultrastructure and function of the lamina propria of mammalian seminiferous tubules. Andrologia 8, 179-185.

Burgos M.H., Vitale-Calpe R. and Aoki A. (1970). Fine structure of the testis and its functional significance. In: The testis. Johnson A.D., Gomes W.R. and Van Demark N.L. (eds). Academic Press. New York. pp 551-649.

Calvo A., Pastor L.M., Gallego-Huidobro J., Horn R. and Pallares J. (1995). Abnormal spermatozoa in the cauda epididymidis of adult and aged hamsters (*Mesocricetus auratus*): a study by electron microscopy. Acta Anat. 154, 186-195.

Calvo A., Martinez E., Pastor L.M., Vazquez J.M. and Roca J. (1997). Classification and quantification of abnormal sperm along the epididymal tract. Comparison between adult and aged hamsters. Reprod. Nutr. Dev. 37, 661-73.

Calvo A., Pastor L.M., Martinez E., Vazquez J.M. and Roca J. (1999). Age-related changes in the hamster epididymis. Anat. Rec. 256, 335-346.

Davidoff M.S., Breucker H., Holstein A.F. and Seidl K. (1990). Cellular architecture of the lamina propria of human seminiferous tubules. Cell Tissue Res. 262, 253-261.

Fawcett D.W., Leak L.V. and Heidger P.M. (1970). Electron microscopic observations on the structural components of the blood-testis

barrier. J. Reprod. Fertil. 10, 105-122.

Fukuda T., Kikuchi M., Kurotaki T., Oyamada T., Yoshikawa H. and Yoshikawa T. (2001). Age-related changes in the testes of horses. Equine Vet. J. 3, 20-25.

Gulkensen K.H., Erdogru T., Sargin C.F. and Karpuzoglu G. (2002). Expression of extracellular matrix proteins and vimentin in testes of azoospermic man: an immunohistochemical and morphometrical study. Asian J. Androl. 4, 55-60.

Hadley M.A. and Dym M. (1987). Immunocytochemistry of extracellular matrix in the lamina propria of the rat testis: electron microscopic localization. Biol. Reprod. 37, 1283-1289.

Heindel J.J. and Treinen K.A. (1989). Physiology of the male reproductive system: endocrine, paracrine and autocrine regulation. Toxicol. Pathol. 17, 411-445.

Horn R., Pastor L.M., Moreno E., Calvo A., Canteras M. and Pallares J. (1996). Morphological and morphometric study of early changes in the ageing golden hamster testis. J. Anat. 188, 109-117.

Johnson L., Petty C.S. and Neaves W.B. (1986). Age-related variation in seminiferous tubules in men. A stereologic evaluation. J. Androl. 7, 316-22.

Johnson L., Abdo J.G., Petty C.S. and Neaves W.B. (1988). Effect of age on the composition of seminiferous tubular boundary tissue and on the volume of each component in humans. Fertil. Steril. 49, 1045-1051.

Levy S., Serre V., Hermo L. and Robaire B. (1999). The effects of aging on the seminiferous epithelium and the blood-testis barrier of the Brown Norway rat. J. Androl. 20, 356-365.

Lustig L., Denduchis B., Ponzio R., Lauzon M. and Pelletier R.M. (2000). Passive immunization with anti-laminin immunoglobulin G modifies the integrity of the seminiferous epithelium and induces arrest of spermatogenesis in the guinea pig. Biol. Reprod. 62, 1505-1514.

Lynch K.M. and Scott W.W. (1950). The lipid content of the Leydig cell and Sertoli cell in the human testis as related to age, benign prostatic hyperplasia, and prostatic cancer. J. Urol. 64, 767-776.

Morales E., Pastor L.M., Horn H., Zuasti A., Ferrer C., Calvo A., Santamaría L. and Canteras M. (2003). Effect of aging on the proliferation and apoptosis of testicular germ cells in the Syrian hamster *Mesocricetus auratus*. Reprod. Fert. Dev., 15, 89-98.

Nipken C. and Wrobel K.H. (1997). A quantitative morphological study of age-related changes in the donkey testis in the period between puberty and senium. Andrologia 29, 149-161.

Nistal M., Paniagua R. and Herruzo A. (1977). Multi-tailed spermatozoa in a case with asthenospermia and teratospermia. Virchows Arch. B Cell Pathol. 30, 111-118.

Nistal M. and Paniagua R. (1984). Infertility in adult males with retractile testes. Fertil. Steril. 41, 395-403.

Paniagua R., Amat P., Nistal M. and Martin A. (1986). Ultrastructure of Leydig cells in human ageing testes. J. Anat. 146, 173-183.

Paniagua R., Nistal M., Amat P., Rodriguez M.C. and Martin A. (1987). Seminiferous tubule involution in elderly men. Biol. Reprod. 36, 939-947.

Paniagua R., Martinez-Onsurbe P., Santamaria L., Saez F.J., Amat P. and Nistal M. (1990). Quantitative and ultrastructural alterations in the lamina propria and Sertoli cells in human cryptorchid testes. Int. J. Androl. 13, 470-487.

Paniagua R., Nistal M., Saez F.J. and Fraile B. (1991). Ultrastructure of the aging human testis. J. Electron. Microsc. Tech. 19, 241-260.

Prijono H. and Schirren U. (1985). Importance of the tubular diameter in testicular biopsy of oligozoospermia. Andrologia 17, 541-545.

Richardson L.L., Kleinman H.K. and Dym M. (1995). The effects of aging on basement membrane in the testis. J. Androl. 16, 118-126.

Russell L.D., Ettlin R.A., Sinha-Hikim A.P. and Clegg E.D. (1990). Mammalian spermatogenesis. In: Histological and histopathological evaluation of the testis. Russell L.D. and Griswold M.D. (eds.). Cache River Press. Clearwater, FL. pp 1-38.

Santamaria L., Martin R., Nistal M. and Paniagua R. (1992). The peritubular myoid cells in the testes from men with varicocele: an ultrastructural, immunohistochemical and quantitative study. Histopathology 21, 423-433.

Santamaria L., Martin R., Codesal J. and Paniagua R. (1995). Myoid cell proliferation in rat seminiferous tubules after ischaemic testicular atrophy induced by epinephrine. Morphometric and immunohistochemical (bromo-deoxyuridine and PCNA) studies. Int. J. Androl. 18, 13-22.

Serre V. and Robaire B. (1998). Segment-specific morphological changes in aging Brown Norway rat epididymis. Biol. Reprod. 58, 497-513.

Sinha Hikim A.P., Bartke A. and Russell L.D. (1988). Morphometric studies on hamster testes in gonadally active and inactive states: light microscope findings. Biol. Reprod. 39, 1225-1237.

Skinner M.K., Tung P.S. and Fritz I.B. (1985). Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. J. Cell. Biol. 100, 1941-1947.

Söderstrom K.O. (1986). Tubular hyalinization in human testis. Andrologia 18, 97-103.

Sohal R.S. and Brunk U.T. (1989). Lipofuscin as an indicator of oxidative stress and aging. Adv. Exp. Med. Biol. 266, 17-26.

Swanson L.J., Desjardins C. and Turek F.W. (1982). Aging of the reproductive system in the male hamster: behavioral and endocrine patterns. Biol. Reprod. 26, 791-799.

Syntin P. and Robaire B. (2001). Sperm structural and motility changes during aging in the Brown Norway rat. J. Androl. 22, 235-244.

Tindall D.J., Rowley D.R., Murthy L., Lipshultz L.I. and Chang C.H. (1985). Structure and biochemistry of the Sertoli cell. Int. Rev. Cytol. 94, 127-49.

Tung P.S. and Fritz I.B. (1984). Extracellular matrix promotes rat Sertoli cell histotypic expression in vitro. Biol. Reprod. 30, 213-229.

Tung P.S. and Fritz I.B. (1986). Extracellular matrix components and testicular peritubular cells influence the rate and pattern of Sertoli cell migration in vitro. Dev. Biol. 113, 119-134.

Wing T-Y. and Christensen A.K. (1982). Morphometric studies on rat seminiferous tubules Am. J. Anat. 165, 13-35.

Wright W.W., Fiore C. and Zirkin B.R. (1993). The effect of aging on the seminiferous epithelium of the Brown Norway rat. J. Androl. 14, 110-117.

Yazama F., Esaki M. and Sawada H. (1997). Immunocytochemistry of extracellular matrix components in the rat seminiferous tubule: electron microscopic localization with improved methodology. Anat. Rec. 248, 51-62.

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