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Distribution of Keratins, Vimentin, and Actin in the Testis of Two South American Camelids: Vicuna *(Vicugna vicugna)* and Llama *(Lama glama)*. An Immunohistochemical Study

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ABSTRACT

The purpose of the present study was to investigate the pattern of distribution of cytokeratins, vimentin and muscular actin in the testis of vicuna *(Vicugna vicugna)* and llama *(Lama glama)* two species of camelids native of the Andean high plateau of South America.

Testicular biopsies of four vicunas and five llamas were used. Animals were healthy breeders. The tissues were processed by standard immunohistochemistry with antipancytokeratin AE1/AE3, antikeratin 18 (K 18), CAM 5.2 (antikeratin 5, 18, and 19), antivimentin, and smooth-muscle-specific antiactin antibodies to track the cytoskeletal pattern of testicular cells. Using AE1/AE3 antibody the immunostaining was found in the epithelial lining of tubuli recti and rete testis. The reaction was relatively stronger in the apical cytoplasm of epithelial cells. The testicular cells of the two species showed no reaction to K 18 and CAM 5.2 antibodies. Antivimentin antibody stained the basal cytoplasm of the Sertoli cells, the Leydig cells, and the epithelial lining of tubuli recti and rete testis. In the last two structures the immunostain was relatively more intense in the basal cytoplasm of epithelial cells. Antiactin antibody stained the peritubular cells and the muscle cells of the lamina propria of tubuli recti and rete testis. The presence in these species of only some keratins found in man, its coexpression with vimentin in epithelial lining of tubuli recti and rete testis and the peritubule organization, so different from other ungulates may reflect a differential adaptation of the cytoskeleton to particular reproductive strategies. Anat Rec 254:330–335, 1999. © 1999 Wiley-Liss, Inc.

Key words: camelids; testis; keratin; vimentin; actin

Vicuna (*Vicugna vicugna*) and llama (*Lama glama*) are two species of South American camelids inhabiting the Andean plateau over 3,500 m. of altitude. Though the llama plays an important economic role as domestic animal for the Andean natives, many facets of its reproductive biology, mostly in the male, are not known. The situation is even worse regarding the male vicuna, a wild camelid producer of high quality wool.

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There are basic descriptions of the testicular anatomy and histology in the llama (for review see Smith et al., 1994) and of its spermatogenesis (Dhelon and von Lawzewitsch, 1987) and also of spermatogenesis and seasonal variations of histological and endocrine parameters in the vicuna (Urquieta et al., 1994; Bustos-Obregón et al., 1997).

In laboratory animals and man, the presence of cytokeratins in the rete testis (Achtstätter et al., 1985; Miettinen et al., 1985; Ramaekers et al., 1985; Feitz et al., 1987; Dinges et al., 1991) and in the tubuli recti (Miettinen et al., 1995) has been demonstrated. Vimentin has been detected in Sertoli cells (Franke et al., 1979; von Vorstenbosch et al., 1984; Achtstätter et al., 1985; Miettinen et al., 1985; Rogatsch et al., 1996), testicular interstitial cells (Achtstätter et al., 1985; Miettinen et al., 1985), rete testis (Ramaekers et al., 1985; Feitz et al., 1987; Dinges et al., 1991), and tumors derived from seminiferous epithelium (Miettinen et al., 1985; Rogatsch et al., 1996). Moreover, smooth muscle specific actin has been found in the peritubular cells (Davidoff et al., 1990; Paranko and Pelliniemi, 1992; Cigorraga et al., 1994). However, in our understanding, no such observations exist for the testis of llama or vicuna.

Therefore, the purpose of the present work was to analyze the pattern of distribution of cytoskeletal keratins, vimentin and muscular actin in Sertoli cells, peritubule, interstitium, tubuli recti, and rete testis in the testis of fertile llama and vicuna, using immunocytochemistry as a tool for better understanding the reproductive histophysiology of these species.

MATERIALS AND METHODS

Materials

Four vicunas and five llamas, mature and healthy, were employed in these studies. The animals were captured and kept in pens at Lauca National Park, in the first Region of Chile, at an altitude of 4,470 m. and were fed lucerne hay and water ad libitum. Microscopic analyses of testes were performed on surgically obtained biopsies. Accidentally, some llama's biopsies included a little zone of rete testis. The animals were sedated and anesthetized prior to surgery. Xilacine hydrochloride (Rompun, Bayer; 1 mg kg^{-1}) and ketamine hydrochloride (Vetejara, Parke-Davis, Ann Arbor, MI; 5 mg kg^{-1}) were injected intravenously via the jugular vein and lidocaine hydrochloride was injected into the scrotum. The condition for selection of material was that the testes showed a normal histological pattern, including full spermatogenesis.

Immunohistochemistry

The testicular samples were immediately fixed in Dubosq-Brasil for 4 hr and embedded in paraffin. Paraffin embedded blocks were cut into sections of 5 um thick, which were processed following avidin-biotin-peroxidase complex (ABC) method for immunohistochemical studies (Hsu et al., 1981). The primary antibodies used for these studies and the optimum dilutions were: 1) Antibody to human broad-spectrum keratin (AE1/AE3), which stains keratins Nos. 1–8, 10, 13–15, and 19, (Hybritech, Liege, Belgium), at 1/200; 2) Antibody to human keratin 18 (K 18; Biomeda Corp., Foster City, CA) at 1/200; 3) CAM 5.2 antikeratin antibody, which stains keratins Nos. 5, 18, and 19 (Biomeda Corp.) at 1/200; 4) Antibody to vimentin, (Dakopatts, Glostrup, Denmark), at 1/100; 5) Antibody to smooth-muscle-specific actin (Biomeda Corp.) at 1/200. All antibodies were raised in mice. Each antibody was assayed in at least three sections of each testicle from each individual. To insure specificity of the immunoreactions, adjacent control sections were subjected to the same immunohistochemical method with the exception that the primary antibodies were replaced by nonimmune serum (Dako).

Briefly, the method is as follows: After deparaffination, sections were hydrated and incubated for 20 min in 0.3% H_2O_2 in PBS to reduce endogenous peroxidase activity. Sections were then digested with either 0.1% trypsin (Merck, Darmstadt, Germany) in 0.01 M phosphate-buffered saline (pH 7.6) for 15 min at 37°C, or in 0.1% pepsin (Sigma, St. Louis, MO) in 0.5 M acetic acid for 20 min at 37°C to enhance antigenic exposure, and incubated overnight at 4°C with the primary antibodies (diluted in PBS containing 1% bovine serum albumin). After incubation with primary antibodies (diluted in PBS containing 1% bovine serum albumin), sections were washed twice in PBS to remove unbound primary antibodies and then incubated with horse antimouse biotinylated immunoglobulin (Biocell, Cardiff, UK) at 1:100 in 20% human serum-PBS buffer, pH 7.6. After 1 hr of incubation with the second antibody, the sections were incubated with the avidin-biotin peroxidase complex (Dako), at 1/200 dilution, for 30 min at room temperature, and developed with diaminobenzidine (Sigma; 30 mg dissolved in 10 ml Tris-HCl buffer, pH 7.4, containing 0.015% H_2O_2). Thereafter, the sections were counterstained with Harris's haematoxylin, dehydrated in ethanol, and mounted in DePex (Probus, Badalona, Spain). As positive control for the immunohistochemical studies, sections from human biopsies of skin, mammary gland, and small intestine were stained simultaneously with the testicular sections of llamas and vicunas.

To quantify the intensity of the immunostain with each antibody, a Nikon (LABOPHOT-2, Nikon Corp.) microscope equipped with a variolume to guarantee even illumination of the field and a COHU, model 4810–5000 video camera (COHU, Inc. Electronics Div.) with manual gain setting were used to obtain images of each section. The video images were digitized by an Internal Tape Back up System (Conner Peripherals, Inc.) image processor, linked to a Tower System 486 DX2 computer. Digital images 512 $\times$ 512 $\times$ 8 bits, were made of each tissue section using the same light intensity and gain settings to assure standardization of images (Brinkley and Morris-Wiman, 1987). Gray values ranged from white (0) to black (255). Two different images were made of each tissue section using a narrow-band-width filter (80A° Optiligth, Cokin, Photo Video Accessories, France) of wavelengths at or near the maximum absorbance for each dye product. A 580 $\pm$ 5 nm filter was used to intensify the visualization of immunostaining. To process the images, a commercial software (PHOTO DE LUXE of U-Lead Systems, Inc., Taiwan) was used. A histogram of the gray levels in each image was obtained, a six-step gray scale produced and colors assigned to each gray value. Computerized analysis of the digitized pictures and the measurements were performed using the software MORPHOMETRY, BI-DIM (Bozzo and Retamal, 1991). Densitometric evaluation was expressed in arbitrary units. The values obtained in the extracellular matrix of the testicular interstitium, referred to in this work as "background," were designated as "control of intensity of immunostaining."

Statistics

The mean ± S.D. for the densitometric evaluation in the testicles were obtained. Two-way analysis of variance tests (Sokal and Rohlf, 1969) were performed to evaluate significant differences for different testicular tissues, treatments, and species.

RESULTS

The testis of llama and vicuna has the same general histological traits common to most mammals. Seminiferous tubules have Sertoli cells (easy to identify by their irregular nuclei and conspicuous nucleoli) and the different cellular components of the germinal line from spermatogonia up to elongated spermatids. The peritubule is formed by the thin basal membrane and one layer of flattened myoid cells. The interstitium is made up of loose connective tissue and groups of Leydig cells plus numerous small blood vessels (Fig. 1). The rete testis of llama is a rather large structure. It consists of anastomotic channels lined by a cuboidal epithelium lying on a well developed lamina propria. Near to the rete testis, some tubuli recti, stroma of septula testis, and seminiferous tubules are found (Figs. 2 and 3).

AE1/AE3 antikeratin antibody elicited a marked immunostaining in the epithelium of tubuli recti and rete testis (Figs. 3 and 4). In the epithelial cells of the rete testis the immunostaining was significantly greater ($P \ll 0.01$) in the apical region (whose digitized value in arbitrary densitometric units was 84.5 ± 2.4) than in the basal region, where this value was 74.0 ± 4.0. The seminiferous epithelium, peritubule and testicular interstitium did not react with this antibody (Table 1). No histological structure of the testis was positive to the antikeratin antibodies 18 (K 18) and CAM 5.2 (that stains keratins 5, 18, and 19).

The antivimentin immunostain was intensively positive in the basal cytoplasm and lateral faces of the Sertoli cells of llama (Fig. 5) and of vicuna (Fig. 6), in the epithelial lining of the tubuli recti and of the rete testis (Fig. 7). Leydig cells showed a positive weak reaction with the antivimentin antibody (Fig. 8). In the rete testis, the whole epithelium was stained, but the immunoreaction was relatively more intense in the basal region of the epithelium. The adluminal cytoplasm of Sertoli cells and the germinal cells did not react with this antibody (Figs. 5 and 6). Muscular antiactin antibody showed immunostaining in the peritubular cells of llama (Fig. 9) and of vicuna (Fig. 10) and in muscular cells of the lamina propria of tubuli recti and rete testis of llama (Figs. 11 and 12).

No positive staining was observed in the negative controls of immunostains for different antibodies employed in this work (Fig. 2; See Materials and Methods).

Table 1 shows the values of the relative intensity of the immunostaining for the different testicular tissues that react to the antibodies. These tissues showed a degree of immunoreaction significantly higher to the background ($P \ll 0.01$ for most cases), whose digitized mean value in arbitrary densitometric units was 30.6 ± 5.0 (See Materials and Methods). In general, no significant interspecific differences were found in the degree of the immunostaining when homologous tissues of both species were compared. In llama, the intensity of antivimentin immunostain was significantly greater in Sertoli cells, than in the Leydig cells ($P < 0.01$), tubuli recti ($P < 0.01$), or rete testis ($P \ll 0.01$). Additionally, the antivimentin immunostain was significantly higher in tubuli recti than in rete testis epithelial cells ($P \ll 0.01$). The other antibodies did not reveal significant differences among different testicular tissues.

DISCUSSION

The present immunohistochemical study demonstrates a pattern of localization of cytoskeletal proteins, such as keratin, vimentin and muscular actin similar in the testis of llama and vicuna. Vimentin is found in Sertoli cells, Leydig cells, and in the epithelium of tubuli recti and rete testis. The latter two structures also have keratin. Actin is found in the peritubule and in the lamina propria of tubuli recti and rete testis. The use of the monoclonal antibody AE1/AE3 (that includes keratins Nos. 1–8, 10, 13–15, and 19), allows detection of keratin in tubuli recti and rete testis of llama, which is absent in the seminiferous epithelium, peritubule, and testicular interstitium, in agreement with similar results reported by other authors (Achtstätter et al., 1985; Miettinen et al., 1985; Dinges et al., 1991). These authors communicated the presence of keratins Nos. 7, 8, 18, and 19 in the human rete testis. These keratins have also been found in most anatomical segments of the human genital tract (Achtstätter et al., 1985; Palacios et al., 1993; Regadera et al., 1993, 1997), making up what seems to be an immunohistochemical pattern of keratins that is characteristic of the human reproductive tract. In addition to the antikeratin complex AE1/AE3, the antikeratin antibodies Nos. 18 (K 18) and CAM 5.2 (which stains keratins Nos. 5, 18, and 19) were used in this work. These antibodies fail to reveal the presence of keratins Nos. 5, 18, and 19 in the testis. This can be due, as proposed by Achtstätter et al. (1985), to the characteristic of the technique that may elicit a weak reactivity of the antibodies used or to the fact that the material was fixed in

Fig. 1. Testis of vicuna showing a seminiferous tubule surrounded by the peritubule and interstitium. Hematoxylin-eosin stain. ×200.

Fig. 2. Immunohistochemical demonstration of AE1/AE3 keratin complex in rete testis epithelial lining of llama (arrows). Negative control in which the primary antigen was omitted. No immunostaining is observed. L, lumen; asterisk, stroma. Compare with Figure 3. ×200.

Fig. 3. Immunohistochemical demonstration of AE1/AE3 keratin complex in rete testis epithelial lining of llama. The immunoreaction is intense in the apical cytoplasm of the cells (arrows). L, lumen; asterisk, stroma. ×200.

Fig. 4. Immunohistochemical demonstration of AE1/AE3 keratin complex in tubuli recti epithelial lining of llama (arrows). ×200.

Fig. 5. Immunohistochemical demonstration of vimentin in the seminiferous tubules of the testis of llama. The immunoreaction stained the basal cytoplasm and the lateral wall of the Sertoli cells. ×200.

Fig. 6. Immunohistochemical demonstration of vimentin in the basal cytoplasm of Sertoli cells of the testis of vicuna (arrows). ×200.

Fig. 7. Immunohistochemical demonstration of vimentin in tubuli recti epithelial lining of the testis of llama (arrow). ×200.

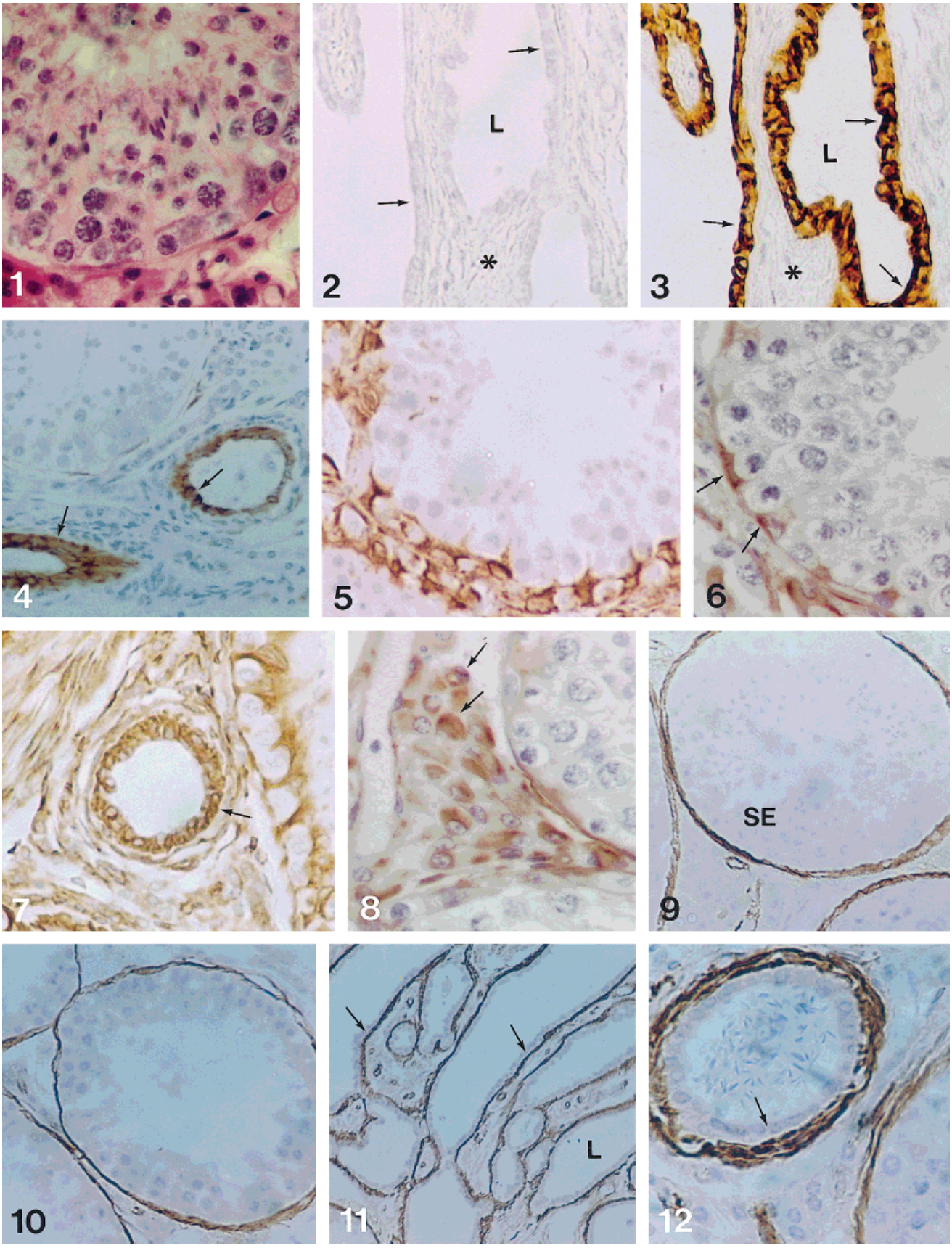
Fig. 8. Immunohistochemical demonstration of vimentin in Leydig cells (arrows) of the testis of vicuna. ×200.

Fig. 9. Immunohistochemical demonstration of muscular actin in the peritubule of the testis of the llama. SE, seminiferous epithelium. ×100.

Fig. 10. Immunohistochemical demonstration of muscular actin in the peritubule of the testis of vicuna. ×100.

Fig. 11. Immunohistochemical demonstration of muscular actin in the rete testis lamina propria of llama testis. Epithelial lining (arrows); L, lumen. ×100.

Fig. 12. Immunohistochemical demonstration of muscular actin in tubuli recti lamina propria of llama testis. Epithelial lining (arrows). ×400.



Figures 1–12.

TABLE 1. Computational densitometry of immunostaining of keratins, vimentin, and actin in different testicular cells (in arbitrary units; mean ± S.D.)†

	Sertoli Cells		Peritubular Cells		Leydig Cells		Tubuli Recti	Rete Testis
Antigens Detected	llama	vicuna	llama	vicuna	llama	vicuna	llama	llama
AE1/AE3 (keratins 1–8, 10, 13–15, 19)	—	—	—	—	—	—	79.0 ± 4.2	79.2 ± 6.3
K18 (keratin 18)	—	—	—	—	—	—		
CAM 5.2 (keratins 5, 18, 19)	—	—	—	—	—	—		
Vimentin	81.7 ± 6.6*,**	63.5 ± 12.7	—	—	60.3 ± 6.8**	60.0 ± 9.1	62.0 ± 0.6*,**	51.5 ± 1.3*
Muscular actin	—	—	71.0 ± 8.8	79.3 ± 2.7	—	—	81.5 ± 7.8	76.1 ± 4.0[a]

†The results are given in absolute values (n = 6). Values with the same superscripts are significantly different at $*P \ll 0.01$; $**P < 0.01$. Other comparisons n.s. Statistical significance was taken at the 99% limit. Two-way analysis of variance.
[a]Values referred to muscular cells of the lamina propria.

formalin, a fixation that may alter the antigenic determinants for cytokeratins. Alternatively, it is possible that the presence in these species of only some of the cytokeratins found in man represents a pattern specific for South American camelids. This situation may correspond to an adaptation of the cytoskeleton to a different reproductive strategy.

In the present work, an intense antivimentin stain was found in the Sertoli cell. Similarly, vimentin has been detected in Sertoli cells of other mammals, including man, by the use of biochemical or immunochemical techniques (Franke et al., 1979; Spruill et al., 1983; von Vorstenbosch et al., 1984; Achtstätter et al., 1985; Miettinen et al., 1985; Davidoff et al., 1990; Rogatsch et al., 1996). The role of vimentin in Sertoli cells is not known.

It is generally agreed that the intermediate filaments provide mechanical stability to the cells (Lazarides, 1988; Janmey, 1991) due to the fibrous nature of their molecules. The high mechanical resistance of vimentin filaments (Janmey, 1991), their spatial distribution in the cytoplasm, frequently anchored to the nuclear membrane in one side and to the cell membrane in the other (Georgatos et al., 1985; Lazarides, 1988; Albers and Fuchs, 1992) and their capacity to polymerize and depolymerize according to the functional needs of the cell, has been proven *in vitro* and *in vivo* (Goldman et al., 1996). All these observations suggest that vimentin may play a significant role in the spatial organization of the Sertoli cells, thus allowing the change of form of Sertoli cells that is needed for the displacement of germinal cells and the opening and closing of the inter-Sertoli tight junctions (a major component of the blood-testicular barrier; Gigula et al., 1976; Meyer et al., 1996).

A variety of mesenchyme-derived cells, including fibroblasts, muscle cells, chondroblasts, microglial cells, and endothelial cells, contain vimentin filaments (Lazarides, 1980). This property seems to be a general characteristic of mesenchyme-derived cells. The presence of vimentin in Sertoli cells, tubuli recti and rete testis in the testis of vicuna and llama confirms the mesodermic origin postulated for these structures (Byskov and Hoyer, 1988).

In the present work it was found that the same epithelial cells of tubuli recti and rete testis that show keratin staining also contain vimentin, thus constituting a unique immunohistochemical model, where two polypeptides from different families of intermediate filaments are expressed in the same cell. The information available on the coexpression of these two families of intermediate filaments in the rete testis is controversial. Some authors have reported the presence of both types of filaments in the human rete testis (Ramaekers et al., 1985; Feitz et al., 1987; Dinges et al., 1991). Other researchers have found only keratin (Achtstätter et al., 1985; Miettinen et al., 1985). Accurate identification of the intermediate filaments present in the histological structures of the testis (including rete testis, which is the origin of a rare carcinoma (Dinges et al., 1991), is of relevance for the histopathological diagnosis of testicular neoplasia.

Actin is the predominant protein in the muscle cells of genital tract, which give a strong positive immunoreaction to the muscle antiactin antibody (Davidoff et al., 1990; Paranko and Prelliniemi, 1992; Palacios et al., 1993; Cigorraga et al., 1994; Regadera et al., 1997). Such a reaction was found in this work in the peritubule and lamina propria of the tubuli recti and rete testis. The peritubule was found to be made of a fine basal membrane and one layer of myoid-like cells, following a structural pattern similar to that of rodents (Bustos-Obregón, 1976). The meaning of this pattern in South American camelids, so different to the organization of the peritubule in other ungulates (Bustos-Obregón, 1976) is not known. It may be speculated that a thinner peritubule and the large number of hyperemic blood vessels seen in the interstitium may represent structural adaptations to facilitate metabolic exchange in the testis for animals adapted to live at high altitude. This presumption is based on the postulate that the peritubular structure is related to testicular metabolism (Bustos-Obregón, 1976), as well as to other conditions, such as testicular pathologies that may alter the regulatory exchanges of the tubules and the testicular interstitium (Davidoff et al., 1990).

The immunostaining with muscle antiactin in tubuli recti and rete testis was seen as a continuous circular sheath underlying the epithelium, indicating the presence of a tissue rich in smooth muscle fibers or other cells with contractile ability, thus confirming previous observations in other mammals, such as rabbit (Jones et al., 1979), bull, ram, and rat (Osman and Plöen, 1978), and also man (Bustos-Obregón and Holstein, 1976; Roosen-Runge and Holstein, 1978). All these observations indicated the presence of a fibromuscular tissue at this site. Moreover, Singh and Bharawaj (1980) described the presence of a circular layer of smooth muscle around the ductuli efferentes in the camel. Based on all these observations we postulate the

presence of a fibromuscular lamina propria that extends from the seminiferous tubules to the epididymis in the camelids. Its contractile activity may help the displacement of spermatozoa in the initial portion of the seminal ducts.

As was described above, the similar pattern of localization exhibited by the antibodies studied in testes of vicuna and llama, suggests a common evolutionary origin for both species and probably similar reproductive strategies.

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