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Research Article

Morphometric Study and Immunolocalization of Androgen Receptors in Epididymis During Postnatal Development in D'Man Lamb Reared Under Arid Environment in Algeria

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Abstract

The aim of this study was to examine the morphometry and the immunolocalization of androgen receptors in the epididymis of D'Man lamb during postnatal development. The epididymis was collected at the slaughtering lamb, aged of 2 to 5 month. The weight of the epididymis increased with a significant difference at 3 months. The tubular diameter of the corpus and the cauda epididymal tubules increased respectively from 2 till 4 months, and from 2 till 5 months. The luminal diameters of the caput epididymal tubules increased significantly at 3 months. The luminal diameters of the corpus and cauda epididymal tubules increased significantly from 3 and 4 months respectively. The epithelial height of the caput and cauda epididymal tubules increased significatively at 5 months. The epithelial height of the corpus epididymal tubules increased significantly at 3 months. The androgen receptor immunostaining was localized in nuclei and cytoplasm of epithelial cells, smooth muscle cells and in the cytoplasm of interstitial cells of the epididymis at each age. In conclusion, both the morphometric changes and androgen receptors immunolocalization during the postnatal development of epididymis indicated the necessity of androgens for postnatal differentiation and maintaining the structure of the epididymis.

Keywords: *Epididymis, Morphometry, Androgen receptor, Immunohistochemistry, Postnatal development, D'Man lamb*

Cezayir'in Kurak İkliminde Yetiştirilen D'Man Kuzularinda Postnatal Gelişim Süresince Epididimiste Androjen Reseptörlerinin İmmunolokalizasyonu ve Morfometrik Bir Çalışma

Özet

Bu çalışmanın amacı D'Man kuzularında postnatal gelişim süresince epididimiste androjen reseptörlerinin immunolokalizasyonunu ve morfometrisini araştırmaktır. 2 ile 5 ay arasında yaşları değişen kuzulardan kesim sonrasında epididimisler toplandı. Epididymis ağırlığı 3 aylık olanlarda anlamlı oranda farklı bulundu. Corpus ve cauda epididimal tüplerin tubular çapları sırasıyla 2'den 4 aylığa ve 2'den 5 aylığa kadar olanlarda artma gösterdi. Caput epididimal tüplerin luminal çapları 3 aylıklarda anlamlı oranda arttı. Corpus ve cauda epididimal tüplerin luminal çapları sırasıyla 3 ve 4 aylıklarda anlamlı oranda arttı. Caput ve cauda epididimal tüplerin epitel yükseklikleri 5 aylıklarda anlamlı oranda artış gösterdi. Corpus epididimal tüplerin epitel yükseklikleri 3 aylıklarda anlamlı oranda artış gösterdi. Androjen reseptör immunboyanması tüm yaş gruplarında epididimisin epitel hücrelerinin çekirdek ve sitoplazmalarında, düz kas hücrelerinde ve intersitisyel hücrelerinin sitoplazmasında lokalize oldu. Sonuç olarak; morfometrik değişimler ve androjen reseptör immunolokalizasyonu epididimisin postnatal gelişimi ve oluşumu süresince androjenlerin gerekli olduğunu göstermiştir.

Anahtar sözcükler: *Epididimis, Morfometri, Androjen reseptör, İmmunohistokimya, Postnatal gelişim, D'Man kuzu*

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INTRODUCTION

Sheep is an important part of the Algerian agricultural economy. D'Man and other second breeds (Hamra, Barbarine, Sidahou, Tazegzawt) represent less than 1% of the Algerian sheep population. D'Man breed acquires its importance from its exceptional reproductive performances and its high adaptation to the oasian environment [1]. The productivity was consistently higher due to generally high fertility and prolificacy [2] with an early onset of puberty at 3 months [3].

The morphohistological change is essential to gain a comprehensive knowledge on the reproductive physiology of epididymis [4,5]. Indeed, epididymal functions can be divided into several general categories: concentration of sperm; functional maturation; storage in a quiescent state until ejaculation, removal of degenerating sperm, provision of appropriate conditions for survival, transport by the myoid cells, protection and maintenance of the blood epididymal barrier [6]. In most species, the epididymis is divided into the caput, corpus and cauda regions [4,5,7]. The activity of these regions is regulated by endocrine, lumicrine, and paracrine factors, the relative importance of which remaining a topic of investigation [8]. The presence of androgen receptors (ARs) during spermatogenesis was investigated in rodent models in which testosterone levels were chemically deleted, or in models with transgenic disruption of ARs [9]. The use of these models made it possible to identify the steps of spermatogenesis requiring ARs, specifically the maintenance of spermatogonia number, integrity of blood-testis barrier, completion of meiosis, adhesion of spermatids and spermiation. Together these studies detailed the essential nature of androgens in the promotion of male fertility [10]. Luminal factors from the testis, in addition to androgens, are important for both the epididymal development [11] and maintenance of adult tissues [12]. The presence of steroids and their receptors, specifically ARs, which are responsible for maintaining epididymal structure and functions throughout the postnatal development [13] has not yet been shown in the epididymis of D'Man lambs. In the prepubertal period several factors can cause epididymal obstruction, such as iatrogenicities due to inguinal herniotomies, inflammatory, tumoral, cystic and similar causes [14]. The effect of these conditions on the testes and epididymal ARs distribution are of major importance. In this study, the objective was to characterize the morphological normal changes and immunolocalization of ARs of the epididymis in 2 to 5 months old D'Man lamb.

MATERIAL and METHODS

Twelve lambs aged from 2 to 5 months reared at El Meniaa experimental station in Algeria (30° 34' N., 02° 52' E.) have been used for this study. For each month, three lambs were weighed and immediately slaughtered; the epididymis was separated from testis and weighed. From each regions of epididymis (caput, corpus and cauda), a sample was fixed in 10% formaldehyde in phosphate buffered saline, dehydrated in a graded series of ethanol, clarified in xylene and embedded in paraffin. The sections were hydrated and stained with Masson's trichroma in order to study general histology. The diameters of tubules and lumen tubules of the epididymis were measured on 10 cross-sections per animal. The height of epithelial cells was measured from the basement to the apical membrane in cross-sections of 10 tubules using a computer program of light microscope Nikon Eclipse E 400 connected to a Nikon DXM 1200 digital camera.

Androgen Receptor Immunohistochemistry

The immunohistochemical studies of ARs were performed using the avidin-biotin complex method (ABC), with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Paraffin sections (3 µm thick) were deparaffinized, hydrated through a graded ethanol series (100%, 95% and 70%), and washed in PBS. Immunohistochemistry was performed on deparaffinized adjacent sections with heat -induced antigen retrieval in citrate buffer (pH 6.0) using *water bath* set at 94°C, as described in the prospectus for the kit "Vector Antigen Unmasking Solutions" (Vector Laboratories, CA, H3300). This step was followed with endogenous peroxidase blocking 3% H_2O_2 in PBS for 5 min at room temperature. All washes between antibody or reagent incubations were rinsed 5 min 2 times at room temperature in PBS, and all the incubations were carried out in a wet chamber. Tissue sections were first submitted to the appropriate serum in order to block the non-specific binding sites. After that, sections processed for ARs labeling were incubated with normal horse serum at room temperature for 5 min, and then with both avidin and biotin sites subsequently blocked (Vector Laboratories, CA, SP-2001). All sections were incubated overnight at 4°C with the primary antibody: a rabbit polyclonal antibody (C-19) raised against a peptide within the C-terminal domain of the human AR (sc-815, Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was diluted at 1 : 200 in PBS. This antibody being currently used to detect the presence of androgen receptors in several mammals, it was used to detect androgen receptors in D'man lamb epididymis. Bound antibodies were visualized by incubating the sections with biotinylated secondary antibody (Vectastain Elite ABC kit-Vector Laboratories, CA, #PK-6200) for 30 min. Labeling of ARs was performed with 3,3'-diaminobenzidine-tetra-hydrochloride chromogenic substrate (SK-4100, DAB substrate kit for peroxidase; Vector Laboratories) and monitored microscopically. Sections were counterstained with hematoxylin (Hematoxylin QS, H-3404; Vector Laboratories, Burlingame, CA, USA). Those sections were dehydrated and mounted. Sections incubated with normal horse serum instead of primary

antibody were used as negative controls. Images were captured using a light microscope (Nikon Eclipse E 400 connected to a Nikon DXM 1200 digital camera).

The results of immunohistochemical staining, evaluated by semiquantitative methods, were given for the epithelial cells (principal, basal and apical cells), the interstitial stromal cells and the peritubular smooth muscle cells of each epididymis compartment. The staining intensity was evaluated at four different levels: ++ / strong, + / moderate, – / negative, +/– / variable.

Statistical Analysis

Results were expressed as Mean ± SEM after verification of their homogeneity. The CV observed was below 20% . Analysis of variance was performed using the One-way ANOVA. Each parameter was analyzed by pairwise comparison using the Tukey HSD test. All analysis was performed using XLSTAT version 2016. The correlation between the average epididymis weight and age was analyzed using a Pearson's Linear Correlation test. $P<0.05$ was considered as significant.

RESULTS

Epididymis Weight

The weight of the paired epididymis related to the age presented in *Fig 1*, increased ($P<0.001$) from 2 to 5 months. At 2 month, the epididymis weight was 2.8±0.3 g, and increased quickly to 10.7±1.4 g at 5 month with a significant increase ($P<0.05$) observed at 3 months. The average epididymal weight was significantly affected by the age ($P<0.001$) and a positive correlation ($r=0.76$, $P<0.05$) was observed between the average epididymis weight and age *(Fig. 1)*.

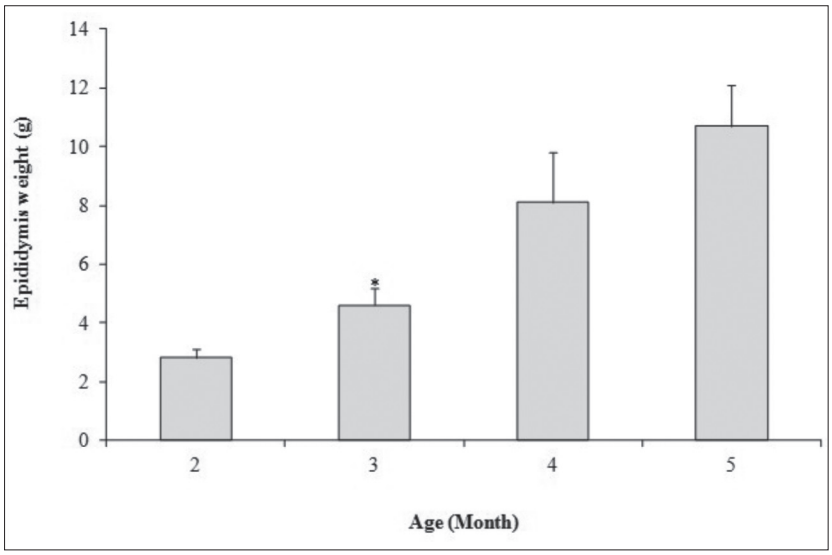

Fig 1. Age-related changes in the epididymis weight (g) in D'Man lamb

Morphometry Development of the Epididymis

The evaluation of the effects of the age on the tubular diameter, luminal diameter, and epithelial height of the epididymis is shown in *Fig. 2*. The measures of tubular and luminal diameters, and the epithelial height showed some regional differences in the three regions of the epididymis *(Fig. 2)*.

The tubular diameter of the caput epididymal tubules presented a high increase ($P<0.001$) from 2 (389±9.7 µm) to 3 (542±11.3 µm) months, then it stabilized at 5 months (550±13.7 µm). In 2 months-old animals, the epididymal tubular diameter was 279±9.9 and 294±9.9 µm, respectively for the corpus and cauda epididymis, increased to 490±7.5 and 467±11.8 µm respectively in 5 months-old animals. The tubular diameter of the corpus and the cauda epididymal tubules increased ($P<0.001$) gradually, respectively between 2 to 4, and between 2 to 5 months *(Fig. 2a)*. During the growth period, there were age effects ($P<0.001$) on the tubular and luminal diameters of the epididymal tubules *(Fig. 2a)*.

The luminal diameters of the caput epididymal tubules increased continuously with the age, with a significant ($P<0.001$) increasing in 3 months-old animals (412±12.8 µm) *(Fig. 2b)*. The epididymal luminal diameters measuring 180±3.7 and 221±7.9 µm, respectively for the corpus and cauda epididymis in 2 months-old animals, increased to 366±8.3 µm and 373±12.8 µm in 5 months-old animals. The luminal diameters of the corpus and caudal epididymal tubules increased significantly ($P<0.001$) from 3 and 4 months respectively *(Fig. 2b)*. During the postnatal growth, there were age effects ($P<0.001$) on luminal diameters of the epididymal tubules *(Fig. 2b)*.

The epithelial height of the caput and caudal epididymal tubules remained unchanged between 2 till 4 months, then it increased significatively ($P<0.001$) in 5 months-old animals *(Fig. 2c)*. The epithelial height of the corpus epididymal tubules increased significatively ($P<0.001$) in 3 months-old animals *(Fig. 2c)*. During the postnatal growth, age effected ($P<0.001$) the epithelial height of epididymal tubules *(Fig. 2c)*.

Immunolocalization of Androgen Receptor During Epididymis Development

ARs immunostaining was observed in all the segments of lamb epididymis *(Fig. 3)*. ARs were localized in the principal cells of the caput, corpus and cauda. The cytoplasm was slightly positive by comparison with nuclei in all the regions. In the epithelium, the ARs immuno-expression was observed in the basal and

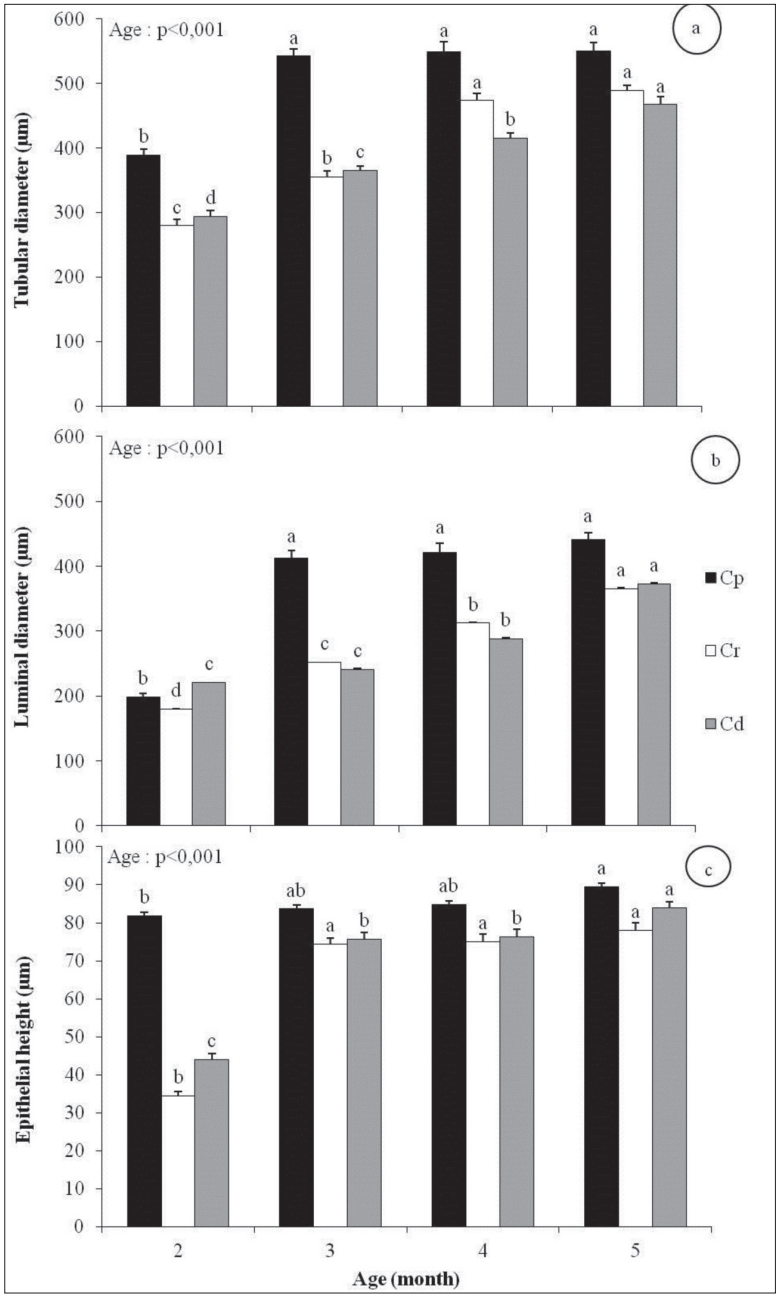

Fig 2. Effect of age on the tubular diameter (a), luminal diameter (b), and epithelium height (c) in epididymis. Bars represent means + SEM (n = 3). Cp: caput, Cr: corpus, Cd: cauda

apical cytoplasm of non-ciliated cells and in apical cytoplasm of ciliated cells.

In the corpus and cauda epididymis, the same pattern of ARs repartition was observed. However, a decreasing intensity of immuno-expression was observed, with the lowest intensity in principal cells of corpus, and cauda epididymis *(Table 1)*. The peritubular connective tissue was negative. Intermittent immunostainings for ARs were also seen in smooth muscle and connective tissue. Sperm in the lumen appeared positive for ARs immunostaining. Nuclei of cells belonging to connective tissue and smooth muscle cells were also positive. Additionally, nuclei of interstitial cell and sperm were positive for antibodies directed against ARs. Luminal sperm in corpus and cauda epididymis showed positive immunostaining for ARs in the cytoplasmic droplet.

No immunoreaction was observed in the caput, the corpus and the cauda epididymidis incubated without any primary antibody. ARs staining in the epithelial cells appeared to be stronger than in the peritubular smooth muscle cells. In the epithelial cells, staining intensity was stronger in the principal cells than in basal and apical cells. The staining intensity of AR positive cells changed depending on the age of animal. The AR immunostaining intensity increased between 2 till 5 months for all the different regions of epididymis.

DISCUSSION

After this study, the epididymis weight increased quickly during the period comprised between 2 and 5 months. The same increase pattern in testis weight was observed in lambs in postnatal development [3]. Several authors reported epididymis weight was increasing with age [15,16].

Table 1. *Average staining intensity of ARs in the different region of epididymis (caput, corpus and cauda) at 2 and 5 months of age during postnatal development in D'Man lamb*

Epithelial Cells	2 Months			5 Months		
	Caput	Corpus	Cauda	Caput	Corpus	Cauda
Principal cells	+	+	+	++	++	++
Basal cells	-	-	-	+/-	+	+/-
Apical cells	+	+	+	++	++	++
Interstitial stromal cells	-	+/-	+	+	+/-	+/-
Peritubular smooth muscle cells	-	-	+/-	+/-	+	-

** Symbols are as follows: ++ strong, + moderate, – negative, +/– variable*

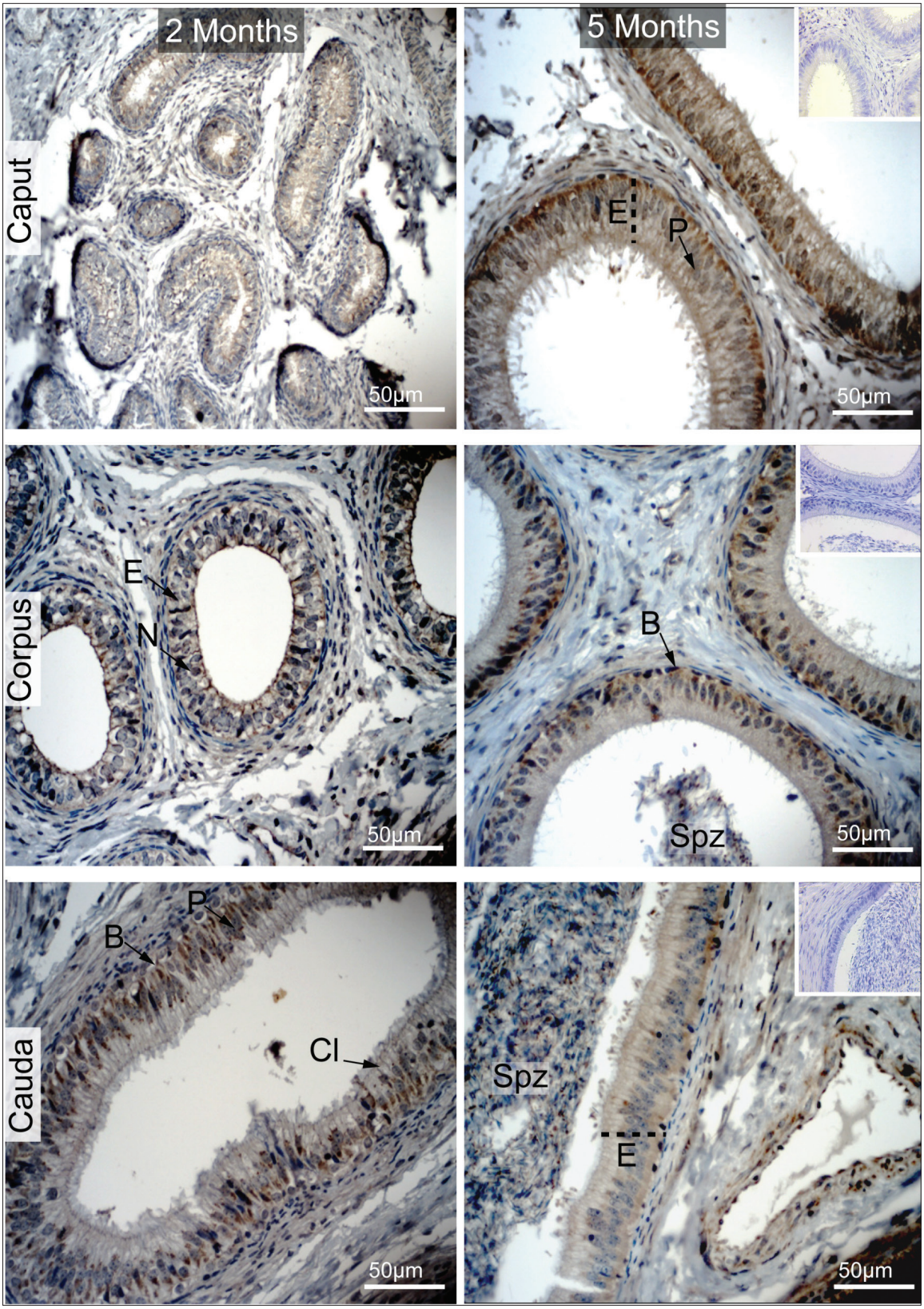

Fig 3. Immunolocalization of androgen receptor in the epididymis of D'Man lamb

AR immunostaining is observed in the nucleus and cytoplasm of epididymal epithelial cells in the caput, corpus and cauda in 2 and 5 months-old D'Man lambs. The images shown here are representative of the results of immunostaining observed in three animals for each age group. No immunostaining was observed in negative controls (inset). E: epithelial cell; P: principal cell; B: basal cell; N: narrow cell; Cl: clear cell; Spz: spermatozoa

At each age, the tubular diameter decreased from the caput to cauda in D'Man lamb. Noviana et al.[17] reported in Kacang goats and local sheep that the diameter in the corpus was smaller than the caput and cauda regions'ones, due to the narrowed and elongated anatomical structure of the corpus epididymis. The tubular diameter increased significantly with the age in the cauda, because spermatozoa were stocked in this epididymis area.

The luminal diameter of the caput epididymal tubules presented a high significant increase in 3 months-old animals. The epididymal luminal diameters decreased from caput then increased in cauda. For the domesticated adult African great cane rat *(Thryonomys swinderianus)* [18] and in age-related study in the rat [19], the epididymal luminal diameters increased progressively from caput to cauda. While the cauda epididymis acted as a sperm reservoir, both the caput and corpus were responsible for sperm maturation [20].

The height of the epithelium of each region increased, as the age increased. The epithelial height of caput was more developed than in corpus and cauda epididymis. In One-Humped Camel *(Camelus dromedaries)* [21], in rat [19], the highest epithelium was seen in the caput and decreased gradually toward the cauda; wards the epididymal duct might mechanically facilitate the passage of the sperms toward the terminal segment [21].

The development of a fully differentiated epithelium is dependent on androgens and also requires the influence of luminal factors from the testis [22]. The epithelial cells of epididymis are able to synthesize some steroid hormones because the cytoplasm has accumulated lipid droplets and contains the active enzymes of steroidogenesis, capable to moderate the *in vitro* synthesis of androgens [23]. Androgens play a crucial role in the proliferation, differentiation and function of the epididymis [24]. Immunocytochemistry identified the epithelium of the epididymis such as a site of ARs expression, while the connective tissue stroma and the blood vessels lacked specific signals throughout the organ. Androgens are also implicated in the regulation of epididymal blood flow [25]. The ARs immunostaining in D'Man lamb epididymis was observed in both the nuclei and cytoplasm of ciliated and non-ciliated epithelial cells, in addition to the peritubular and some stroma cells. The epididymal localization of the ARs was reported for various species [26,27]. However, the presence of ARs epididymis is well documented in adult ram [26-28] than during postnatal development of lambs. Additionally, sperm in the lumen appeared positive for ARs. The heterogeneous signal distribution for ARs expression along the ram epididymis did not change depending on age as reported in the rat [29]. During postnatal development, the luminal secretion of androgens is essential for the maintenance of epithelial cell identity [30], and for the normal development and function of the stromal cells [31]. Gur and Timurkaan [14] reported the progressive degenerative alterations occurred in the seminiferous tubules after prepubertal epididymal ligation. These degenerative changes included increase at the seminiferous tubule diameter and basal membrane thickness, decrease at the germinal epithelium thickness, depletion of spermatids and presence of multinucleated spermatids [32]. Both the regionalized differentiation of the epididymis and the variation in the luminal fluid composition take place under the control of androgens [33].

The morphometric changes and immunolocalization of androgen receptors during the postnatal development of epididymis indicated the necessity of androgens for postnatal differentiation and maintaining the structure of the epididymis.

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