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Immunophenotyping of Rabbit Testicular Germ and Sertoli Cells Across Maturational Stages

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Summary

During testicular maturation, both Sertoli cells (SCs) and germ cells (GCs) switch from an immature to a mature immunophenotype. The reexpression of markers of immaturity in adults has been reported in cancer and in other testicular pathologies, in men as well as in animal species. Naturally affected with testicular cancer, rabbits have long been used in human reproductive research, but reports on the expression of testicular cell markers in this species are few and data about the immunophenotype of normal postnatal SCs and GCs are lacking. The aim of this study was to investigate the immunophenotype of SCs and GCs in the rabbit, from neonatal to adult age, using the antibodies anti-Müllerian hormone (AMH), vimentin (VIM), CKAE1/AE3 (cytokeratins [CKs]), desmin (DES), inhibin alpha (INH-α), placental alkaline phosphatase (PLAP), and periodic acid–Schiff (PAS) staining. In SCs, VIM was constantly expressed, and AMH and CKs expression was limited to neonatal and prepubertal age, whereas DES, INH-α, PLAP, and PAS were constantly negative. GCs were negatively stained for PLAP, PAS, and for the other markers. Results revealed analogies with human testicular immunophenotype, suggesting that rabbits could represent a potential experimental model for the study of human testicular pathology. (J Histochem Cytochem 64:715–726, 2016)

Keywords

germ cells, immunohistochemistry, rabbit, Sertoli cells, testis

Introduction

In recent years, research has demonstrated a decline in human male reproductive health: Incidence of testicular cancer, poorer semen quality, undescended testes, and demand for assisted reproduction have increased over the last 40 years.[1]

Clinical and epidemiological research has evidenced that all the aforementioned disorders may be interrelated and may be symptoms of a single underlying entity: testicular dysgenesis syndrome (TDS).[2] TDS is suggested to be the result of disruption of embryonal programming and gonadal development caused by in utero exposure to estrogen-like chemicals defined as endocrine disruptors (EDs), which are progressively becoming more concentrated and widespread in our environment, such as phthalates.[3]

Fetal testis appears to be the crucial EDs target: The functions and development of Leydig cells (LCs) are reduced and this has an adverse effect on the seminiferous compartment, affecting both Sertoli cell (SC) and germ cell (GC) development and causing variable impairment of spermatogenesis and a predisposition to neoplastic transformation. In these

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pathological conditions, SCs are characterized by an altered immunophenotype with features of cellular dedifferentiation.[4] GCs are also affected, being completely absent or proliferating as gonocytes, the GC progenitors[2,5] from which carcinoma in situ (CIS) can derive.

Rats have been largely used to study EDs-induced (in particular phthalates induced) effects on fetal testes; however, the incidence of testicular cancer in rats is extremely low, and rats have a shorter life expectancy than men and dogs in which these tumors spontaneously occur and in which the histological features of TDS, in combination with seminoma, have been reported.[3,6,7]

The rabbit (*Oryctolagus cuniculus*) is commonly used in experimental biomedical research, and having a life span of approximately 7 to 10 years, it represents a good animal model for the study of testicular neoplasms. Interestingly, in the only available study about the effects of phthalates in rabbits, the authors described antiandrogenic effects in male rabbit fetuses with a case manifesting cryptorchid testes with CIS-like cells.[8] Moreover, rabbits can be spontaneously affected by seminomas[9–12] and, as well as men, are prone to develop other testicular neoplasms,[13,14] including CIS[15] and gonadoblastoma.[16]

IHC has been used to study mature, immature, and neoplastic testes both in human and in veterinary medicine. Different studies revealed that in testicular cancers, there is a reexpression of markers of immaturity. In particular, in SC tumors, reexpression of desmin (DES), anti-Müllerian hormone (AMH), cytokeratins (CKs), and inhibin alpha (INH-α)[17–21] has been observed. In GC tumors, the reexpression of markers typical of gonocytes, such as placental alkaline phosphatase (PLAP), periodic acid–Schiff (PAS) staining, and tyrosine-protein kinase kit (c-kit, CD117), has been reported.[6,22–24]

To date, a definite animal model for studying human testicular cancer is still lacking. Rabbits have several characteristics (long life span, spontaneous testicular tumors) that suggest this species is a good model; however, in comparison with other experimental species, rabbit testes have been rarely studied. Currently, there are only a few reports in the literature concerning expression of immunohistochemical testicular markers in rabbits,[16,25,26] whereas studies on normal SCs and GCs in postnatal rabbit testes, focused on the their functional and morphological maturation from neonatal to adult age, are completely lacking.

The aim of this study was to characterize the immunophenotype of normal SCs and GCs during the maturation of rabbit testes from neonatal to adult age.

Materials and Methods

Samples

Formalin-fixed, paraffin-embedded normal testes from 30 New Zealand (NZ) rabbits were retrieved from the departmental archives.

Samples were clustered in three categories of age:

- Category 1 = neonatal (from 0 to 7 days of age)
- Category 2 = prepubertal (from 25 to 74 days of age)
- Category 3 = adults (from 10 to 40 months of age).

Categories 1, 2, and 3 included 12, 15, and three pairs of NZ rabbit testes, respectively.

In addition, two pairs of prepubertal testes (category 2) from two Belgian Hare rabbits and eight pairs of adult testes (category 3) from six Colored Dwarf and two Turingia Chamois rabbits, present in the archive, were also considered. No rabbits were sacrificed for this study. Gonads from adult NZ rabbits were collected at the abattoirs after rabbits had been slaughtered in accordance with Council Regulation (EC) No. 1099/2009. Testes from prepubertal or neonatal NZ white rabbits were collected directly in the rabbitries from animals which had died of hyperacute diseases or trauma. All the other testes were derived from routine castration.

Histology, Histochemistry, and IHC

For each sample, multiple serial sections (5 µm) were obtained. Two sections were stained with hematoxylin and eosin and PAS, respectively; the other sections were immunohistochemically labeled. IHC was performed with the standard avidin–biotin-peroxidase complex (ABC) procedure with a commercial kit (Vectastain Standard Elite; Vector Laboratories, Burlingame, CA). Sections were dewaxed, treated with 0.3% H_2O_2 in methanol for 20 min, and rehydrated. Details of the antibodies used and the antigen retrieval method applied are listed in Table 1. Sections covered by primary antibodies, diluted in Tris buffer, were incubated at 4C overnight. After washing in Tris buffer, the sections were covered with antimouse IgG biotinylated antibody (diluted 1:200) for monoclonal antibodies or with antigoat IgG biotinylated antibody (diluted 1:200) for AMH and incubated at room temperature for 30 min. After washing, the peroxidase-conjugate ABC (diluted 1:100) was allowed to react at room temperature for 30 min. The immunohistochemical reaction was developed with 3-amino-9-ethylcarbazole (Vector Laboratories) for 10 min according to

Table 1. Antibodies Used in IHC (Antigen Retrieval, Dilutions, and Sources).

Antibodies	Clone	Antigen Retrieval	Dilution	Positive Controls
Vimentin[a] (VIM)	3B4	MW 650 W, 10 min, pH 6, citrate buffer	1:1000	Interstitial fibrocytes
Inhibin alpha[b] (INH-α)	R1	MW 650 W, 10 min, pH 6, citrate buffer	1:40	Normal mature rabbit ovary (granulosa cells)
Cytokeratins[c] (CKAE1/AE3)	AE1/AE3	Pepsin,[c] 37C, 14 min	1:2000	Epithelial cells from rete testis and/or epididymis
Desmin[d] (DES)	NCL-L-DES-DERII	Pepsin,[c] 37C, 14 min	1:150	Myoid peritubular cells
Anti-Müllerian Hormone (C-20; AMH)[e]		MW 650 W, 10 min, pH 6, citrate buffer	1:30,000	Normal neonatal canine testes
Placental alkaline phosphatase (PLAP)[a]	8A9	MW 650 W, 10 min, pH 8, EDTA buffer[c]	1:25	Myoid peritubular cells

Abbreviation: MW, microwave.
[a]Dako Corporation, Carpinteria, CA.
[b]Serotec Corporation, Oxford, UK.
[c]Zymed, San Francisco, CA.
[d]Novocastra, Newcastle, UK.
[e]Santa Cruz Biotechnology, Inc, CA, Dallas, TX.

the manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin.

For each immunohistochemical test, one section of normal neonatal canine testes and one of normal adult rabbit ovaries were included as positive controls for AMH[21] and INH-α, respectively. For the other antibodies, internal positive controls were available. They consisted of interstitial fibrocytes for vimentin (VIM), epithelial cells of *rete testis* and/or epididymis for CKAE1/AE3 (CKs), and myoid peritubular cells and vessel walls for DES and PLAP (Table 1). For PAS staining, the basal membranes of seminiferous tubules and vascular walls were used as internal positive controls.

The percentage of immunolabeled cells was assessed semiquantitatively and scored as follows: – (negative), + (less than 10%), ++ (11%–40%), +++ (41%–80%), and ++++ (81%–100%), in line with a previous report.[27]

Labeling intensity was defined as weak, medium, and strong staining.

Western Blot

Because of the lack of data about immunohistochemical detection of INH-α in rabbit gonads, the presence of INH-α protein in rabbit ovary and testes was further investigated by Western blotting. Briefly, 100 mg of adult NZ rabbit ovary and testes from 30-day-old NZ rabbits was mechanically homogenized in six volumes (w/v) of lysis buffer with a protease inhibitor cocktail (Sigma-Aldrich), as previously described.[28] After centrifugation, the protein content of the supernatant was quantified at 280 nm. Aliquots of 150 µg (total protein) were loaded onto each lane of a 12% SDS-PAGE gel. Before gel separation, 1-µl β-mercaptoethanol (Sigma-Aldrich) was added to each sample. After electrophoretic separation, the proteins were electrotransferred to nitrocellulose membrane. Immunolabeling was performed with primary antibody (1:200 dilution for 2 hr at room temperature), whereas an antimouse IgG labeled with peroxidase was used as secondary antibody (GE Healthcare Life Sciences; 1:1000 dilution for 1 hr at room temperature). Immunoreactive bands were visualized by enhanced chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Results

Histology

Category 1: Neonatal Testes. Numerous small seminiferous tubules, separated by a moderate amount of interstitial connective tissue, were evident within the testicular parenchyma. Tubules had no lumina and were lined by SCs. Scattered early GCs were present admixed with SCs. SCs had a scant amount of pale eosinophilic cytoplasm and round-to-oval basally located dense nucleus.

Early GCs, morphologically consistent with spermatogonia, were round, with abundant pale eosinophilic cytoplasm, large vesicular nucleus, and one to two centrally located nucleoli. Consistent with what has been described for neonatal rabbit testes,[29] scattered intratubular pyknotic GCs were also evident (Fig. 1A). Tubules of rete testis were clearly recognizable in all cases.

Category 2: Prepubertal Testes. Seminal tubules were characterized by larger dimensions and greater

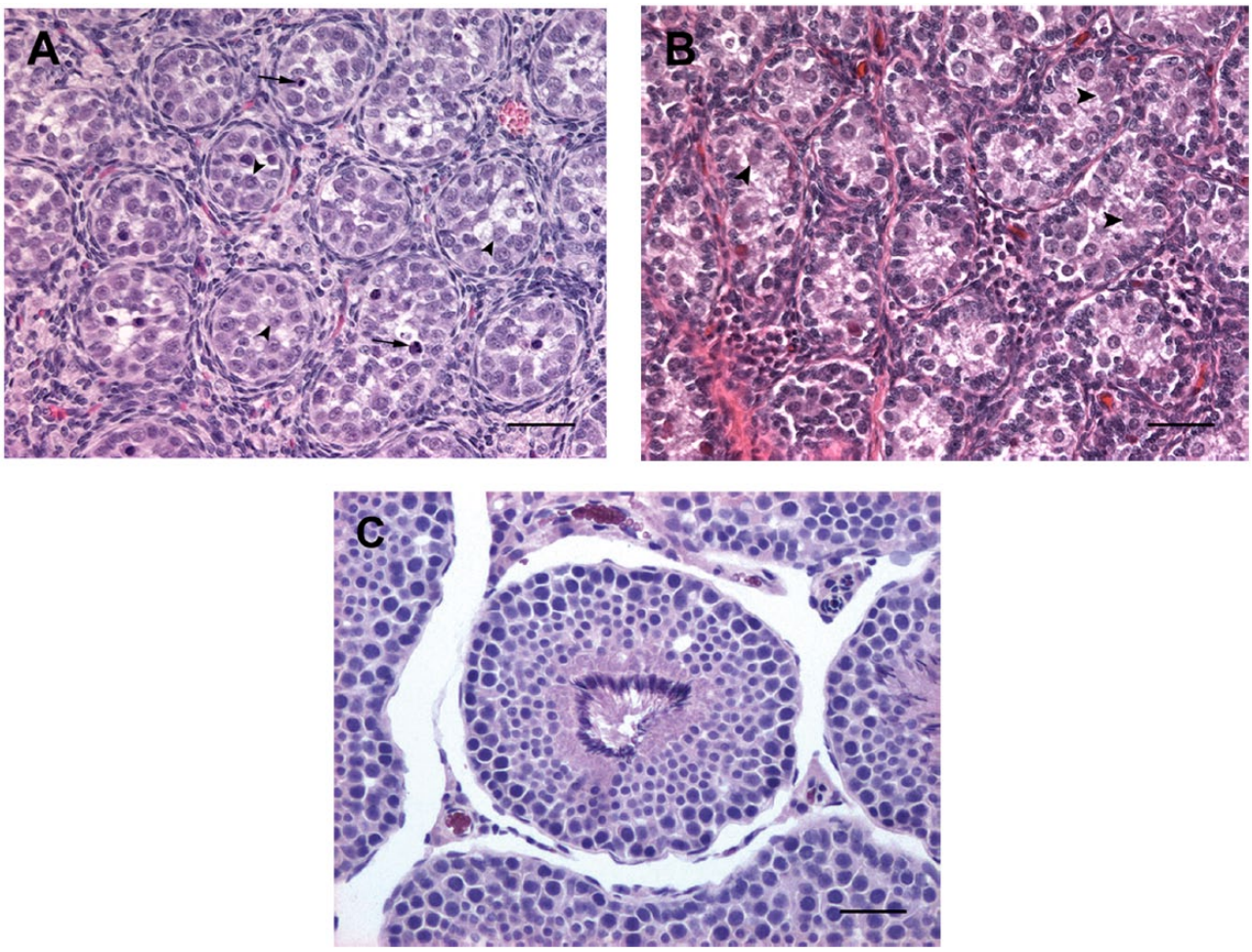

Figure 1. Histological features of rabbit testes. A, Neonatal testis, histological section. The testis is characterized by small seminiferous tubules with no lumina, lined by Sertoli cells (SCs) and filled with rare immature germ cells (GCs) (arrowheads). Pyknotic nuclei are indicated (arrows). Hematoxylin and eosin (H&E) staining. Magnification, 200×. B, Prepubertal testis, histological section. The testis has larger tubules, lined by SCs admixed with GCs consistent with spermatogonia and a few spermatocytes (arrowheads). H&E staining. Magnification, 200×. C, Adult testis, histological section. The testis is characterized by seminiferous tubules exhibiting all stages of seminal epithelium. H&E staining. Magnification, 200×. Scale bars: A–C = 35 µm.

amounts of GCs than neonatal testes. SCs rested on the basement membrane and were characterized by low columnar cytoplasm protruding into the lumen and dense, oval, basally located nuclei. GCs, morphologically consistent with spermatogonia and few spermatocytes, were distributed among SCs (Fig. 1B). Occasional karyorrhectic and pyknotic GCs were also evident. Rete testis was clearly recognizable in all samples.

Category 3: Adult Testes. In the adult testes, seminiferous tubules exhibited a well-defined central lumen, and they were lined by SCs, closely associated with all the different stages of seminal epithelium (Fig. 1C). Spermatozoa were abundantly present within the lumen of the epididymis.

Histochemistry and IHC

Both SCs and GCs were processed with the aforementioned antibody panel. SCs revealed to be positive for VIM, CKs, and AMH, according to the age of the rabbits, whereas GCs were negatively labeled for all the used markers. The results are summarized in Table 2.

PAS Staining. SCs and GCs were always PAS negative in all the samples examined. The tubular basal membranes and vascular walls considered as internal controls were PAS positive in all the specimens.

Vimentin. VIM was constantly, diffusely, and strongly expressed in the cytoplasm of SCs in all testes from neonatal to adult age (Fig. 2A and B, Table 2). The percentage of labeled cells was scored as ++++ (81%–100%) in

Table 2. Immunohistochemical Results of Sertoli Cells in Rabbit Testes, From Neonatal to Adult Age.

Case	Age (Days)	Breed	AMH	INH-α	CKs	DES	VIM	PLAP
Category 1: Neonatal rabbits								
1	0	NZ	++++	−	++++	−	++++	−
2	0	NZ	++++	−	+++	−	++	−
3	0	NZ	++++	−	−	−	++	−
4	0	NZ	−	−	++++	−	++++	−
5	0	NZ	++++	−	−	−	+++	−
6	0	NZ	++++	−	−	−	++	−
7	0	NZ	++++	−	++++	−	++++	−
8	7	NZ	+++	−	−	−	++++	−
9	7	NZ	++++	−	++++	−	++++	−
10	7	NZ	++++	−	+++	−	++++	−
11	7	NZ	++++	−	++++	−	++++	−
12	7	NZ	+++	−	+	−	++++	−
Category 2: Prepubertal rabbits								
13	25	NZ	+++	−	−	−	+++	−
14	35	NZ	++++	−	++	−	++++	−
15	35	NZ	++++	−	++	−	++++	−
16	45	NZ	++++	−	++	−	++++	−
17	50	NZ	++	−	−	−	++++	−
18	60	NZ	−	−	+++	−	++++	−
19	60	NZ	−	−	−	−	++++	−
20	60	NZ	−		−	−	++++	
21	60	NZ	−	−	−	−	++++	−
22	60	NZ	++++	−	+	−	++++	−
23	60	NZ	−	−	+	−	++++	−
24	60	NZ	−	−	−	−	++++	−
25	60	NZ	++++	−	−	−	++++	−
26	60	NZ	−	−	−	−	++++	−
27	60	NZ	−	−	++	−	++++	−
28	67	Belgian Hare	−	−	−	−	+++	−
29	74	Belgian Hare	−	−	+	−	++++	−
Category 3: Adult rabbits								
30	10 months	NZ	−	−	−	−	++++	−
31	10 months	NZ	−	−	−	−	++++	−
32	10 months	NZ	−	−	−	−	++++	−
33	10 months	NZ	−	−	−	−	++++	−
34	10 months	NZ	−	−	−	−	++++	−
35	11 months	Turingia Chamois	−	−	−	−	++++	−
36	11 months	Turingia Chamois	−	−	−	−	++++	−
37	12 months	Colored Dwarf	−	−	−	−	++++	−
38	12 months	Colored Dwarf	−	−	−	−	++++	−
39	24 months	Colored Dwarf	−	−	−	−	++++	−
40	24 months	Colored Dwarf	−	−	−	−	++++	−
41	40 months	Colored Dwarf	−	−	−	−	++++	−
42	40 months	Colored Dwarf	−	−	−	−	++++	−

− (negative), + (<10%), ++ (11%–40%), +++ (41%–80%), and ++++ (81%–100%). Abbreviations: AMH, anti-Müllerian hormone; INH-α, inhibin alpha; CKs, cytokeratins; DES, desmin; VIM, vimentin; PLAP, placental alkaline phosphatase; NZ, New Zealand rabbits.

the majority of cases. In the first category, case numbers 2, 3, and 6 were scored as ++ (11%–40%), and in the second category, case number 28 was scored as +++ (11%–40%). GCs were always negatively labeled in all samples examined.

CKAE1/AE3. In neonatal testes, SCs were diffusely and strongly labeled for CKs in four of seven samples of 0-day rabbits (Fig. 3A). Three out of four cases were scored as ++++ (81%–100%), whereas in the case number 2, the percentage of labeled SCs was +++

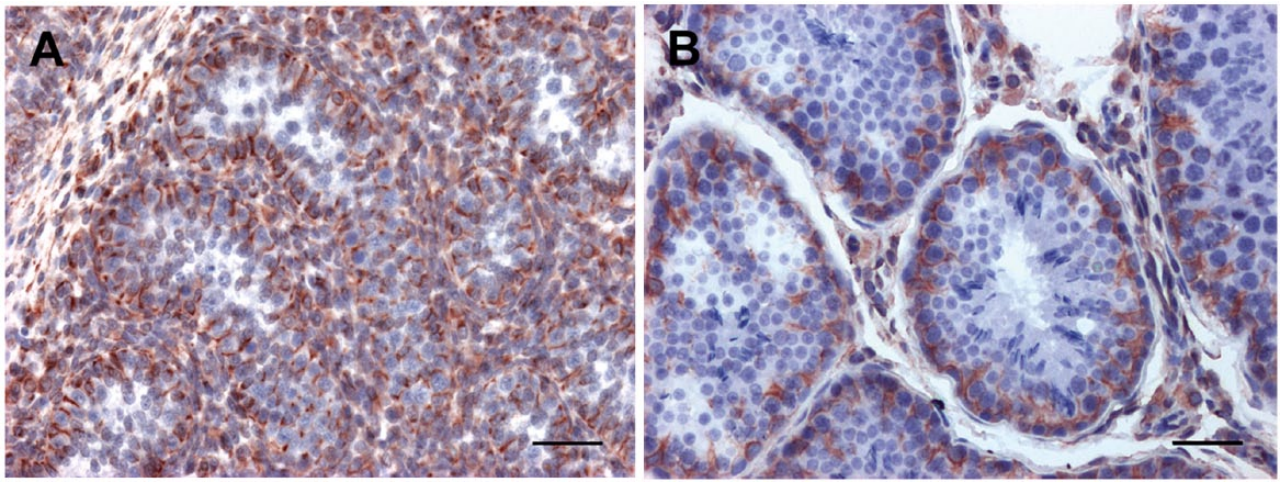

Figure 2. Immunohistochemical staining for vimentin (VIM). Neonatal (A) and adult (B) rabbit testes. Diffuse and strong expression of VIM in the cytoplasm of Sertoli cells and within the interstitium. 3-amino-9-ethylcarbazole. Magnification, 200×. Scale bars: A, B = 35 µm.

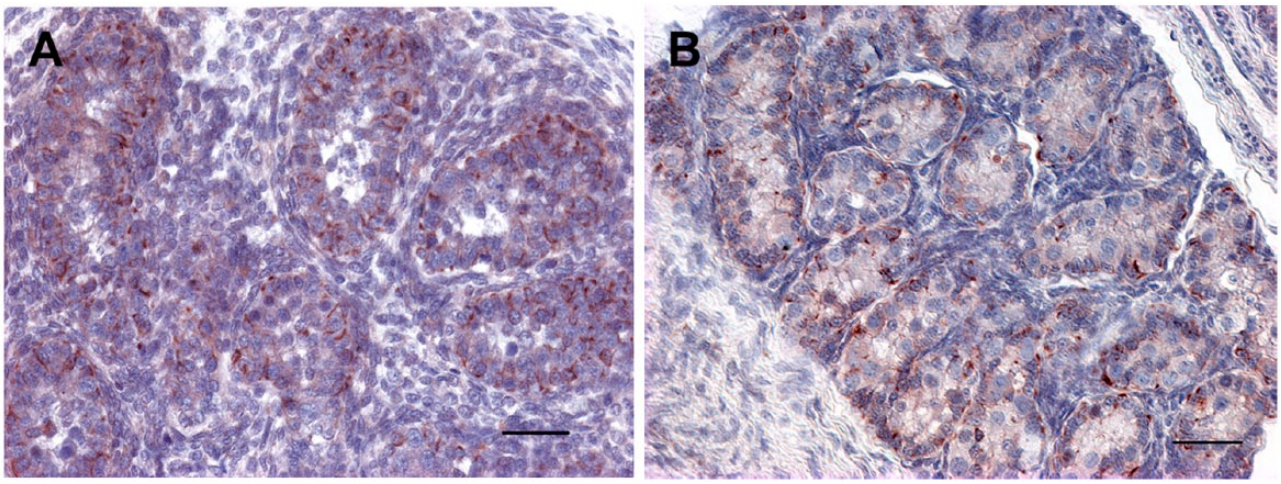

Figure 3. Immunohistochemical staining for cytokeratins (CKs). Neonatal rabbit testis (A). Diffuse expression of CKs in the cytoplasm of Sertoli cells (SCs). Prepubertal rabbit testis (2 months of age; B). Scattered positive SCs are evident. 3-amino-9-ethylcarbazole. Magnification, 200×. Scale bars: A, B = 35 µm.

(41%–80%). In testes from 1-week-old rabbits, SCs were CKs positively labeled in four of five cases (varying from + to ++++). The intensity of the immunohistochemical signal was considered "medium," less intense than that observed in neonatal testes. In prepubertal testes, CKs immunostaining was present in eight of 17 samples. In seven of these eight samples, positive SCs varied from less than 10% to 40% (+/++; Fig. 3B). In only one case (case number 18), the percentage of labeled cells was higher, varying from 41% to 80% (+++). In the prepubertal rabbits, the intensity of the staining was recorded as "weak." In adult testes, SCs were always CKs negative.

Epithelial cells from epididymis and rete testes were always CKs labeled, independently of ages, whereas neonatal and prepubertal GCs and adult seminal line were always negative.

INH-α. All testes examined were constantly negative for INH-α (Fig. 4A), whereas normal rabbit ovarian granulosa cells, used as positive controls, were markedly and diffusely positive (Fig. 4B).

Desmin. DES was exclusively expressed by myoid peritubular cells, the internal positive control, in all testes (Fig. 5), whereas both SCs and GCs were constantly negative, in all ages considered.

AMH. AMH immunostaining was observed in SCs of neonatal and prepubertal testes. In details, in six of

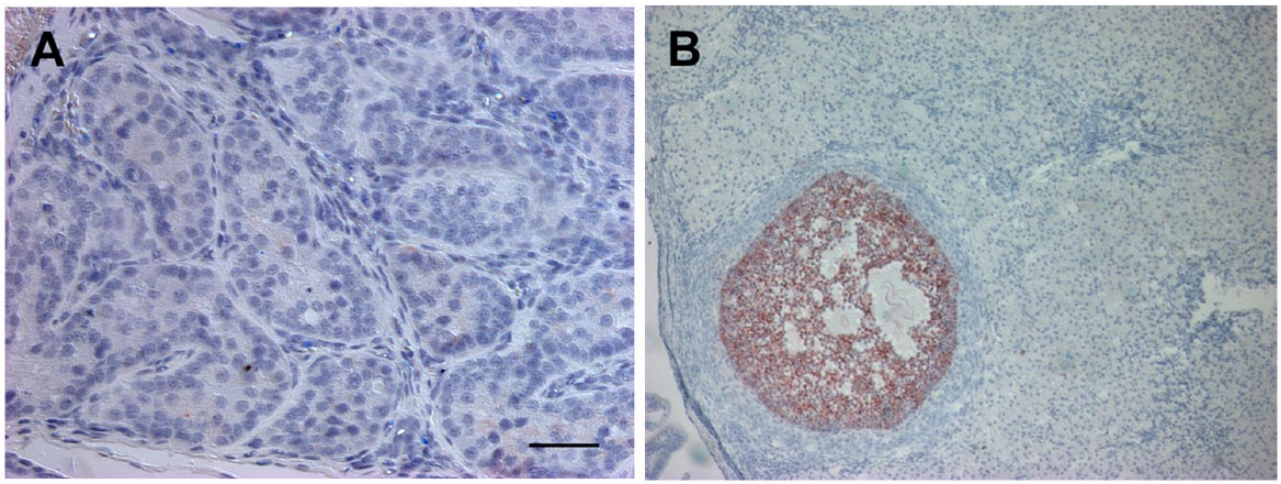

Figure 4. Immunohistochemical staining for inhibin alpha (INH-α). Prepubertal rabbit testis (2 months of age; A). Sertoli cells are negatively labeled for INH-α. Magnification, 200×. Adult rabbit ovary (B). Diffuse and strong expression of INH-α in granulosa cells. Magnification, 50×. 3-amino-9-ethylcarbazole. Scale bars: A = 35 µm; B = 125 µm.

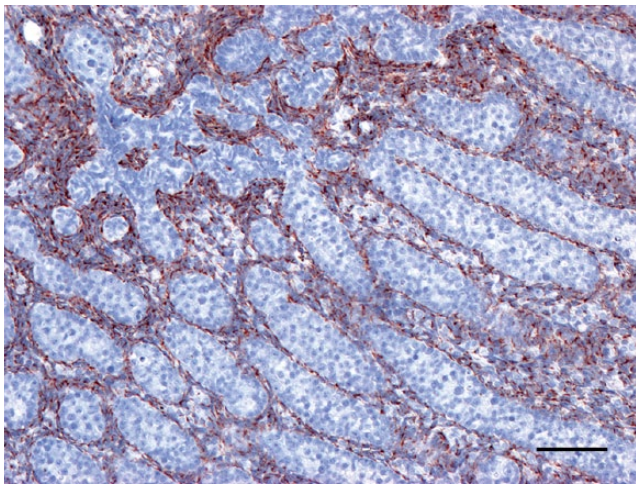

Figure 5. Immunohistochemical staining for desmin (DES). Prepubertal testis (2 months of age). Peritubular myoid cells are diffusely positive for DES, whereas seminal tubules and rete testis are negative. 3-amino-9-ethylcarbazole. Magnification, 100×. Scale bar = 70 µm.

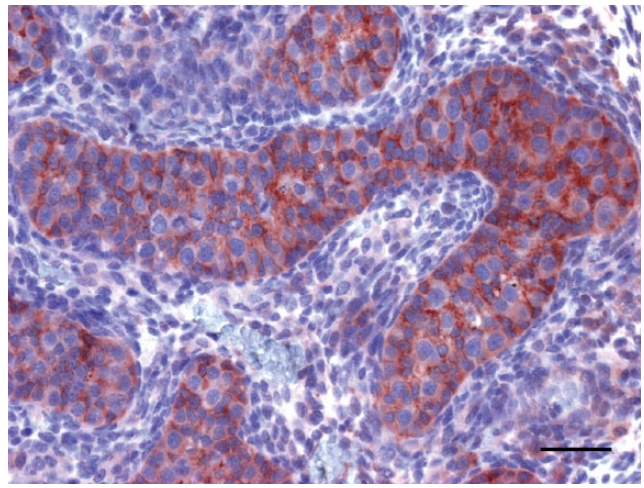

Figure 6. Immunohistochemical staining for anti-Müllerian hormone (AMH). Neonatal rabbit testis. Diffuse and strong expression of AMH in the cytoplasm of Sertoli cells. 3-amino-9-ethylcarbazole. Magnification, 200×. Scale bar = 35 µm.

seven neonatal testes (0 days) and in five of five 1-week-old rabbit testes, SCs were diffusely (41%–100%) and strongly positive for AMH (+++/++++; Fig. 6). In prepubertal testes, the percentage of positive cells and the intensity of the immunostaining decreased with the increasing of the age (Table 2). In only two rabbits of 60 days (case numbers 22 and 25), SCs were diffusely (++++) but weakly immunostained with AMH, whereas the oldest rabbits included in this category were completely negative. In the adult testes, SCs were constantly negative for AMH.

GCs were always AMH negative, independently of age.

PLAP. In all samples, PLAP was exclusively expressed by the internal positive controls, that is, myoid peritubular cells and vessel walls, whereas SCs and GCs were always negative, independently of age (Fig. 7).

Western Blot

Immunoblotting with the INH-α antibody was positive for ovary but not for testes (Fig. 8). Two bands were observed in the extract obtained from ovary with a molecular weight around 44 and 36 kDa. In the extract obtained from testes, the 44-kDa band almost disappeared and the 36-kDa band was not detected.

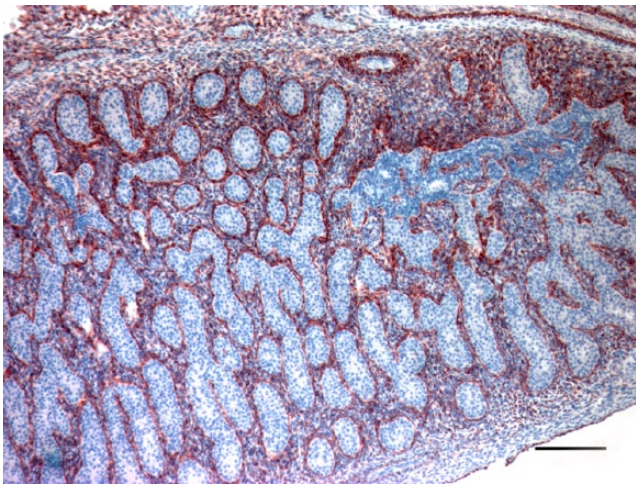

Figure 7. Immunohistochemical staining for placental alkaline phosphatase (PLAP). Neonatal rabbit testis. Diffuse and strong expression of PLAP in the peritubular myoid cells and vascular walls. Both Sertoli cells and germ cells are negative. 3-amino-9-ethylcarbazole. Magnification, 50×. Scale bar = 125 µm.

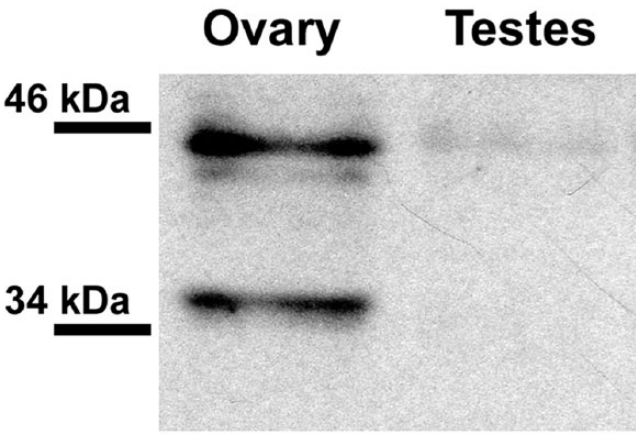

Figure 8. Western blot of rabbit ovary and testes using the inhibin alpha antibody. Two bands are observed in rabbit ovary with a molecular weight around 44 and 36 kDa. In the extract obtained from testes, the 44-kDa band almost disappears and the 36-kDa band is not detected.

Discussion

The rabbit species is widely used in scientific research as a model for neoplastic and metabolic diseases,[30] for environmental toxicity testing,[8,31] and for research on human male reproductive system toxicology.[32]

On the other hand, during the last few years, the acceptance of the rabbit as pet has increased: They have a relatively long life span, and are inquisitive, sociable, and relatively inexpensive to keep compared with other species. Consequently, veterinary interest in pet rabbits and their pathologies is increasing.

In veterinary literature, spontaneous testicular preneoplastic and neoplastic lesions have been described in rabbit species,[12,14,16] suggesting that rabbits could represent a possible model for the study of human testicular tumors. IHC is widely used in oncology; however, the study of neoplastic SCs and GCs cannot disregard the characterization of the immunophenotype of their normal counterpart. For these reasons, in the current study, immunohistochemical characterization of normal GCs and SCs from neonatal to adult age was performed.

To this end, PAS stain and a panel of six immunohistochemical markers, commonly used in the study of human and animal testicular pathology, were used. All the antibodies used showed good reactivity in the rabbit species. Antibodies gave without exception a clearly detectable immunohistochemical signal in association with the expected staining of control structures.

Except for INH-α, all markers were expressed in the testicular samples examined. INH-α stained the granulosa cells of rabbit ovary, confirming the cross-reaction of this antibody with rabbit antigen, but INH-α was not detected in rabbit SCs and/or in LCs, in contradiction of what is reported in other species.[33–35] After these immunohistochemical results, Western blotting was performed to further validate the antibody in the rabbit species and to check for the presence of INH-α in rabbit ovarian and testicular tissue. In rabbit ovary, INH-α antibody produced two protein bands, around 44 and 36 kDa, respectively. In the extract obtained from testes, the 36-kDa band was not present and a faint signal of the 44-kDa band was observed, confirming the immunohistochemical findings in which INH-α labeling was observed in ovarian and not in testicular samples.

Inhibin is a heterodimeric protein consisting of α and β subunits. Two related isoforms are identified, inhibin A (α/βA) and inhibin B (α/βB) secreted in circulation by the gonads, both with the capacity to suppress secretion of Follicle-stimulating hormone (FSH) by the pituitary gland.[36]

In veterinary endocrinology, INH-α has been investigated among species by using immunoblotting, IHC, and bioassays, demonstrating its expression in testicular samples from boars and calves.[37–39]

Moreover, with IHC, the site of expression of INH-α has been demonstrated to be different among species. In men, rats, and sheep, INH-α has been detected in both LCs and SCs.[33,40,41] Depending on the species, SCs or LCs can be the prominent site of expression of INH-α. In men, primates, swine, sheep, and laboratory rodents, SCs have been identified as the predominant site of production of INH-α,[33,35,39,42–44] whereas in horses, INH-α is more expressed in LCs than in SCs.[34,45]

Concerning rabbits, no data about INH-α expression in testes or in blood plasma were available in the literature. In the present study, INH-α was not expressed in rabbit male gonads, independently of age. A possible hypothesis, which needs validation in further studies, is that rabbit testes produce a very low amount of INH-α and a preponderance of βB and βA subunits.

Concerning the other markers used, VIM was always expressed in rabbit testes, independently of age, and was restricted to SCs, consistent with their origin in the mesenchymal urogenital ridge.[46,47] Therefore, in the rabbit, as in the human species,[48] VIM is also a structural marker for SCs.

CKs and AMH were expressed by SCs only in immature testes, whereas in mature testes, they were constantly negative. Interestingly, for both markers, the percentage of positive SCs and the intensity of the immunohistochemical signal were inversely related to the age of the rabbits. CKs were strongly and diffusely expressed in neonatal SCs. The percentage of positively stained SCs declined progressively in the prepubertal samples, and in 2-month-old rabbits, only occasional positively stained SCs were still evident. AMH paralleled CKs staining, but it seemed to be lost earlier than CKs expression (Table 2). AMH was constantly expressed by neonatal and 7-day-old rabbits, but was less intense in 50-day-old rabbits and was completely lost in 2-month-old rabbits.

CKs have been demonstrated to be markers of early immaturity, transiently expressed in SCs, in men as in rodents, only in the early fetal stages.[47,49–51] In dogs, CKs have not been extensively studied in fetuses but have been demonstrated to be absent in normal immature and mature postnatal testes. However, SCs have been demonstrated to be immunolabeled for CKs in pathological conditions such as SC tumors and testicular atrophy.[20,27] The results of the present study confirm that CKs could be considered markers of immaturity for rabbit SCs as well. However, in contrast to other species, in rabbits CKs are not exclusively expressed in SCs in fetal stages, but their expression apparently persists postnatally until 2 months of age.

AMH is produced by SCs and represents the earliest functional specific marker for these cells, being expressed in developing testes, as demonstrated in humans[52] and in several other animal species such as bovines, swine, dogs, and horses.[53–56] Although Müllerian duct regression is completed during fetal life, SCs continue to produce AMH postnatally: until puberty in men[48,54]; limited to the first few weeks of life in goats, cats, rats, and dogs; and until 10 to 18 months of age in bulls and horses.[21,54,55,57] AMH is absent in normal adult testes but can be reexpressed in pathological conditions such as testicular atrophy, androgen insensitive syndromes, 5α-reductase type 2 deficiency, and SC tumors.[20,27,58]

As far as the rabbit species is concerned, previous studies have demonstrated the immunohistochemical expression of AMH in fetal testes.[26,55] In the present study, AMH was detected in neonatal and prepubertal testes, its expression decreased with age, and it was totally absent in adult testes. These findings demonstrated that for the rabbit species, AMH can also be considered a reliable marker of immature SCs.

DES expression was never observed in SCs in the present study. In human testes, DES has been demonstrated in fetal ancestors' SCs, at the level of mesonephros and in immature SCs between weeks 11 and 14 of gestation.[46,47] Afterward, SCs condensate on the surface of the mesonephros and undergo transient epithelial transformation, losing DES and acquiring CKs expression. For this reason, the negative results obtained with DES in postnatal rabbit testes were expected and confirmed that DES could be considered a marker of SC immaturity also in the rabbit species.

Concerning GCs, two different markers of immaturity (PAS and PLAP) were tested and failed constantly to stain GCs, independently of the age of the rabbits.

Histochemically, the progenitors of GCs (gonocytes) can be recognized, in human species, by PAS staining for the presence of glycogen[59,60] and, with IHC, by the expression of PLAP.[61] PAS staining and PLAP expression are lost before birth, when gonocytes become spermatogonia.[62] PLAP can be reexpressed in pathological conditions of the adult testis, as demonstrated in human and canine classical seminoma[6,23,63,64] and TDSs.[2,7]

Regarding rabbit species, no data were available about PAS staining or PLAP immunolabeling in normal testes. However, PLAP was recently successfully demonstrated in neoplastic GCs in two testicular gonadoblastomas.[16] The results of the present study confirm that, as demonstrated in men[62] in normal postnatal rabbit testes, no immature GCs referring to primordial GCs (gonocytes) are present.

In our study, we did not perform staining with OCT4, another marker of human germ stem cell population, which is used to identify gonocytes. However, in a previous study, Daniel-Carlier et al.[26] demonstrated a significant expression of the OCT4 gene in fetal rabbit testes, associated with nuclear stain in fetal GCs, but obtained negative immunofluorescence results on postnatal testicular samples. To explain this result, the authors hypothesized that pluripotent stem cells were too rare to be microscopically visualized.

In conclusion, the results of the present study, obtained from normal immature and mature rabbit

testes, contributed to better definition of the temporal sequence of markers expressed in this species, revealing analogies with its human counterpart.

Further studies focused on the phenotype of neoplastic rabbit testes could help to clarify whether rabbits could be considered a good animal model for human testicular cancer.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

GG and VG selected cases from rabbitries and from pet rabbits; BB and SCC performed the IHC and histochemistry; ATM carried out the Western blot analysis studies; VG designed the study; and BB, CG, and VG drafted the manuscript. All authors have read and approved the final manuscript.

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