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Case Report
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Sertoli cell tumour in a pet rabbit (*Oryctolagus cuniculus*): histological and immunohistochemical characterization

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Abstract
The present study describes a case of a spontaneous, unilateral Sertoli cell tumour (SCT) in a 6-year-old pet rabbit. The rabbit was presented with a palpable, unilateral, subcutaneous left inguinal mass, consistent with the suspected clinical diagnosis of neoplasia developing within the retained testis in the inguinal canal. The intrascrotal contralateral testis was palpable, but reduced in volume. The rabbit underwent orchiectomy and both the testes were collected, formalin-fixed, and submitted for histopathological examination. Microscopically, the enlarged testis was effaced by an intratubular SCT in which numerous intratubular microliths were evident. The contralateral testis was severely atrophied. Immunohistochemical stains showed neoplastic Sertoli cells that were diffusely positive for vimentin and anti-Müllerian-Hormone and multifocally positive for cytokeratins and desmin. Eighteen months after the surgery, the rabbit showed no clinical signs of disease. This is the first report of a spontaneously occurring rabbit SCT histologically described and immunohistochemically investigated.
Keywords: Immunohistochemistry, Rabbit, Sertoli cell tumour, Testes.

Introduction

Testicular neoplasms are not commonly reported in rabbits. The most prevalent are interstitial cell tumours (Leydig cell adenoma) (Flatt and Weisbroth, 1974; Zwicker and Killinger, 1985), followed by seminoma (Brown and Stafford, 1989; Anderson *et al*., 1990; Roccabianca *et al*., 1999). This is the first histological and immunohistochemical description of a Sertoli cell tumour (SCT) affecting a retained testis in an adult pet rabbit. The paper includes histological and immunohistochemical characterization of the neoplasm and focuses on the comparative features of SCTs and Sertoli cells among domestic animals.

Case Details

A 6-year-old, intact male dwarf pet rabbit (*Oryctolagus cuniculus*), weighting 1.7 kg, was referred due to a wide, soft, subcutaneous mass located in the left inguinal region. The mass was scarcely mobile, and no scrotum was identifiable on the left side. On palpation of the scrotum, only a small right testis was identified.
Ultrasonography investigation revealed a subcutaneous, 3.7 x 1.7 cm, partially fluid-filled mass, with heterogeneous echogenicity. No other alterations were detected in the thorax and abdomen and an ultrasound-guided fine-needle aspiration of the mass was obtained. The cytology samples were poorly cellular due to severe hemodilution, and considered inconclusive. Radiographic examination of the thorax showed no abnormalities.
A neoplastic lesion in the left testis was suspected, and castration was suggested. The rabbit was admitted for bilateral orchiectomy under general anaesthesia. Blood samples were collected, and complete blood count and chemistry were performed. Hematologic and biochemical analyses were within normal limits for the species. The rabbit was premedicated with 0.1 mg/kg medetomidine plus 10 mg/kg (Sedastart, Le Vet B.V., Oudewater - Netherlands), ketamine plus 5 mg/kg (Lobotor, ACME, Cavriago (RE) - Italy) and butorphanol (Nargesic, ACME, Cavriago (RE) - Italy) subcutaneously, then placed in an oxygen enriched chamber. After 20 minutes, when sedation was achieved, an intravenous (IV) catheter was placed in the lateral auricular vein, through which an injection of enrofloxacin (10 mg/kg) (Baytril, KVP Pharma, Kiel - Germany) was given. In addition, 1 mg/kg of meloxicam (Meloxoral, Le Vet B.V., Oudewater - Netherlands) was injected IV plus 10 ml of Ringer Lactate solution (B. Braun Medical SA, Barcelona - Spain). Anaesthetic induction was obtained with propofol (2 mg/kg IV) (Propovet, Fresenius Kabi AB, Uppsala - Sweden). An endotracheal tube was placed visualizing the glottis through an otoscope. The rabbit was then intubated, and anaesthesia was maintained with 1% or 2% of isoflurane (ISOFLO, Zoetis inc., Parsippany - USA).
The rabbit was placed in dorsal recumbency and the surgical site was shaved and disinfected. The

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subcutaneous mass was identified and an incision of the skin was made laterally, parallel to the mass. Lidocaine (2mg/kg) (Lidocaina 2%, Zoetis Manufacturing and Research Spain, Girona - Spain) was utilized as a local block of the right spermatic cord and subcutaneously in the areas of the skin incision. The subcutaneous tissue was bluntly dissected and the mass isolated. No particular adhesions were noticed. Grossly, the mass was anatomically and macroscopically consistent with an enlarged, undescended left testicle. The epididymis and spermatic cord were clearly identifiable. Blood vessels were then ligated (with a 3-0 polyglactin 910 suture) and the abnormal left testis removed. Subcutaneous tissue and skin were routinely closed with a continuous suture (with a 3-0 polydioxanone suture). On the right side, a routine orchiectomy was performed by scrotal approach, with open technique. Both testes were submitted for histologic examination.

Atipamezole (Sedastop, Le Vet B.V., Oudewater - Netherlands) was intramuscularly administered at the end of the procedures (0.05 mg/kg).

Post-operative care included IV fluid therapy (10 ml of Ringer Lactate solution[f] once) and recovery in warm cage for a few hours. Analgesia was provided by meloxicam (0.5 mg/kg twice a day orally; Meloxoral, Le Vet B.V., Oudewater - Netherlands), for 5 days after surgery. The rabbit was discharged the next day and healed without complications. The rabbit was examined every three months after surgery, and was always eating with appetite, passing normal stools and maintaining a good body condition at 18 months after the initial diagnosis. No sign of illness was detected during the follow up clinical assessments.

Histopathology

Samples were processed for histology and embedded in paraffin wax. Sections 5 µm thick were obtained and stained with haematoxylin and eosin (HE). Histologically, the right testis was severely and diffusely atrophic: seminiferous tubules were lined by Sertoli cells (SCs) and scattered, rare round germ cells, consistent with spermatogonia, were present. The left testis was completely effaced by an expansile, unencapsulated, densely cellular neoplasm, composed of tubules, irregular in shape and diameter, surrounded by thick basement membranes and lined by 1-2 layers of tall, slender palisading neoplastic cells morphologically consistent with SCs. Neoplastic SCs, similar in size to normal ones, were 20–30 µm in diameter, with variably distinct cell borders, high nuclear-cytoplasmic ratio, scant amount of eosinophilic cytoplasm, and a basally located nucleus with reticular chromatin pattern and one prominent, centrally located, magenta nucleolus.

Anisokaryosis and anisocytosis were moderate, and mitotic figures ranged from 0 to 1 per high power field (400×). Occasionally, basally located, rare round germ cells (consistent with spermatogonia) were interspersed with the neoplastic SCs. Numerous tubules were also expanded by the presence of large, multiple, laminated round basophilic mineral concretions (microliths), multifocally surrounded by SCs in a radiating pattern (Fig. 1).

Multifocally, scattered intratubular Call-Exner bodies (neoplastic SCs arranged in rosettes surrounding microcavities filled with hyaline eosinophilic amorphous material) were also observed (Fig. 1, inset). Based on anatomic location and histological features of the neoplastic lesion, a diagnosis of intratubular SCT with multiple intratubular microliths developing with in a retained inguinal testis was made.

To further characterize this neoplasm, a panel of immunohistochemical markers commonly employed in human and veterinary testicular pathology was applied.

Immunohistochemistry

Serial microtomic sections from the neoplastic testis were obtained, mounted on polylysine coated slides (Menzel-Gläser, Braunschweig, Germany), and immunohistochemically labelled.

Immunohistochemistry (IHC) was performed with the standard avidin-biotin-peroxidase complex (ABC) procedure with a commercial kit (Vectastain Standard Elite; Vector Laboratories, Burlingame, California, USA). Sections were dewaxed, treated with 0.3% H_2O_2 in methanol for 20 minutes and rehydrated. Details of the antibodies used and the antigen retrieval method applied are listed in Table 1. Negative controls were obtained by replacing the respective primary antibody with normal rabbit or mouse serum (non-immune serum, Agilent, Santa Clara, CA, USA). Sections, covered by primary antibodies diluted in Tris buffer, were incubated at 4° C overnight. After washing in Tris buffer, the sections were covered with anti-mouse Immunoglobulin G (IgG) biotinylated antibody (diluted 1:200) for monoclonal antibodies, and with anti-goat IgG biotinylated antibody (diluted 1:200) for anti-Müllerian-Hormone (AMH) (Vector Laboratories, Burlingame, CA), and incubated at room temperature for 30 minutes. After washing, the peroxidase-conjugate ABC (diluted 1:100) was allowed to react at room temperature for 30 minutes. The immunohistochemical reaction was developed with 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories, Burlingame, CA) for 10 minutes according to the manufacturer's instructions. Sections were counterstained with Mayer's haematoxylin.

As in a previous report, for each immunohistochemical test, one section of normal neonatal rabbit testes and one of normal adult rabbit ovary were included as positive controls for AMH and Inhibin- α (INH-α), respectively (Banco *et al.*, 2016). For the other antibodies, internal positive controls were available (Table 1).

Table 1. Antibodies employed in immunohistochemistry (antigen retrieval, dilutions and sources).

Antibodies	Clone	Antigen retrieval	Dilution	Positive controls
Vimentin* (VIM)	3B4	MW 650 W, 10 min pH 6 citrate buffer	1:1000	Interstitial fibrocytes
Inhibin-α‡ (INH-α)	R1	MW 650 W, 10 min pH 6 citrate buffer	1:40	Normal mature rabbit ovary (granulosa cells)
Cytokeratins† (CKs AE1/AE3)	AE1/AE3	Pepsin† 37 °C, 14 min	1:2000	Epithelial cells from rete testis and/or epididymis
Desmin§ (DES)	NCL-L-DES-DERII	Pepsin† 37 °C, 14 min	1:150	Myoid peritubular cells
Anti-Müllerian Hormone (C-20) (AMH)□	-	MW 650 W, 10 min pH 6 citrate buffer	1:30000	Normal neonatal rabbit testes

MW= Microwave; Min= Minutes; *: Dako Corporation, Carpinteria, CA, USA; ‡: Serotec Corporation, Oxford, UK; †: Zymed, San Francisco, CA, USA; §: Novocastra, Newcastle, UK; □: Santa Cruz Biotechnology, Inc, CA, USA.

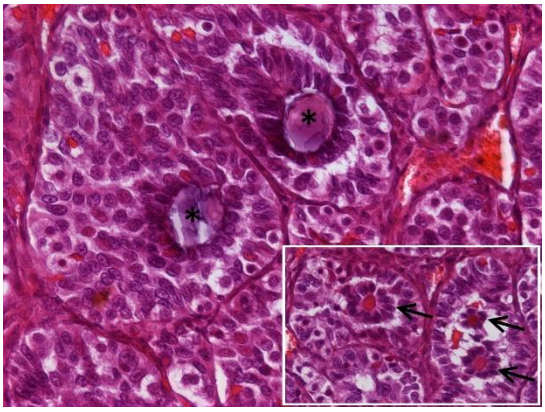

Fig. 1. Rabbit, retained left inguinal testis effaced by Sertoli cell tumour (SCT) composed of tubules surrounded by thick basement membranes and lined by 1-2 layers of tall, slender palisading neoplastic cells. Neoplastic Sertoli cells (SCs) had a basally located nucleus with reticular chromatin pattern and one prominent, centrally located, magenta nucleolus. Anisokaryosis and anisocytosis were moderate, and mitotic figures ranged from 0 to 1 per high power field (400×). Numerous tubules were also expanded by the presences of large, multiple, laminated round basophilic mineral concretions (microliths, asterisk), multifocally surrounded by SCs in a radiating pattern (H&E, 200X). ***Inset*:** Multifocally, intratubular Call-Exner bodies (arrows) (neoplastic SCs arranged in rosettes surrounding microcavities filled with hyaline eosinophilic amorphous material) were also observed (H&E, 200X).

The percentage of immunolabelled cells was assessed semi-quantitatively and scored as follows: - = negative; + = less than 10%; ++ = 11-40%; +++ = 41-80%; ++++ = 81-100%, in line with a previous report (Banco *et al*., 2016).

Vimentin (VIM) and AMH were diffusely expressed in the cytoplasm of neoplastic SCs (++++, 81-100%) (Fig. 2 and Fig. 3). Desmin (DES) (Fig. 4) and cytokeratins (CKs) (Fig. 5) were multifocally expressed, and the percentage of positive cells was assessed as +++ (41-80%) and ++ (11-40%), respectively. INH-α was not expressed by the neoplastic SCs.

Discussion

Testicular neoplasms are infrequently reported in rabbit species (Anderson *et al*., 1990). In addition, male pet rabbits are usually neutered in the first years of life for prevention of breeding, reduction of urine spraying, and to decrease social aggression. Among testicular tumours in lagomorphs, interstitial cell tumours (Leydig cell adenoma) are the most represented (Flatt and Weisbroth, 1974; Zwicker and Killinger, 1985), followed by seminoma (Brown and Stafford, 1989; Anderson *et al*., 1990; Banco *et al*., 2017) and simultaneous development of bilateral testicular tumour with dissimilar histology (a seminoma and interstitial cell tumour) (Roccabianca *et al*., 1999; Veeramachaneni and Vandewoude, 1999). Moreover, a case of carcinoma in situ (Veeramachaneni and Vandewoude, 1999), considered a pre-neoplastic lesion of seminoma, a case of metastasizing seminoma (Banco *et al*., 2012), a case of granular cell tumour (Irizarry-Rovira *et al*., 2008), and two cases of testicular gonadoblastoma have been recently described in rabbits (Suzuki *et al*., 2011).

The presence of SCT in rabbit species is signaled (Ness, 1998), but papers about the histological and immunophenotypical characterization are not present in literature.

Among domestic animals, SCT is considered rare, except in the dog, where SCT is frequently described in cryptorchid testes, but has been occasionally reported also in the stallion, ram, cat, and bull (Agnew and MacLachlan, 2017). Histologically, the SCT described in this report had comparable morphological features described in other domestic animals (Kennedy *et al*., 1998; Agnew and MacLachlan, 2017) and in human beings as well (Sesterhenn *et al*., 2004).

In the current case, the tumour was expansile, had an intratubular growth, was densely cellular, with a low mitotic count, and scattered Call-Exner bodies. These structures are considered a diagnostic feature of granulosa cell tumours, most often evident in the microfollicular pattern, and have been widely described, in ovarian pathology, both in human and veterinary medicine (Kennedy *et al*., 1998; Kurman *et al*., 2014).

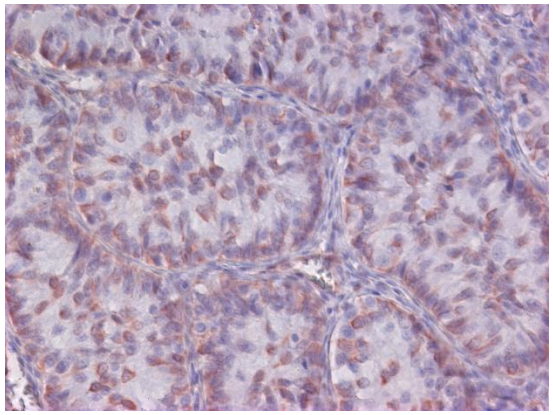

Fig. 2. Rabbit, retained left inguinal testis effaced by SCT. Immunohistochemical staining anti- vimentin (VIM). Numerous neoplastic SCs (red stained) were characterized by diffuse intracytoplasmic expression of VIM (AEC chromogen, 200X).

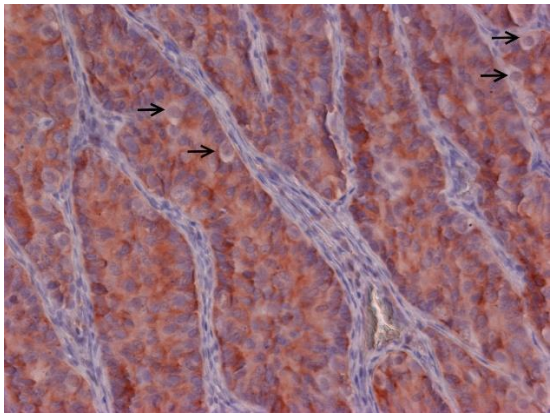

Fig. 3. Rabbit, retained left inguinal testis effaced by SCT. Immunohistochemistry anti- anti- Müllerian-Hormone (AMH). Diffuse intracytoplasmic staining of neoplastic SCs was evident. Scattered round germ cells (consistent with spermatogonia), interspersed with the neoplastic SCs, were unstained (long arrows) (AEC chromogen, 200X).

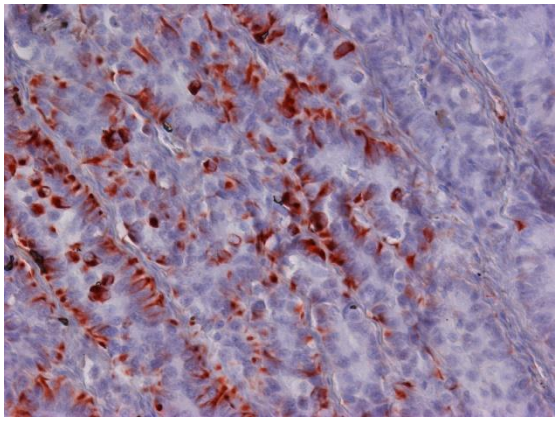

Fig. 4. Rabbit, retained left inguinal testis effaced by SCT. Immunohistochemistry anti-desmin (DES). Intracytoplasmic staining of neoplastic SCs, multifocally evident within neoplastic tubules (AEC chromogen, 200X).

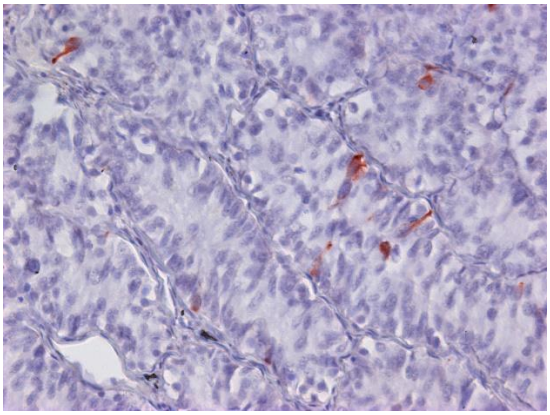

Fig. 5. Rabbit, retained left inguinal testis effaced by SCT. Immunohistochemistry anti- cytokeratins (CKs). Intracytoplasmic staining of scattered neoplastic SCs (AEC chromogen, 200X).

Regarding testicular pathology, Call-Exner bodies have been also reported in bovine and canine SCTs as well (Ladds and Saunders, 1976; Hauser and Wild, 1978; Bazzo *et al*., 2002; Masserdotti *et al*., 2008; Banco *et al*., 2010). The function of Call-Exner bodies is still debated, but it has been hypothesized that they could represent an attempt to form basement membranes (Hauser and Wild, 1978).

The current SCT was also characterized by a diffuse presence of intratubular, multilaminar, mineralized concretions, interpreted as microliths, a feature occasionally described in veterinary medicine, reported as "ovum-like bodies" in the seminiferous tubules of ectopic testis from a goat, a cat, and a rabbit almost a century ago (Crew and Fell, 1922). Such intratubular microliths have been also reported more recently in retained equine testes by McEntee (1990) and also in a testis from a clinically healthy cynomolgus monkey (Shirai and Evans, 2018).

In human medicine, testicular microlithiasis is an uncommon entity of unknown etiology that results in formation of intratubular calcifications within the seminiferous tubules. The mechanism of testicular microliths formation is not certain. They are thought to originate from degenerating intracellular debris, surrounded by concentric layers of stratified collagen fibers, followed by calcification of the glycoprotein material (Vegni-Talluri *et al*., 1980; Nistal *et al*., 1995). Testicular microlithiasis has been reported associated with several pathological condition, including cryptorchidism, testicular dysgenesis, infertility, testicular atrophy, testicular torsion, Kleinfelter syndrome, hypogonadism, testicular germ cell cancer (Vegni-Talluri *et al*., 1980; Winter *et al*., 2016), and even in normal testes (Shanmugasundaram *et al*., 2007). Regarding human SCTs, they account for less than 1% of all testicular tumours, and intratubular

microliths can be rarely found associated to this condition (Sesterhenn *et al*., 2004; Brehm *et al*., 2006). Immunohistochemistry is increasingly used in veterinary oncology both for diagnostic and research purposes, but there are only a few reports in the literature concerning the immunophenotype of rabbit testicular neoplasm (Marino *et al*., 2003; Maratea *et al*., 2007; Irizarry-Rovira *et al*., 2008; Suzuki *et al*., 2011; Banco *et al*., 2017). Recently, an immunohistochemical study focused on the phenotype of SCs across the maturational phases of rabbit testicular development from neonates to adults has been published (Banco *et al*., 2016). Results showed that rabbit SC cytoplasm is characterized by the expression of different immunohistochemical markers, paralleling the physiological, functional, and morphological maturation of the testis, from neonatal, to pubertal, and adult age. In normal rabbit testes, CKs and AMH were expressed by SCs only in immature testes (neonatal and prepubertal), while mature SCs were negative for these markers (Banco *et al*., 2016). In the current SCT, more than 80% of neoplastic SCs showed a diffuse staining for AMH, and also the expression of CKs was observed in a lesser percentage of neoplastic SCs (varying from 11-40%). These results suggest that, during neoplastic transformation, rabbit neoplastic SCs revert to an immature immunophenotype. In addition, in the current case, the expression of DES was demonstrated in numerous neoplastic SCs, ranging from 41-80%. In human testes, DES has been demonstrated in foetal ancestors' SCs, at the level of mesonephros and in immature SCs between weeks 11 and 14 of gestation. Afterwards, SCs undergo transient epithelial transformation, losing DES and acquiring CKs expression (Pelliniemi *et al*., 1993; Rogatsch *et al*. 1996). In postnatal rabbit testes, DES was not observed in SCs (Banco *et al*., 2016), and was hypothesized that, also in this species, DES could be expressed by SCs only during the embryogenesis of the male gonad, as a marker of SCs immaturity. Interestingly, as observed in immunohistochemical studies on canine SCTs (Yu *et al*., 2009; Banco *et al*., 2010), DES was expressed in a limited number of canine SCTs, suggesting that DES, similar to the aforementioned CKs and AMH, could be considered a marker of SC regression to an undifferentiated immunophenotype.

VIM, constantly expressed by normal rabbit SCs (from neonates to adults) (Banco *et al*., 2016), was expressed by numerous neoplastic SCs (81-100%) of the present case, paralleling the results obtained in human and canine SCTs (Sesterhenn *et al*., 2004; Yu *et al*., 2009; Banco *et al*., 2010). This finding suggest that VIM, a cytoskeletal protein, represents a structural marker conserved in SCs across their maturational stages as well as during neoplastic transformation.

Conclusion

To the best of authors' knowledge, this is the first report of a testicular SCT in a pet rabbit immunohistochemically investigated. Immunohistochemical results confirmed that neoplastic SCs expressed markers of immaturity, such as CKs, DES, and AMH, as already described both in human and canine species. Further studies, on a larger number of rabbit SCTs are needed to clarify if this species could play a role as a spontaneous model for human SCT.

Conflict of interest

The Authors declare that there is no conflict of interest.

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