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An immunohistochemical study of the presence and distribution of neuronal and glial markers in simple testicular cysts in the ostrich (*Struthio camelus*)

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Summary

Simple testicular cysts are rare in birds and mammals. However, the condition has recently been reported in the ostrich (*Struthio camelus*), an economically important farmed bird. The innervation of normal and cryptorchid testes, unlike the simple testicular cysts of birds and mammals, has received considerable attention. This study, therefore, immunohistochemically demonstrates the presence and the general distribution pattern of neuronal and glial markers in the simple intratesticular cyst, and its associated structures, of fourteen adult ostriches using antibodies to neurofilament protein, S-100, neuron-specific enolase and protein gene product 9.5. The LSAB+ Kit® (DakoCytomation, Denmark) immunostaining protocol was used in this study. The normal seminiferous peritubular tissue showed few or no immunoreactive nerve fibres. A greater density of neurofilament protein, S-100, neuron-specific enolase and protein gene product 9.5 immunopositive nerve fibres were observed in the tunica albuginea adjacent to the cyst, as well as in the peritubular connective tissue of cystic seminiferous tubules. In addition, the tunica adventitia of blood vessels within the interstitial space of the cystic seminiferous tubules displayed neurofilament protein, S-100 and protein gene product 9.5 immunoreactive nerve fibres of varying intensity and pattern. Protein gene product 9.5 immunostaining was also observed in the multinucleated giant cells of both the normal and the cystic seminiferous tubules. The cystic portion of the testis appears to have a richer innervation than the normal portion of the same testis. The richer innervation of simple testicular cysts in the ostrich is similar to that observed in the cryptorchid testis of mammals.

Key words

Ostrich; simple testicular cysts; innervation; immunohistochemistry.

Introduction

Simple testicular cysts are rare in both birds and mammals, but have been reported in turkey poults (Dewar and Siller, 1971), domestic fowl (Siller *et al.*, 1972), cat and fox (Gelberg and McEntee, 1983), horse (Schumacher *et al.*, 1994; Palmer *et al.*, 1995), dog (Wakui *et al.*, 1997), man (Mancilla-Jimenez and Matsuda, 1975; Heetderks and Hommerson, 1988; Dmochowski *et al.*, 1989; Nistal *et* al., 1989; Garcia *et al.*, 1999; Ceylan *et al.*, 2004; Mondaini *et al.*, 2006) and ostrich (Aire *et al.*, 2003).

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Simple testicular cysts, according to Dmochowski *et al.* (1989), Garcia *et al.* (1999) and Aire *et al.* (2003), are located within the parenchyma of the testis, contain clear, transparent fluid in the lumen, and are lined by a single layer of flat or cuboidal epithelial cells in a separate fibrous wall from that of the tunica albuginea. Furthermore, simple cysts of the testis lack both teratomatous elements in the cyst wall or testicular parenchyma, as well as chronic inflammation and fibrosis in the rest of the testis (Dmochowski *et al.*, 1989). Siller *et al.* (1978), Rothwell (1978), Aire *et al.* (2003) and Mondaini *et al.* (2006) have described the histological and ultrastructural changes associated with simple cysts of the testis in man and various species of birds.

The innervation of the testicular capsule and peritubular tissue of normal as well as cryptorchid testes has received considerable attention, using ultrastructural, histological, histochemical and immunohistochemical techniques (Bennett and Malmfors, 1970; Tingari and Lake, 1972; Hargrove and Ellis, 1976; Carvalho *et al.*, 1986; Davidoff *et al.*, 1999; Sienkiewicz *et al.*, 2000; Marettová *et al.*, 2002; Saleh *et al.*, 2002). However, there is, apparently, no information on the innervation of the simple intratesticular cyst of birds and mammals. The present study, therefore, characterises the neurofilament protein, S-100, neuron-specific enolase and protein gene product 9.5 immunoreactivity in the tunica albuginea, the peritubular tissue, the simple cyst and the normal parenchyma of the testis in the ostrich (*Struthio camelus*), an economically important farmed bird that is associated with low reproductive performance (Deeming and Ar, 1999).

Materials and Methods

An archival collection of twelve paraffin-embedded simple testicular cyst tissues and two additional fresh samples were used in this study. Briefly, fourteen testes were collected from sexually mature male ostriches slaughtered at the local abattoirs. Testicular tissues containing multifocal, fluid-filled, unilocular cysts were immediately immersed in an ample volume of Bouin's fluid. The tissue samples were subsequently carefully examined in the laboratory and appropriate tissue samples (testicular capsule and parenchyma composed of intermingled intratesticular simple cysts and normal seminiferous tubules) from each testis were removed and fixed further in fresh Bouin's fluid for a total of 12 to 24 hrs.

The Bouin's fluid-fixed testicular tissue was processed routinely and embedded in paraffin wax. Tissue sections of 5 μm thickness were mounted on slides precoated with polylysine, deparaffinized and treated with 3% (v/v) hydrogen peroxide in distilled water for five minutes to inhibit endogenous peroxidase. After blocking the non-specific reaction, the sections were subsequently microwaved at 750 W for two cycles of seven minutes each in citrate buffer (pH 6). Thereafter, the slide was allowed to cool at room temperature for 20 minutes before being rinsed in phosphate buffered saline (PBS) containing bovine serum albumin (pH 7.6) for 5 minutes and then incubated with neurofilament protein (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom; type M of 160 kD; code number NCL-NF160-NN18), S-100 (DakoCytomation, Glostrup, Denmark ; Z 0311), protein gene product 9.5 (DakoCytomation; Z 5116), and neuron specific enolase (DakoCytomation; M 0873); primary antibodies were applied at dilutions of 1:25, 1:400, 1:35 and as ready-to-use solution, respectively, for one hour at room temperature, following the protocol of LSAB+® kit

(DakoCytomation). After rinsing in PBS, each slide was incubated for 15 minutes, at room temperature, in a biotinylated secondary antibody, then rinsed in PBS and incubated at room temperature for another 15 minutes in peroxidase-labelled streptavidin. The antigen localization was visualised by incubation of the sections with 3,3-diaminobenzidine (LSAB+® kit). The sections were counterstained with haematoxylin for 30 seconds before being dehydrated in graded concentrations of ethanol.

Negative control sections were incubated with either normal mouse or rabbit serum in the absence of primary antibody. Positive controls were performed with histological sections of the brain.

Results

Testicular capsule, peritubule and interstitium

NORMAL SEMINIFEROUS TUBULES OF THE TESTIS

The peritubule and the interstitium of the normal seminiferous tubules were, generally, immunonegative for the pan-neuronal and glial nerve markers used in this study. The tunica media of their accompanying blood vessels were similarly negative at immunoreactions.

SIMPLE INTRATESTICULAR CYSTS

Neurofilament protein (NP)

Large and intensely neurofilament protein-immunoreactive nerve bundles were present throughout the tunica albuginea of the testicular capsule, either freely or accompanying the blood vessels (Fig. 1) of simple intratesticular cysts. Numerous medium-sized, but intensely NP-immunostained nerve fibres were distributed in the peritubular boundary tissue of cystic seminiferous tubules (Fig. 1). Some nerve fibres were also observed to run freely in the intertubular connective tissue of adjacent cystic seminiferous tubules. Very strongly NP immunopositive nerve fibres were equally observed to run along the tunica media of blood vessels found in the interstitial spaces of cystic seminiferous tubules (Fig. 1). The pericystic fibrous tissue capsule also showed few medium-sized strongly NP immunoreactive nerve fibres (Fig. 1).

S-100

Several strongly S-100 immunopositive nerve fibres were associated with the tunica albuginea of the testicular capsule, the intertubular connective tissue and the peritubular connective tissue of the cystic seminiferous tubules (Fig. 2). Strongly S-100 immunoreactive nerve fibres were also observed in the tunica media of blood vessels in the interstitium of the cystic seminiferous tubules (Fig. 2).

Neuron-specific enolase (NSE)

The tunica albuginea showed nerve bundles that strongly immunoreacted for NSE (Fig. 3). Strongly NSE immunopositive nerve fibres were observed in the tunica media of the intertubular blood vessels of simple intratesticular cysts (Fig. 3).

Protein gene product 9.5 (PGP 9.5)

Nerve bundles that were intensely immunopositive for PGP 9.5 run within the tunica albuginea (Fig. 4). Strongly immunopositive nerve fibres were observed amongst the peritubule and the interstitium of simple cysts (Fig. 4). In addition, the tunica media of
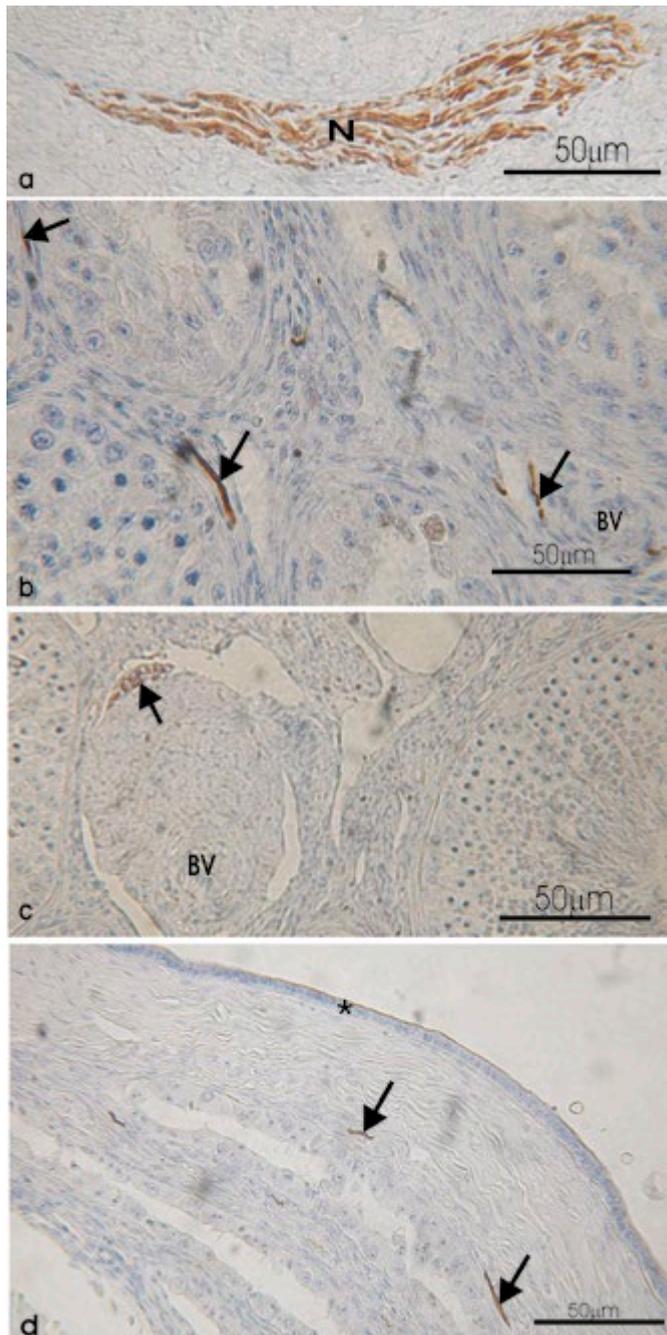
Figure 1 – Testicular capsule (a), peritubular tissue (b), blood vessel (c) and wall of simple cyst (d) immu-nostained for neurofilament protein (NP). Arrows = nerve fibres. Asterisk = epithelial lining of mature cyst. BV = blood vessel. N = nerve bundle.
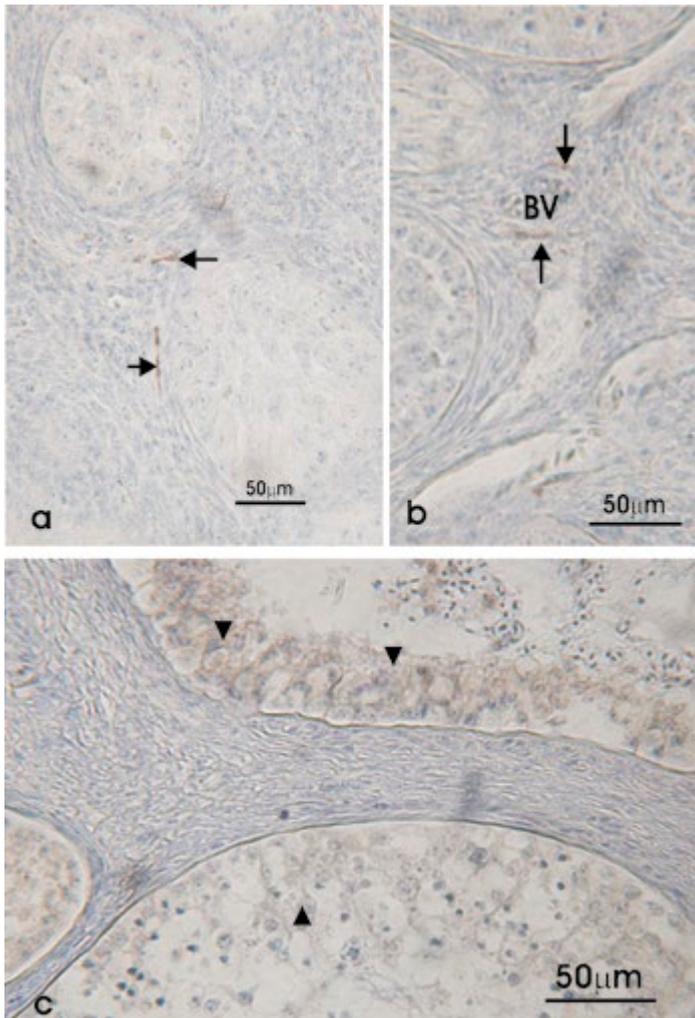
Figure 2 – [=original Fig. 4]: Peritubular tissue (a), blood vessel in the interstitial space (b), and maturing cystic seminiferous tubules (c) immunostained for S-100. Arrows = nerve fibres. Arrowheads = cytoplasmic processes of Sertoli cells. BV = blood vessel.

blood vessels within the intertubular connective tissues in the proximity of such cystic seminiferous tubules displayed PGP 9.5 immunoreactive nerve fibres (Fig. 4).

Parenchyma

NORMAL SEMINIFEROUS TUBULES
Neuron-specific enolase (NSE)
Weak to moderate NSE-immunostaining was exhibited by the cytoplasm of Sertoli cells lining the normal seminiferous tubules as shown in Figure 3.
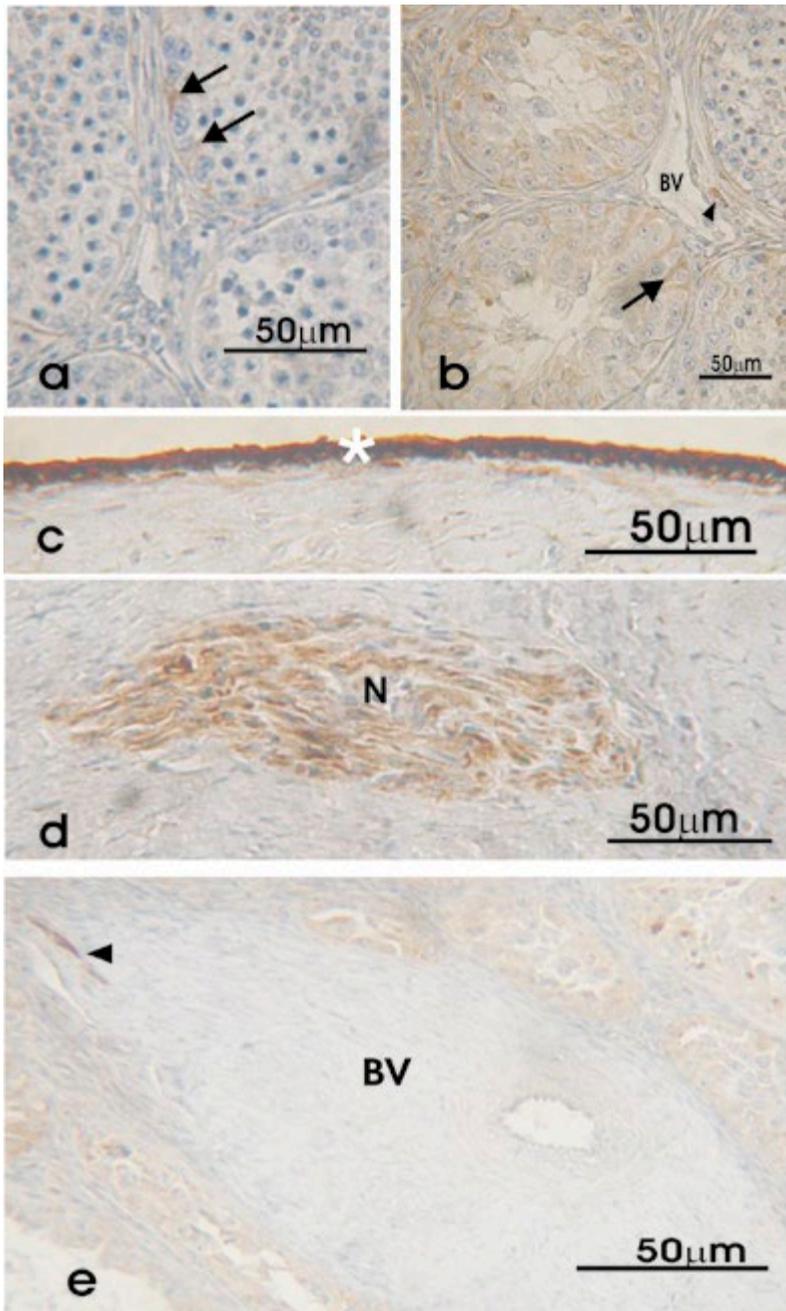
Figure 3 – [=original Fig. 2]: Normal seminiferous tubules (a), degenerating seminiferous tubules (b), wall of simple cyst (c), testicular capsule (d) and blood vessel within the interstitial space (e) immunostained for neuron-specific enolase (NSE). Arrows = cytoplasmic processes of Sertoli cells. Arrowheads = nerve fibres. Asterisk = epithelial lining of matured cyst. BV = blood vessel. N = nerve bundle.
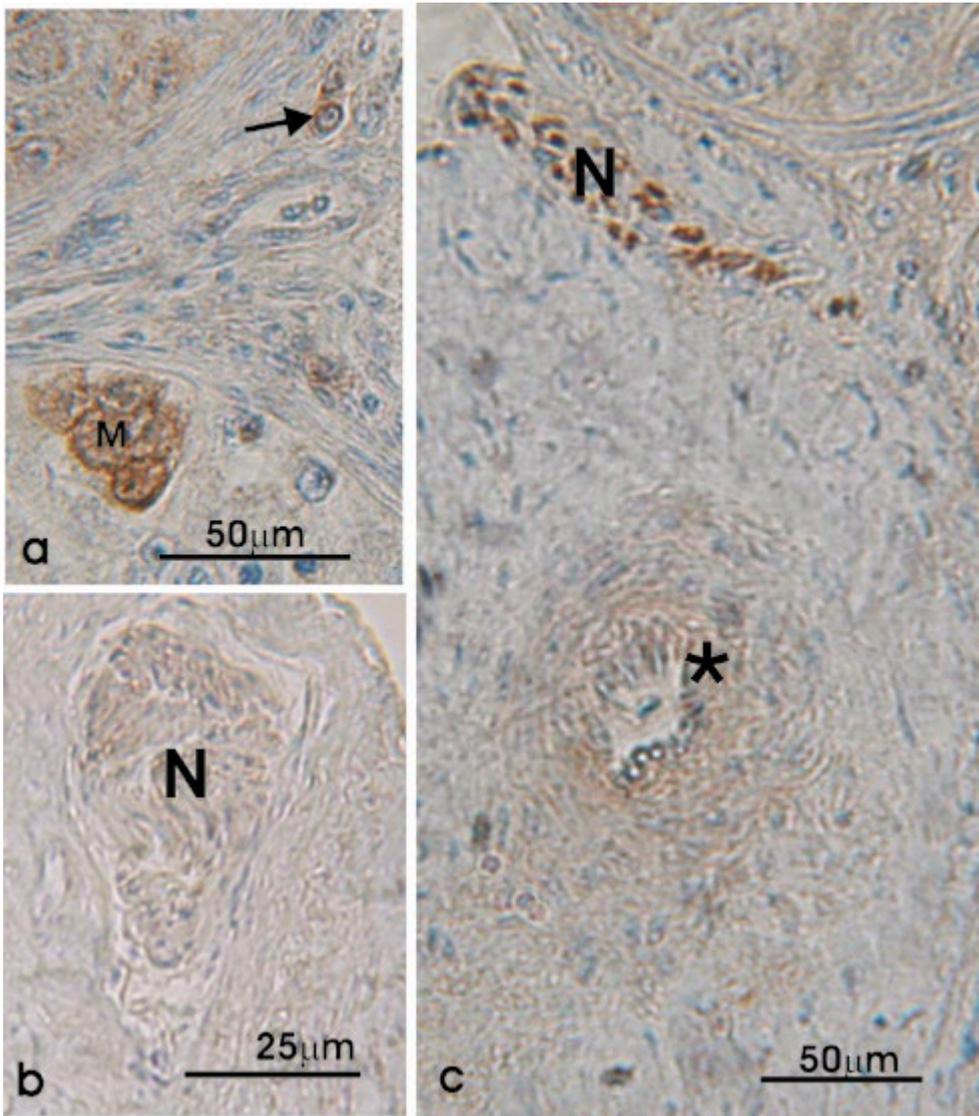
Figure 4 – [=original Fig. 3]: Part of seminiferous tubule (a), testicular capsule (b) and blood vessel in the interstitial space (c) immunostained for protein gene product 9.5 (PGP 9.5). Arrow = Leydig cell. Asterisk = blood vessel. M = multinucleated giant cell. N = nerve bundle.

Protein gene product 9.5 (PGP 9.5)

An intense immunostaining for PGP 9.5 was observed in the multinucleated giant cells of the normal seminiferous tubules as shown in Figure 4.

SIMPLE INTRATESTICULAR CYSTS

Neurofilament protein

Sertoli cells and the germinal epithelium of the atrophic seminiferous tubules, maturing and matured simple cysts were immunonegatively stained for neurofilament protein.

S-100

The cytoplasm of epithelial cells lining cysts at their early and mid-formative stages was strongly S-100 immunopositive. However, the simple cuboidal epithelium of the wall of the full-formed/mature cyst was S-100 immunonegative. The cytoplasmic processes of Sertoli cells of cysts at their maturing stages were weakly to moderately S-100 immunopositive (Fig. 2).

Neuron-specific enolase (NSE)

Weak to moderate NSE-immunostainings were exhibited by the cytoplasm of Sertoli cells lining the immature cysts at their early formative stages as shown in Figure 3. However, very strong NSE-immunostaining was shown by the cytoplasm of the simple cuboidal epithelium, composed of Sertoli cells, lining the wall of matured/full-formed cyst (Fig. 3) and the multinucleated giant cells of cysts at their mid-formative stages.

Protein gene product 9.5 (PGP 9.5)

Multinucleated giant cells of cystic seminiferous tubules were intensely immunostained for PGP 9.5 as shown in Figure 4. The germinal cell, unlike the Sertoli cell, of cysts at the early as well as the mid-formative stages were moderately to strongly immunostained for PGP 9.5.

Discussion

The majority of samples used in this study were obtained from the museum specimens of Aire *et al.* (2003). The testicular cysts and the normal seminiferous tubules observed in this study, therefore, displayed formative stages and morphological features similar to those described by Aire *et al.* (2003).

The innervation of the normal testis has been described in mammals and birds (Bennett and Malmfors, 1970; Tingari and Lake, 1972; Hargrove and Ellis, 1976; Carvalho *et al.*, 1986; Marettová *et al.*, 2002; Saleh *et al.*, 2002), breeding and non-breeding primates (Tokunaga *et al.*, 1999) as well as the cryptorchid testis (Davidoff *et al.*, 1999; Sienkiewicz *et al.*, 2000), using various investigative techniques. Marettová *et al.* (2002) described the innervation of the testicular capsule of pigeons, using S-100 only. The innervation of the testicular capsule of the domestic fowl, however, was not described by both Bennett and Malmfors (1970) and Tingari and Lake (1972). Equally, the innervation of simple cysts of the testis of either mammals or birds has not been described previously. The present study has shown that glial and neuronal markers are variably immuno-expressed in the cystic testis of the ostrich as was observed in the immature ovary of the female ostrich (Kimaro and Madekurozwa, 2006). The study has also shown that the simple testicular cysts of the ostrich are more immunoreactive to the neuronal and glial markers than the normal seminiferous tubules of the same testis.

The nerve fibres/bundles of simple testicular cysts of the ostrich were best immunolabelled by NP in this study. The reason for the increased density of immunoreac-

tive nerve fibres in the simple cysts of seminiferous tubules relative to the normal seminiferous tubules of the ostrich is not clear. However, both Sienkiewicz *et al.* (2000) and Saleh *et al.* (2002) demonstrated an increased distribution of nerve fibres in a cryptorchid testis of the pig and in a normal winter testis of the non-breeding/non-sexually active camel, respectively. Wrobel and Brandl (1998), on the other hand, reported age-dependent changes in the testicular innervation of the pig. Sienkiewicz *et al.* (2000) opined, and Saleh *et al.* (2002) confirmed, that the increased testicular innervation is not as a result of increased density of the tissue but rather may be indicative of a possible existence of an inverse relationship between nervous and endocrine activities in the testis. Such an inverse relationship between nervous and endocrine activities may be responsible for the differences in immuno-expression of glial and neuronal markers between the normal and cystic seminiferous tubules in this study.

The increased density of PGP 9.5 immunoreactive nerve fibres within the peritubular connective tissue of cystic seminiferous tubules, relative to the normal tubules, confirms the earlier observation of Sienkiewicz *et al.* (2000) that the nerve fibres in hormonally inactive testes of pigs are more PGP 9.5 immunoreactive than those found in the hormonally active pigs.

Although the associated structures of the simple cyst of the testis of the ostrich were more S-100 immunopositive than those of the normal seminiferous tubules, the cystic testes of the ostrich, in this study, were generally less S-100 immunoreactive than those observed in the normal testis of the pigeon, a bird reported by Marettová *et al.* (2002) to have sparsely distributed S-100 immunoreactive nerve fibres in the tunica albuginea. This variation could be due to the pronounced species-specific differences that Sugimura *et* al. (1990) observed to exist in the intensity of S-100 immunoreactivity in vertebrates.

A quick comparison between the distribution of NP-immunoreactive nerve fibres in the cystic testis of ostrich, in this study, and the normal testis of the guinea-pig and rat (Carvalho *et al.*, 1986) shows that the cystic testis of the ostrich has a richer innervation than that of either mammal.

Multinucleated giant cells of the testis of the ostrich are an accumulation of degenerating germ cells during the involuting/regressing phase of the reproductive cycle (Madekurozwa *et al.*, 2002). The increased density of PGP 9.5 immunoreaction within the cytoplasm of germinal cells and multinucleated giant cells of cystic seminiferous tubules, relative to the normal tubules, confirms the earlier observation of Tokunaga *et al.* (1999) that the cytoplasm of the spermatogonia of non-breeding primates was more PGP 9.5 immunoreactive than that found in the breeding primates. PGP 9.5 is a neuron-specific cytoplasmic marker. Doran *et al.* (1983) reported that the central nervous system has the greatest concentration of PGP 9.5. This study has demonstrated that PGP 9.5 is the best marker of germ cells in a cystic testis while the NSE is best suited for the identification of the Sertoli cells. The reliability of S-100, as a marker for Sertoli cells in a cyst wall, is dependent on the stage of cyst formation, being greater in a forming than in a mature cyst.

Atrophic seminiferous tubules of simple testicular cysts appear to have a richer innervation than that of normal seminiferous tubules of the same testis. The increased innervation of the inactive seminiferous tubules of simple testicular cysts of the ostrich is similar to that observed in the cryptorchid testis of mammals.

Acknowledgements

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