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LOCALIZATION, DENSITY AND SHAPE OF SEROTONIN - AND CHROMOGRANIN A-POSITIVE NEUROENDOCRINE CELLS IN FELINE BULBOURETHRAL GLANDS

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ABSTRACT

The current study aimed to investigate the presence, type and localization of endocrinocytes containing serotonin and chromogranin A (NE) in feline bulbourethral glands and their role in the functioning of those organs.
Bulbourethral glands of 8 sexually mature, clinically healthy male European shorthair cats (*Felis catus*) aged 1−2 years and weighing 2.8−4 kg were studied. Collected specimens were processed by routine techniques and embedded in paraffin. Immunohistochemical assay was carried out with primary antibodies against human serotonin and chromogranin A using the avidin-biotin peroxidase technique.
Neuroendocrine cells (NE) were mainly observed in the tubular epithelium. In the glandular stroma, only single serotonin-positive endocrinocytes (SNE) were found. The density of serotonin-expressing endocrinocytes was higher than that of chromogranin A-expressing cells (ChNE), while the latter were larger and of a various shape than the former. The morphology of studied neuroendocrine cells was of both open-type and closed-type.
We propose that neuroendocrine cells in feline bulbourethral glands were involved in the protection of glandular epithelial cells, in homeostatic regulation of glands‘ secretion and semen and urinary excretion.

Key words: bulbourethral glands; neuroendocrine cells; cat

INTRODUCTION

Neuroendocrine cells (NE) are a specialized group of nervous cells, producing neuropeptides. Endocrinocytes are identified by the presence of neurosecretory granules and their potential to express a numerous markers (serotonin, chromogranin A or B, somatostatin etc.). They are involved in the homeostatic regulations of glands where they are located. Morphologically, they are polymorphic cells with irregular processes located within glandular epithelial cells. A number of authors have observed serotonin-positive and chromogranin A-positive endocrinocytes of low density. They are situated among adjacent epithelial cells reaching the alveolar lumen (open-type) or remaining entirely surrounded by glandular epithelium (closed-type) without reaching the lumen (Ismail *et al*., 2002; Patnaik *et al*., 2005; Aprikian *et al*., 2006).

Neuroendocrine cells are a common finding in prostate gland, prostatic urethra and urinary bladder mucosa in men (Fetissof *et al*., 1983, Battaglia *et al*. 1994, Guy *et al*. 1998). A large amount of neuroendocrine cells (paraneurons) are observed in the prostatic and urethral epithelium in sheep (Vittoria *et al*., 1990). Depending on

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neuropeptides in their secretory granules, four types of endocrinocytes were identified: serotonin-, chromogranin A-producing, producing both peptides and somatostatin-producing. The presence of serotonin in urinary organs and accessory genital glands in sheep was related to semen and urinary emissions (Vittoria *et al*., 1990).

Serotonin- and chromogranin A-positive endocrinocytes are detected in prostate and bulbourethral glands in boars, bulls, horses and donkeys, as well as in prostate glands of rats, guinea pigs, dogs and cats (Angelsen *et al*., 1997), but there are no data about their occurrence in feline bulbourethral glands.

The purpose of the present investigation was to explore the presence, type and localization of serotonin- and chromogranin A-containing endocrinocytes in feline bulbourethral glands and to suggest their role in the function of these organs.

MATERIAL AND METODS

The investigation was performed with 8 sexually mature, healthy male European shorthair cats (*Felis catus*), at the age of 1-2 years, weighing 2.8 to 4 kg.

The study was approved by the institutional committee of animal care. The experiment was performed in strict compliance with the ethical guidelines for humane treatment of animals as per European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasburg, 16.05.1986), the European Convention for the Protection of Pet Animals (Strasburg, 13.11.1987) and Law on Animal Protection in the Republic of Bulgaria –Section IV- Experiments with animals.

Cats were euthanized with intravenous injection of 200 mg Thiopental® (50 mg/kg i v) (thiopental sodium 1000 mg in 5 ml sterile isotonic solution; Biochemie, Austria) into the cephalic vein (Posner and Burns, 2009) according to the Guidelines of the American Veterinary Association Panel on Euthanasia.

The material was obtained immediately after approach to the pelvic cavity and removal of bulbourethral glands. Organ specimens were fixed into 10% neutral formalin and embedded in paraffin. Immunohistochemical staining for detection of serotonin- and chromogranin A-positive neuroendocrine cells (NE) was done by the avidin-biotin peroxidase method. Tissue sections of 5 µm were dehydrated, incubated in 10 % sucrose solution for 4 h and rinsed with 0.1 M phosphate buffer (pH 7.4). For inhibition of endogenous peroxidase, tissue sections were dipped in 1.2 % hydrogen peroxide in methanol for 30 min and rinsed with 0.1M phosphate buffer, pH 7.4, for 15 min. Then they were incubated with normal mouse serum (DAKO) for 30 min to block non-specific binding. Further, the slides were incubated with primary mouse/rabbit anti-serotonin and anti-chromogranin A antibodies for 24 h and rinsed with 0.1M phosphate buffer, reincubated with biotin-conjugated secondary anti-mouse/anti-rabbit antibodies (DAKO LSAB® 2 system, HRP K0675) for 4 h and finally with streptavidin-HRP complex (DAKO LSAB® 2 system, HRP K0675) for 4 h.

The immune reaction was visualized in a solution of 3 mg 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis MO, USA) in 15 ml 0.1M phosphate buffer, pH 7.4 and 36 ml 30 % hydrogen peroxide for 10–20 min. Tissue sections were dehydrated and embedded in Entellan.

The negative control was run on the same way with incubation of tissue sections with non-immune serum instead of the primary antibody (Gulubova & Vlaykova, 2008; Gulubova *et al.*, 2008).

The following immune reagents were used:
- polyclonal rabbit antibody against human chromogranin A (N1535, DAKO A/S Denmark)
- monoclonal mouse antibody against human serotonin (M0758, DAKO A/S Denmark) diluted 1:100
- Detection system: Immunostaining kit DAKO LSAB® 2 System, HRP (K0675, DAKO)
- DAKO® DAB Chromogen tablets (S3000, DAKO)

Light microscopy was performed with a light microscope Leica DM 2500 (Germany), and results were photographed with a digital camera Leica DSC 290 (Germany).

The density of neuroendocrine cells (NE/mm^2) and dimensions (in µm) of glandular endocrinocytes were determined with a standardized eyepiece micrometer.

The statistical analyses were performed with StatView™ v. 4.53 for Windows (1995) software. We used 24 organ specimens from bulbourethral glands of investigated animals and made descriptive statistics of values for density, length and width of serotonin-positive (SNE), and chromogranin A- positive (ChNE) neuroendocrine cells.

RESULTS

Serotonin- and chromogranin A-positive neuroendocrine cells (NE) were mainly observed in the tubular epithelium of glandular parenchyma (Figs. 1 and 4). In the stroma, only single serotonin-positive endocrinocytes (SNE) were seen (Figs. 2 and 3).

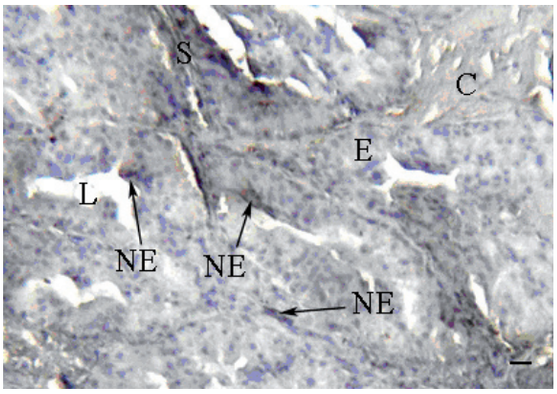

Fig. 1: Bulbourethral glands (organ specimens of 8 sexually mature, healthy male European shorthair cats (*Felis catus*), 1-2 years, weighing 2.8 to 4 kg; fixed into 10 % neutral formalin and embedded in paraffin; immunohistochemical staining - avidin-biotin peroxidase method): serotonin-positive neuroendocrine (NE) cells, localized in apical parts of glandular tubular epithelium (E); tubular lumen (L), stroma (S), capsule (C). Bar = 20 μm

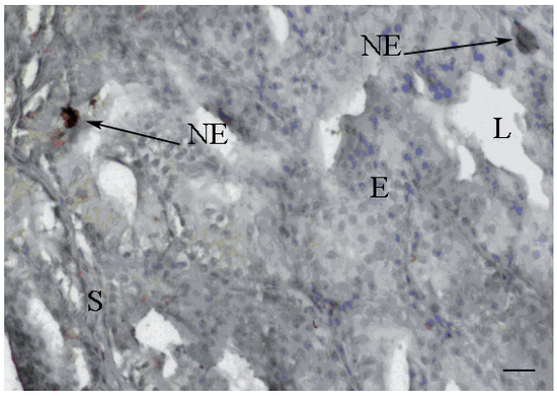

Fig. 2: Bulbourethral glands (organ specimens of 8 sexually mature, healthy male European shorthair cats (*Felis catus*), 1-2 years, weighing 2.8 to 4 kg; fixed into 10 % neutral formalin and embedded in paraffin; immunohistochemical staining - avidin-biotin peroxidase method): serotonin-positive endocrinocytes (NE), localized in basal parts of glandular tubular epithelium (E); stroma (S), tubular lumen (L), Bar = 15 μm

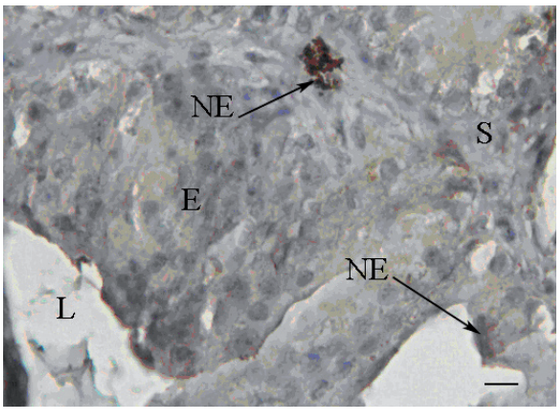

Fig. 3: Bulbourethral glands (organ specimens of 8 sexually mature, healthy male European shorthair cats (*Felis catus*), 1-2 years, weighing 2.8 to 4 kg; fixed into 10 % neutral formalin and embedded in paraffin; immunohistochemical staining - avidin-biotin peroxidase method): serotonin-positive endocrinocytes (NE), localized in apical parts of glandular tubular epithelium (E) and glandular stroma (S); tubular lumen (L). Bar = 10 μm

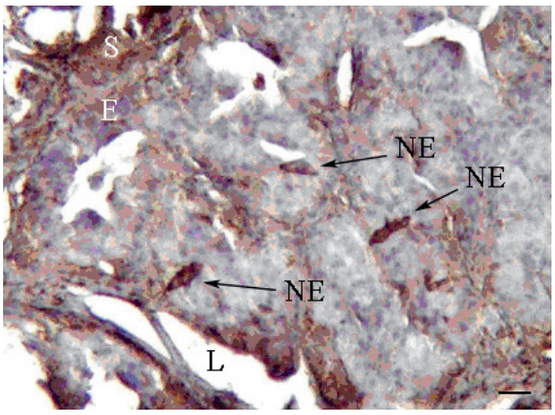

Fig. 4: Bulbourethral glands (organ specimens of 8 sexually mature, healthy male European shorthair cats (*Felis catus*), 1-2 years, weighing 2.8 to 4 kg; fixed into 10 % neutral formalin and embedded in paraffin; immunohistochemical staining - avidin-biotin peroxidase method): chromogranin A-positive neuroendocrine cells (NE), localized in basal and apical parts of glandular tubular epithelium (E); stroma (S); tubular lumen (L). Bar = 10 μm

Observed serotonin-positive cells (SNE) were with an oval, elongated oval or irregular shape and small processes (Figs. 2 and 3). Some of them were situated among the basal parts of adjacent epithelial cells, near the basal membrane (Figures 1 and 2). Another part was located among apical glandular parts and contacted the tubular lumen (Figs. 1 and 3). Serotonin-positive endocrinocytes were of both closed-type (their processes did not reach the tubular lumen) and open-type (with processes reaching the tubular lumen) (Figs. 1 and 2).

Chromogranin A-positive neuroendocrine cells (ChNE) were with elongated oval, elongated irregular or triangular shape (Figs. 5 and 6). They possessed small processes (Figs. 4 and 6). Chromogranin A-positive endocrinocytes were situated in glandular tubules and exhibited only intraepithelial localization (Figs. 4 and 5). They were also located among the basal and apical parts of secretory epithelial cells, the latter being in contact with tubular lumen (Figs. 4 and 5). The morphology of chromogranin A-expressing neuroendocrine cells was both closed-type and open-type (Figs. 4 and 6).

The average density of serotonin-positive endocrinocytes (SNE) in bulbourethral glands' epithelium of cats was 1.333 ± 0.48 SNE /mm^2. It was significantly higher than that of chromogranin A-positive cells (ChNE) (0.792 ± 0.42 ChNE/mm^2, p = 0.001, paired t-test) (Fig. 7).

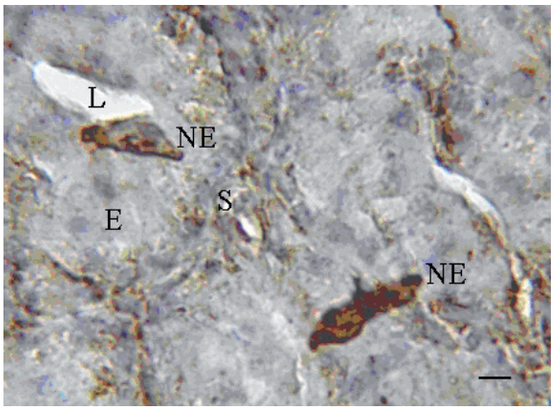

Fig. 6: Bulbourethral glands (organ specimens of 8 sexually mature, healthy male European shorthair cats (*Felis catus*), 1-2 years, weighing 2.8 to 4 kg; fixed into 10 % neutral formalin and embedded in paraffin; immunohistochemical staining - avidin-biotin peroxidase method): chromogranin A-positive neuroendocrine cells (NE), localized among the basal and apical parts of glandular tubular epithelium (E); stroma (S); lumen (L). Bar = 5 μm

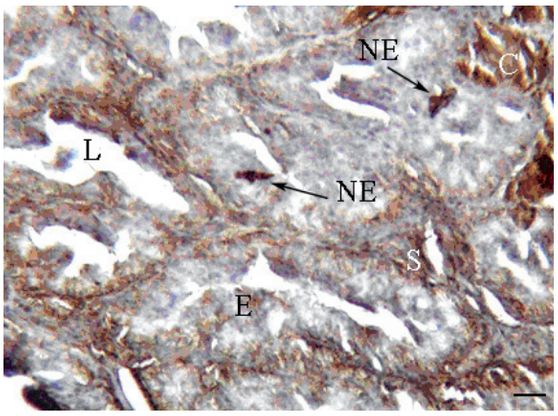

Fig. 5: Bulbourethral glands (organ specimens of 8 sexually mature, healthy male European shorthair cats (*Felis catus*), 1-2 years, weighing 2.8 to 4 kg; fixed into 10 % neutral formalin and embedded in paraffin; immunohistochemical staining - avidin-biotin peroxidase method): chromogranin A-positive neuroendocrine cells (NE), localized in glandular tubular epithelium (E) adjacently to the tubular lumen (L); stroma (S), capsule (C). Bar = 15 μm

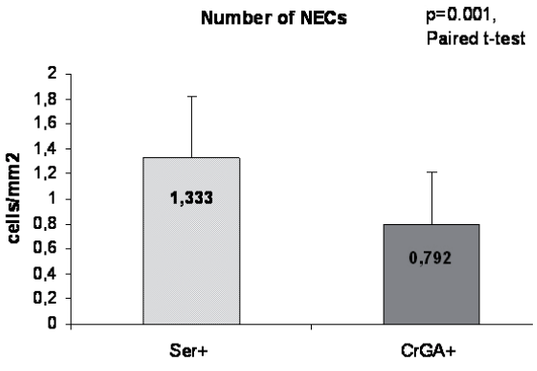

Fig. 7: Density of serotonin-positive (Ser) and chromogranin A-positive (CrGA) cells in the epithelium of feline bulbourethral glands. Values are presented as mean ±SD.

The length of serotonin-positive endocrinocytes (SNE) varied from 4.23 µm to 8.93 µm (mean 6.042 ± 1.30 µm), whereas their width from 4.1 µm to 5.9 µm (mean 4.750 ± 0.94 µm). These were significantly higher than respective values of chromogranin A-positive neuroendocrine cells (ChNE) for both dimensions ($p<0.0001$, paired t-test). The length of chromogranin A-positive cells varied from 7.23 µm to 13.15 µm (mean 9.833 ± 1.97 µm), and the width between 5.2–6.7 µm (mean 5.750 ± 0.74 µm) (Fig. 8).

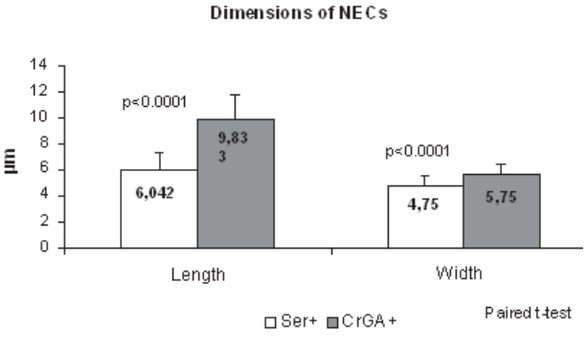

Fig. 8: Dimensions of serotonin-positive (Ser) and chromogranin A-positive (CrGA) cells in the epithelium of feline bulbourethral glands. Values are presented as mean ± SD

DISCUSSION

This is the first report concerning the serotonin- and chromogranin A-positive neuroendocrine cells (NE) in feline bulbourethral glands. Previous studies by our group (Dimitrov *et al*., 2010) as well as others (Angelsen *et al*. 1997) have described such cells in feline prostate gland.

Both types of neuroendocrine cells were with predominantly epithelial localization, except for serotonin-expressing that were rarely encountered in the glandular stroma as well. The density of serotonin-positive endocrinocytes (SNE) was bigger as compared to that of chromogranin A-positive cells (ChNE), but the latter were of a larger size (length and width) and more variable shape.

The localization and polymorphic morphology of endocrinocytes in feline bulbourethral glands observed in this study corresponded to research carried out in human and canine prostate glands (Ismail et al., 2002; Patnaik *et al.*, 2005; Aprikian *et al*., 2006). Similar to the two types of neuroendocrine cells (closed and open) as reported by the above mentioned authors, the same endocrinocyte types were identified in feline bulbourethral glands as well.

We share the opinion of Ismail *et al.* (2002), Patnaik *et al.* (2005) and Aprikian *et al*. (2006) that neuroendocrine cells were probably involved in the regulation of the homeostasis of glands‘ secretion.

The localization and the density of neuroendocrine cells observed in feline bulbourethral glands could be assumed as a normal finding similar to results reported in human prostate, prostatic urethra and urinary bladder mucosa (Fetissof *et al*., 1983). These researchers provided evidence that the presence of neuroendocrine cells was essential for the development of neoplasms in accessory genital glands and urinary organs. Taking into consideration data communicated by Fetissof *et al*. (1983), Battaglia *et al*. (1994) and Guy *et al.*, (1998) we therefore assume that the occurrence of neuroendocrine cells in the parenchyma of bulbourethral glands could be important for the development of neoplastic growth in cats as well.

Our results with regard to the shape and localization of some of endocrinocytes in close vicinity of the basal membrane or tubular lumen confirmed data reported for ovine prostate and urethra (Vittoria *et al.*, 1990). The researchers described four types of endocrinocytes with a disseminated localization in the urethro-prostatic complex of sheep and highest density in tissues near to the colliculus. The presence of serotonin-positive neuroendocrine cells in the urinary organs and accessory genital glands of sheep was thought to be related to urine and semen emissions (Vittoria *et al*., 1990).

Similar to the findings of Vittoria *et al*. (1990), we have shown that endocrinocytes in feline bulbourethral glands were disseminated in the epithelium and lacked a focal localization. Therefore, an analogous role of neuroendocrine cells in semen, urine and mucinous discharge could be also assumed in cats.

The presence of serotonin- and chromogranin A-positive neuroendocrine cells in feline bulbourethral glands adds further information to the results of Arrighi *et al.* (2004) about the presence of these cells in bulbourethral glands of boars, bulls, horses and donkeys.

Studies on the role of neuroendocrine cells in men have affirmed that their neurosecretory products potentiated the proliferation and enhanced the antiapoptotic capacity of epithelial cells (Hansson and Abrahamson, 2001). Some of neuropeptides, produced by human accessory genital glands, play the role of a growth factor by activating membrane receptors via a paracrine route or activating androgen receptors (Noordzij *et al.*, 1995). Some neuropeptides produced by endocrinocytes in the human epididymis are essential for spermatogenesis and the function of male reproductive organs. These neuropeptides exhibit not only endocrine and paracrine, but also an exocrine activity occurring in the glandular fluids composition (Martin *et al*., 2000).

CONCLUSION

Taking into consideration the assumptions about the role of endocrinocytes in the function of male reproductive organs (Martin *et al*., 2000) we believe that they could play a similar role in male cats as well. It could be therefore hypothesized that the observed two immunohistochemical types of neuroendocrine cells in bulbourethral glands are essential for spermatogenesis, semen composition, and protection of glandular epithelial cells.

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