Presence and localization of apelin and its cognate receptor in the testis of the dog using immunohistochemical and molecular biology technique.

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Short Report

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Abstract

Apelin, a member of the adipokine family, is a new endogenous peptide and it’s involved, by interacting with a specific receptor, in the control of human and laboratory animals’ male reproduction. As far as we know, no data is available about the presence of the apelinergic system in dog testicles. Therefore the aim of this study was to show, for the first time, its presence and distribution in the canine testis by immunohistochemistry and molecular biology studies. For this purpose, five fertile and healthy male dogs were used and subjected to elective orchiectomy. The immunohistochemical reaction evidenced the presence of apelin and its receptor in the canine testis. In particular, apelin appeared localized in spermatids and spermatozoa with a positive signal in the “acrosomal bodies”. Regarding to the apelin receptor, a positive immunoreaction was evidenced in the cytoplasm of the cells localized in the basal portion of the seminiferous tubules, likely Sertoli’s cells, and in the cytoplasm of Leydig cells. The RT-PCR analysis showed the presence of transcripts for apelin and apelin receptor in all the samples examined. In conclusion our result allows us to hypothesize that the role of the paracrine and endocrine apelinergic system reported in laboratory animals can also be found in the dog.

Introduction

Apelin (APLN), a member of the adipokine family, is a new endogenous peptide isolated for the first time from the bovine stomach extract (Tatemoto et al., 1998). This pleiotropic molecule performs its function by binding to a G-protein coupled receptor, called APJ (or APLNR), that is structurally very similar to the type 1 angiotensin receptor (Tatemoto et al., 1998). The peptide precursor of apelin is composed of a chain of 77 amino acids. Starting from this precursor it is possible to obtain, through enzymatic catalysis, many molecular forms with different biological functions: apelin-13, apelin-17, apelin-36, and pyroglutamate-apelin-13 (Tatemoto et al., 1998; Tatemoto et al., 2001). The shorter forms of apelin have shown greater biological potency than the longer ones (Tatemoto et al., 2001). The presence of APLN and its receptor have been shown in many tissues of numerous species of mammals. Infact the apelinergic system, including transcript and protein, is expressed in specific hypothalamus and cerebroventricular regions of the brain, pituitary gland, skeletal muscle, kidney, spinal cord, thyroid gland, adipose tissue, lungs, heart, and reproductive tract (Medhurst et al., 2000; Carpéné et al., 2007; Falcao-Pires et al., 2010; Mercati et al., 2018). In particular in veterinary medicine, the apelin was isolated in the ovary of cows (Roche et al., 2017), sows and in bitch’s placenta (Troisi et al., 2020). Therefore, it is supposed that it has a significant function in biological events such as folliculogenesis, cellular proliferation or apoptosis, and release of steroid hormones (Estienne et al., 2019) and it’s involved in the angiogenesis process during pregnancy (Troisi et al., 2020). Lately, the presence of APLN and its receptor have been evidenced in the male gonad of human and laboratory animals (Medhurst et al., 2003; Estienne et al., 2019; Kawamata et al., 2001; Pope et al., 2021); thus it has been hypothesized that they have an important function in the regulation of the reproductive activity. To our knowledge, there is no data about the presence of the apelinergic system in domestic animals. Wherefore, the purpose of this research was to evidence, for the
first time, its presence and distribution in the canine testis by immunoistochemical and molecular biology studies.

2. Material And Methods

2.1. Animals

Testis tissues were collected from 5 mixed-breed dogs (age from 2 to 5 years old) but with an average weight of 30 kg. The subjects were registered in the Teaching Hospital of Department of Veterinary Medicine, University of Perugia for elective orchiectomy with the written consent of their owners.

2.2. Surgical procedures

The orchiectomy was carried out under general anaesthesia using the following protocol: premedication with methadone 0.2-0.4 mg/kg IM (or IV) and medetomidine 1-5mcg/kg; induction with preoxygenation and propofol 4 - 6 mg/kg IV; maintenance through isoflurane, adjusting the vaporiser setting according to anaesthetic depth; 1 IV Ringer 10 ml/kg/h. Routine surgical technique was used.

2.3. Tissue collection and processing

Upon orchiectomy, testicular tissue samples were promptly removed and thoroughly washed with saline. Within a few minutes, under stereoscopic magnification, tissue samples were quickly reduced and sent for subsequent examination. For the molecular biological studies, the samples were rinsed with RNase-free water and then frozen at -80 C for later evaluation of gene and protein expression. For the immunohistochemical analysis, other tissue samples of testes were fixed by immersion in 4% (w/v) formaldehyde in PBS (pH 7.4) for 24 h at room temperature and subsequently processed following routine tissue preparation procedures (Dall’Aglio et al., 2014).

2.4. Reagents

For immunohistochemical analysis (IHC), the rabbit polyclonal anti-APLN antibody (NBP2-31176) was from Novus Biologicals (Novus Biologicals, USA); the mouse monoclonal anti-APLNR antibody (sc-517300) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the normal goat serum (s-1000), the two secondary biotin-conjugated antibodies, goat anti-mouse (BA-9200 and goat anti-rabbit (BA-1000), as well as the ABC Kit and DAB, were from Vector Laboratories (Vector Laboratories, Burlingame, CA, USA). Finally, the Eukitt (03989) was from Sigma-Aldrich.

2.5. Immunohistochemistry

Five micrometer thick serial sections were collected on poly-lysine-coated glass slides and processed for the immunohistochemical reaction, following antigen retrieval with a microwave oven and using 10 mM citric acid, pH 6.0, (three cycles at 750W, each lasting 5 min). All subsequent steps were carried out in a moist chamber at room temperature to prevent evaporation of the reagents. To avoid non-specific binding of the primary antibodies, after proper cooling the sections were pre-incubated for 30 min with the
specific normal goat serum (1:10). Subsequently, serial sections were incubated overnight with anti-APLN rabbit polyclonal (1:100) and anti-APLNR mouse monoclonal (1:100) primary antibodies. The next day, after washing in PBS, the sections were incubated with specific secondary biotin-conjugated antibodies, a goat anti-rabbit and a goat anti-mouse respectively (both 1:200), for 30 min then, after another washing in PBS, with the ABC KIT, again for 30 min. Finally, the tissue sections were rinsed in PBS and the reaction was developed using diaminobenzidine (DAB) as the chromogen. At the end of the immunoreaction, the sections were rinsed in PBS, counterstained with hematoxylin, dehydrated and mounted in Eukitt. Sections in which the primary antibodies were omitted were used as a control of unspecific staining. All tissue analyses were carried out on coded slides using a light microscope (Nikon Eclipse E800) connected to a digital camera (Dxm 1200 Nikon digital camera). For processing images, an image analysis system was used. The settings for image capture were standardized by subtracting the background signals obtained from the matched tissue sections which had not reacted with the primary antibodies and which were used as immunohistochemical controls (Dall’Aglio et al., 2012). However, these controls were not quantified given the prevalently qualitative nature of the immunohistochemical technique.

2.6. Molecular biology

2.6.1. Reagents

Deoxyribonuclease I (DNAase I Amp. Grade), Superscript III Reverse Transcriptase (Superscript III First-Strand Synthesis System), and DNA ladders were obtained from Life Technologies Italia (Monza, Monza Brianza, Italy). Reagents for isolation and purification of total RNA (TRIzol), Taq DNA polymerase (Platinum), RNasefree tubes, water and deoxyNTPs, primers for APLN and APLNR, were also acquired from Life Technologies. NucleoSpin Gel and PCR cleanup were from Macherey-Nagel Inc (Bethlehem, PA, USA). 2.6.2. RNA extraction and RT-PCR

Total RNA was extracted from the testicular tissue (100 mg each) of five different male dogs. Five micrograms of total RNA were reverse transcribed in 20 mL of Superscript III First-Strand Synthesis System using random hexamer according to the protocol provided by the manufacturer. Genomic DNA contamination was checked by developing the polymerase chain reaction without reverse transcriptase. The multiplex PCR amplification was performed as previously described [9,21] with the use of 1.0 mL of complementary DNA as a template for APLN and APLNR primers (Table 1). Cycling conditions consisted of an initial denaturizing cycle at 94°C for 75 s, followed by 35 cycles for each target gene at 94°C for 15 s, 60°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 10 min. Within each experiment, the complete set of samples was processed in parallel in a single PCR, using aliquots of the same PCR master mix. The amplified PCR-generated products (18 mL of 25 mL total reaction volume) were analyzed by electrophoresis on 2% agarose gel using ethidium bromide staining (Troisi et al., 2020).

4. Results
4.1 Immunohistochemistry

The immunohistochemical reaction evidenced the presence of apelin and its receptor in the canine testis. In particular, apelin appeared localized into spermatids and spermatozoa with a positive signal in the “acrosomal bodies” (arrows, Fig. 1a). Regarding the apelin receptor, a positive immunoreaction was evidenced in the cytoplasm of the cells localized in the basal portion of the seminiferous tubules, likely Sertoli’s cells (arrow), and in the cytoplasm of Leydig cells (asterisk). (Fig. 1b)

4.2 Molecular biology

Transcripts for APLN and APLNR were evidenced in all the samples examined. The APLN and APLNR PCR product matched the expected size (171 and 97 bp, Fig. 2A and B, respectively).

| Gene  | Primers                        | bp  |
|-------|--------------------------------|-----|
| APLN  | F CTCCTGCAACTCTGGCTAC          | 171 |
|       | R GTGGGAGACAAAGGAATCA          |     |
| APLNR | F AGTCAGGTAGCATGACAGCAC        | 97  |
|       | R AGCCTCAAGAAGGAAGGAAGCAC      |     |

Discussion

In human medicine, the adipokines regulate metabolic process (Bertrand et al., 2015), including reproduction (Dupont et al., 2015) in male and female, by control of the activity of hypothalamic-pituitary gonadal axis in both normal and pathological condition (Campos et al., 2008).

The presence and role of apelin and the APJ receptor in some animal reproduction has been widely reported (Roche et al., 2017; Troisi et al., 2020). On the contrary, as far as we know, few reports are available regarding the male reproductive and apelinergic system (Bertrand et al., 2015; Dupont et al., 2015; Campos et al., 2008). Our data, in agreement with those reported in humans and mice (Medhurst et al., 2003), showed the presence of apelin and its receptor in dog testis. In particular, the immunohistochemical presence of the apelin in spermatids and spermatozoa, as well as the apelin receptor in the cytoplasm of cells localized in the basal portion of the seminiferous tubules, likely Sertoli’s cells, was reported. These observations suggest that apelin, through paracrine actions, could be involved in the sperm functionality as well reported by Thomas et al. (Thomas et al., 2013).
Furthermore, in our study the presence of APLNR in the cytoplasm of Leydig cells could allow us to hypothesize that it may affect testosterone secretion, in endocrine manner, as well as was reported in laboratory animals. At this regard, the presence and distribution of APJ receptor was demonstrated in the cells from the anterior pituitary, hypothalamic paraventricular and supraoptic nucleous. In addition, these latest results are further supported by Sandal et al. (Sandal et al., 2015) which showed that the intracerebroventricular infusion of a high dose of apelin-13 is able to cause significant suppression of LH release, thus lowering serum testosterone levels and the number of Leydig cells. Finally more recently, Milirani D. et al. (Milirani et al., 2021) showed that pharmacological inhibition of apelin receptor can improve testicular steroidogenesis.

On the basis of these observations, it’s possible to hypothesize that the role of the apelin system in control of reproduction is also present in dogs.

The data obtained with molecular biology, carried out for the first time for this animal species, testify the presence of the APLN / APLNR system in the examined dog testes as reported in testis of adult rat (Brzoskwinia et al., 2020).

Our immune histochemical and molecular biology results could provide useful information to study the possible role of the APJ and APJ receptor in the course of reproductive pathologies in the dog which could represent a further animal model for comparative human pathology. Until now, in fact, the rat is the only animal model for the comparative study of human reproduction pathology. As this regard, Akkan e al. (Akkan et al., 2002) demonstrated that the expression of apelin in testicular tissue of rats significantly increased in course of surgical-induced varicocele.

**Declarations**

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**Conflicts of Interest:** The authors declare no conflict of interest

**Availability of data and material:** not applicable

**Code availability:** not applicable

**Authors contributions:** Polisca, Orlandi, Pastore and Troisi visited animals and collected samples; Dall’Aglio carried out the immunohistochemical studies; Maranesi carried out the molecular biology studies; Troisi, Dall’Aglio and Polisca prepared the manuscript.

**Ethic approval:** Authors declare that Ethics Committee Approval was not required for this study because all the enrolled dogs underwent orchiectomy at the Veterinary Hospital of the Department of Veterinary Medicine of Perugia, with the written consent of their owners, and did not undergo any other experimental procedures or manipulation.
Consent to participate: not applicable

Consent for publication: not applicable

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Figures

Figure 1
Immunostaining for apelin (a) and apelin receptor (b) in the canine testis. Apelin (a) stained the acrosomal bodies of mature cells (arrows). The apelin receptor (b) stained the basal line of germinative epithelium (arrow) and Leydig cells in the connective tissue (asterisk).

Figure 2

Gene expression of *APLN* (panel A) and *APLNR* mRNA (panel B) in testicular tissue (T) of five dogs. Representative agarose gel electrophoresis stained with ethidium bromide to verify matching between expected and obtained PCR products. For every PCR, a negative control (CTR-) were included, LD=100 bp DNA ladder.