

The immunohistochemical presence and distribution of ghrelin, apelin and their receptors in dog ovaries

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Abstract

The activity of ghrelin, apelin and their receptors has been correlated to the control of some infectious diseases, besides the hypothesis of their role in the control of some peripheral organs, among which ovaries. The aim of the present work was to highlight the presence and distribution of ghrelin, apelin and cognate receptors in the ovaries of pregnant bitches, by means of immunohistochemical techniques. Apelin, its receptor and the receptor of ghrelin were highlighted in the corpora lutea, with a particular localization in the cytoplasm of some luteal cells. Instead, a positive reaction for ghrelin was evident in the walls of small arteries in the connective tissue. These results allowed us to hypothesize that these molecules intervene in the control of ovaries in pregnant bitches, suggesting autocrine/paracrine mechanisms of regulation.

Short Communication

Recently, numerous studies have devoted great attention to the mechanisms that regulate appetite and energy metabolism, focusing in particular on some molecules with hormonal activity such as leptin, orexins, endocannabinoids, ghrelin and apelin.¹⁻⁵ Many studies in humans and laboratory and domestic animals have shown that these molecules have a ubiquitous distribution: they are not limited to the digestive and nervous systems, but also extend to organs and systems not anatomically and functionally related to appetite control, such as the reproductive system.⁶ Moreover and also for this reason, their functional activity is not only limited to the food intake control but it is extended to the control of the functional activity of the organs where they were evidenced. In a very intriguing way, their activity has been correlated to the control of some infectious diseases: in particular the APJ receptor has been seen as a coreceptor for HIV and in this way apelin protein, linking to the receptor, has been seen

to block the HIV infection,⁷ while the ghrelin protein has been involved in the control of gastric mucosal defense from *Helicobacter pylori*.⁸

Given the presence of apelin, ghrelin and cognate receptors in the female genital tract, as observed in the ovaries of cattle, pig and sheep,⁹⁻¹² one may hypothesize that these molecules are involved in the functional control of ovaries and therefore that they serve as a point of contact between the animal's nutritional status and the functionality of the genital tract. As the primary source of progesterone during pregnancy, the ovary and in particular the corpora lutea seem to be key organs regulating the reproductive cycle in dogs. While several luteotrophic or luteolytic factors have been characterized,¹³ the overall understanding of canine CL physiology, especially during pregnancy, remains limited. To date, no studies have explored the distribution of ghrelin, apelin and their receptors in the ovary and corpus luteum of pets. Therefore, the aim of this work was to detect the presence and identify the location of these molecules and their corresponding receptors in dog ovaries, starting with investigation on pregnant bitches, to acquire useful information for the complete understanding of the functionality of this organ.

The study was conducted using six mixed-breed pregnant dogs admitted to the day-hospital service at the Veterinary Teaching Hospital of the University of Perugia and subjected to a routine clinical examination. The weeks of gestations were calculated on the basis of fetal crown-rump length.¹⁴ According to the clinical history provided by their owners, none of the animals had any pregnancy complications. With the written consent of their owners, the animals were subjected to ovario-hysterectomy by spaying. The surgical procedures were carried out as previously described,⁵ under general anesthesia. Specifically, all bitches were pre-medicated with medetomidine (Sedator® Ati srl azienda terap, Italy) at a dose of 10 g/kg and butorfanol (Dolorex® Intervet Italia srl) at a dose of 0.2 mg/kg. General anesthesia was induced with approximately 4–6 mg/kg of propofol (Rapinovel® Intervet Italia Srl) and maintained with isoflurane vaporized in oxygen. After three days of hospitalization, the dogs were returned to the kennel.

When the ovaries were removed, they were immediately fixed by immersion in a 4% formaldehyde solution for 24 h at room temperature, dehydrated and subsequently processed for embedding in paraffin wax. Five µm thick serial sections were collected on poly-L-lysine-coated glass slides and processed for the immunohistochemical

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reaction following antigen retrieval with a microwave oven using 10 mM citric acid, pH 6.0, (three cycles, 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, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, serial sections were incubated overnight with anti-Apelin (ab59469), anti-APJ receptor (ab140508), anti-Ghrelin (ab129383) and anti-Ghrelin receptor (ab188986) polyclonal antibodies.

The next day, after washing in PBS, the sections were incubated with the specific secondary biotin-conjugated antibody, a goat anti-rabbit (1:200; Vector Laboratories, Burlingame, CA, USA), for 30 min.

After another washing in PBS, the sections were processed for 30 min using the ABC KIT (ABC, Vector Elite Kit, Vector

Laboratories) to visualize the antigen-antibody complex.

Finally, the tissue sections were rinsed in PBS and the reaction was developed using diaminobenzidine (DAB, Vector Laboratories) as a chromogen. At the end of the immunoreaction, the sections were rinsed in PBS, counterstained with hematoxylin, dehydrated and mounted in Canada Balsam (BDH, Poole, Dorset, England). Positive and negative controls were included in the study.

Immunohistochemical reactions showed strong positivity for apelin, ghrelin and their receptors in some ovarian structures. In particular, a positive immunoreaction for apelin and its receptor and for the ghrelin receptor was evident in the corpora lutea with a peculiar localization in the cytoplasm of some luteal cells (Figure 1A,B,C). Instead, regarding ghrelin, a positive reaction for this molecule was evident within the wall of small arteries localized both inside the corpora lutea and in the connective tissue (Figure 1D). The immunopositive reaction was not observed in other ovarian structures or in the sections used as negative controls.

The female gonad, the ovary, is a complex organ that undergoes cyclic variations of its structure mainly as the result of hormonal stimulation.^{15,16}

Besides being the target of the action of hormones produced in more or less distant tissue structures, the ovary is itself able to produce and receptors for these substances. Most of these hormones and their mechanism of action are known. Recently, interest has shifted to other hormonal substances (leptin, orexins, and endocannabinoids), some of them newly discovered (apelin and ghrelin), initially identified as molecules involved in appetite and metabolism control. More detailed studies in humans and in laboratory or domestic animals have highlighted that these molecules have a ubiquitous distribution, also often involving the reproductive organs. Our study evidenced the presence of the hormonal molecules apelin and ghrelin and their cognate receptors in pregnant bitch ovaries, in line with findings of these molecules in the ovaries of sheep, pig and bovine. Thus these results demonstrate that these molecules are also present in the canine ovary, even if with a distribution not always overlapping what already evidenced in other animal species.

Given the presence of these molecules in some of the ovarian structures, one can hypothesize that apelin, ghrelin and their receptors are involved in the functional control of the female genital tract and maybe also in the infectious diseases control of the organ through an autocrine/paracrine mech-

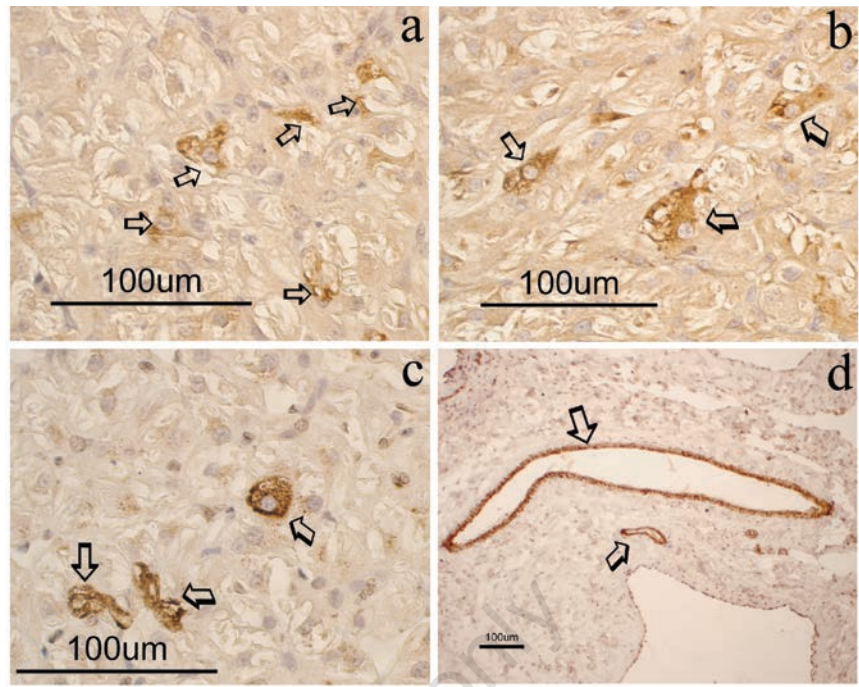


Figure 1. Apelin, ghrelin and their receptors-immunoreactivity in the dog ovary. A) Apelin immunopositivity in the dog ovary: apelin immunoreaction is localized in the corpora lutea, in particular in some of the large luteal cells (arrows). B) Apelin-receptor immunopositivity in the dog ovary: Apelin receptor immunoreaction is localized in the cytoplasm of some large luteal cells (arrows). C) Ghrelin receptor immunopositivity in the dog ovary: Ghrelin receptor immunoreaction is localized in the cytoplasm of some luteal cells (arrows). D) Ghrelin immunopositivity in the dog ovary: strong ghrelin immunoreaction inside the wall of arteries localized in the corpus luteum and in the connective tissue (arrows).

anism of regulation.

Furthermore, their presence in the reproductive system confirms that there is a strong link between the animal's nutritional status and the functionality of the genital tract. It would be very interesting to see further studies in this regard that complete the investigation of the whole estrus cycle.

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