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Temporal expression of *GDF-9* and *BMP-15* mRNAs in canine ovarian follicles

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ABSTRACT

This study aimed to investigate the expression profiles of growth differentiation factor 9 (*GDF-9*) and bone morphogenetic protein 15 (*BMP-15*) mRNA in canine oocytes and follicular cells throughout development at the different phases of the estrus cycle. Ovarian structures (follicles and CL) and plasma progesterone concentration were used to confirm the physiological status of each donor. Denuded oocytes and their follicular cells were recovered from follicles (n = 675) distributed into 4 types (preantral, small antral ~0.2–0.39 mm, medium antral ~0.4–5.9 mm, and large antral ~6–8 mm). Total RNA was extracted and reverse transcribed, and the levels of expression for these 2 genes were determined using a quantitative real-time polymerase chain reaction technique; the data were evaluated by ANOVA. Relative expressions levels of *GDF-9* and *BMP-15* transcripts were detected in the oocyte and follicular cells in all follicular stages evaluated, showing differential changes ($P < 0.05$) during development over the estrus cycle. The expression patterns of both transcripts were highly correlated between follicles cells and oocytes ($r > 0.8$; $P < 0.05$ for *GDF-9* and *BMP-15*), although *GDF-9* was expressed at higher levels ($P < 0.05$) in the oocyte compared with the follicle cells. All cell types showed more *GDF-9* mRNA abundance at early developing stages, mainly in the anestrus phase, and declining levels in the later stages ($P < 0.05$), whereas *BMP-15* mRNA levels increased ($P < 0.05$) in follicular cells and oocytes from the preantral to the later stages, and remained constant during the final preovulatory stage. In conclusion, these two genes were detected in follicular cells and oocytes and were differentially expressed during the follicular development across the estrus cycle.

1. Introduction

The oogenesis and folliculogenesis processes rely on interactions between the oocyte and the somatic cells involving both gap junctions and paracrine factors. Among these paracrine intraovarian factors, growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), which are members of the transforming growth factor beta superfamily of cytokines, induce follicular somatic cells to undergo proliferation and differentiation during follicular development through a paracrine signaling pathway [1,2]. These factors are involved in follicular growth from the primordial follicle to ovulation [3] and are considered as essential regulators of follicular cell processes those are fundamental to oocyte maturation [4,5].

Studies using different animal models to investigate the expression of *GDF-9* and *BMP-15* genes and their encoded proteins, fully describe the physiology and significance of these factors. In rodents, these factors are produced primarily in the ovaries, where they are localized exclusively to the oocytes of growing follicles [6–8]. There are also evidences in species with different ovulation-rate phenotype to support the idea that within

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the cumulus-oocyte-complexes, *GDF-9*, and *BMP-15* mRNA are specific to the oocytes [9]. However, other studies have described that *GDF-9* and *BMP-15* genes or their encoded proteins are expressed also in cumulus, mural granulosa, or theca cells in species such as bovine [10,11], ovine [12,13], porcine [14], human [15], and also canines [16–19]. These divergent findings may relate to interspecies differences, the status of follicle atresia, the estrous cycle, or to methodologic variations, which would account for certain contradictory reports on the same species.

Canine follicular development differs from that of most domestic animals; however, the mechanisms controlling such differences remain unknown. Characteristic examples of these differences are the formation of primordial follicles after birth [20] in contrast to many other mammals in which this event occurs during fetal development [21], and the lengthy process of cytoplasmic and nuclear oocyte maturation [22,23], starting before ovulation and ending 48–72 hours after ovulation [24]. Therefore, dogs oocytes are ovulated arrested at the dictyoten stage of meiosis [16,22]. The final maturation process in the oviducts depends greatly on the prior period inside the follicles [25] because oocyte meiotic competence is acquired during follicular growth [26]. The lack of knowledge regarding the follicular period makes it important to study the regulation of follicular development leading to ovulation in this species.

During follicular development, the oocyte synthesizes and accumulates the RNAs and proteins that are necessary for its growth and maturation [27]. The changes in the pattern of gene expression during development could be explained by the utilization of mRNAs, which were accumulated during oocyte growth [5]. The expression of the *GDF-9* and *BMP-15* genes is essential for folliculogenesis and acquisition of the oocyte meiotic and developmental competence [10,28]. Therefore, the differences between competent and incompetent oocytes might depend on differential gene expression patterns. Recently, we described that GDF-9 and BMP-15 proteins are expressed differentially during follicular growth over the estrus cycle in bitches [19], showing a declining secretion of these factors in preovulatory follicles, which in turns may be related with the special mucification pattern in canine. Previously, we found in the *in vitro* studies that the expression of GDF-9 in canine cumulus-oocyte-complexes had a negative correlation with cumulus expansion [16]. Canine GDF-9 has an extra N-glycosylation site which could be related to a corresponding reduction of protein secretion [29], as N-linked glycan's play a role in regulating post-translational processing to produce the mature protein [30]. To date, no information is available on the expression of these genes during folliculogenesis in dogs. Therefore, it is of particular interest to investigate through follicular and oocyte analysis, the transcripts pattern of *GDF-9* and *BMP-15* at different stages of folliculogenesis and during the preovulation process in dogs. The immaturity of the canine oocyte at ovulation may be due, at least in part, because the canine follicular cells and oocyte are upregulated or downregulated transcriptome in different way during follicular development. Therefore, to know the stage of follicular growth in which these relevant genes are expressed in the canine ovarian follicles, the aim of this study was to evaluate the mRNA abundance of the *GDF-9* and *BMP-15* genes in oocytes and follicle cells from the preantral to preovulatory follicle stages establishing temporal changes in mRNA expression during follicular development throughout the estrus cycle.

2. Material and methods

All procedures were approved by the Ethic and Animal Care Committees Faculty of Veterinary Sciences, University of Chile and the Research Ethics Committee of the Chilean National Foundation for Scientific and Technological Research.

2.1. Follicle recovery

Canine ovaries were collected from 35 adult bitches (aged 1–6 years) at different stages of the estrus cycle undergoing routine ovariohysterectomy at the local Veterinary Hospital of the Faculty of Veterinary Sciences.

After removal of the ovaries, the stage of the estrus cycle of each bitch was assessed by evaluating the presence of developing follicles or CL [26]. In addition, the reproductive status were estimated by blood plasma progesterone (P4) concentrations assessed by ELISA [31] (Table 1) using a PHomo Microplate Reader (Autobio Labtec Instruments, Zhenghaidong, China) with a progesterone canine kit (Prog ELISA Kit, MyBioSource; San Diego, CA, USA). The sensitivity of the assay was 0.23 ng/mL, and the mean intra-assay and interassay coefficients of variation were 6.1% and 7.4%, respectively.

Each ovary was placed in a disposable Petri dish (Falcon; Becton Drive, Biosciences Franklin Lakes, NJ, USA) with sterile PBS, trimmed of parenchymal tissues and cut into small pieces at room temperature (21 °C). Each individual follicle was measured with a graticule and mechanically isolated free of stromal tissue by microdissection under a stereoscopic microscope (Motic SMZ-171, Vancouver, Canada) using 26-gauge needles fitted to 1-mL syringes (Nipro Corporation, Miami, FL, USA) and a surgical blade. The follicles were dissected from the ovarian cortex according to the developing stage into 4 types: preantral (surrounded by more than one layer of granulosa cells up to the onset of antrum formation), small antral (~0.2–0.39 mm), medium antral (~0.4–5.9 mm), and large antral or preovulatory follicles (~6–9 mm). Abnormal follicles, based on their morphologic characteristics such as irregularities in the basal membrane, irregular darkening of the oocyte, and the surrounding granulosa cells or debris in follicular fluid were discarded. Follicular cells (theca, granulosa, and some cumulus cells) were collected without separating each cell type from the different follicles by scraping the granulosa and theca cells layers and also by the aspiration of the intrafollicular contents from dissected antral follicles. The oocytes from each follicle were completely denuded *via* gentle pipetting with a fine bore glass pipette in PBS under a dissecting microscope.

A total of 240 preantral (n = 80 in each phase of the estrus cycle); 240 small antral (80 in each phase of the estrus cycle), and 150 medium antral follicles (50 in each