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Differential expression of GDF-9 and BMP- 15 during follicular development in canine ovaries evaluated by flow cytometry

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ABSTRACT

Growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) play important functions in follicular and oocyte development in many species. This study evaluated the dynamic expression of GDF-9 and BMP-15 in canine follicles cells using flow cytometry analysis. Follicular cells were removed from three sizes of antral follicles (small, medium and large) from ovaries of bitches throughout the estrus cycle. Cells were incubated with anti-human GDF-9 polyclonal and anti-mouse BMP-15 monoclonal antibodies. A size and complexity discriminatory gate was used for the cytometryc analysis in the initial dot plot and, additionally, a CD45 marker for leukocyte and propidium iodide (PI) were used for erythrocyte and debris discrimination. The evidence corroborated the presence of both proteins in canine follicle cells, but these proteins were not expressed equally during follicular development. The results analyzed by ANOVA showed that GDF-9 expression decreased ($P < 0.05$) during follicular growth in anestrus and proestrous/estrous, but increased in diestrus ($P < 0.05$). The expression levels of BMP-15 rose ($P < 0.05$) from small to medium sizes in anestrous without changing at diestrus. Small antral follicles expressed the highest values of GDF-9 at anestrus while only BMP-15 showed higher value in small antral follicles at proestrous-estrus compared to diestrus and anestrus. Both proteins decreased in proestrous/estrous ($P < 0.05$) with increasing follicle size, registering the lowest levels in large follicles. The flow cytometric assay was able to assess GDF-9 and BMP-15 expression in canine follicular cells, showing that these proteins were differentially expressed during follicular development, possibly related to the special features of canine reproduction.

Introduction

Oocyte development and follicle growth are closely regulated by an ordered and complex series of signaling events throughout folliculogenesis. The interplay between oocytes and the somatic cells of ovarian follicles determines the developmental ability of these cells. During this process, meiotic competence of the oocyte is gained gradually (Eppig, 2001; Fair, 2003; Hussein et al., 2006) and gap

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junctional communication within the developing follicle, both between oocytes and granulosa cells and among the follicular cells themselves, maintains the follicle in a functionally integrated state (Kidder and Vanderhyden, 2010; Huiyu et al., 2013). However, little is known about the physiological control of follicular growth in canines, despite being of pivotal importance in oocyte development in vivo and in vitro. The subsequent in vitro maturation (IVM) of oocytes, that remains unsuccessful in this species (De los Reyes et al., 2005, 2011; Chastant-Maillard et al., 2011), depends strongly on its prior period inside the follicles. In fact, the compromised developmental competence of IVM in canines can be attributed to the lack of knowledge of the ability of the oocyte's intrinsic requirements during follicular maturation.

The communication of oocyte and follicular cells is characterized by the secretion of several growth factors such as those members of transforming growth factor beta superfamily (TGF-β), which plays an important function in proliferation and differentiation of a variety of cell types. Many studies have shown that members of the TGF-β superfamily can regulate granulosa cell proliferation and differentiation (Sudiman et al., 2014; Cheng et al., 2015). Growth differentiation factor (GDF) 9 and bone morphogenetic protein (BMP) 15 are members of the TGF-β superfamily of proteins, and thus are similar at a structural level (Knight and Glister, 2006; Otsuka et al., 2011). Studies indicate that GDF-9 and BMP-15 are both present in follicles throughout different stages of follicular development and in many species these factors influence ovarian follicular growth. Findings in sheep, humans and rodents show that BMP-15 and GDF-9 can be considered to be new targets for fertility regulation in mammals (Gilchrist et al., 2006; Persani et al., 2014), as they participate in signaling pathways that control the development of ovarian follicles. These proteins are thought to affect granulosa cell proliferation independently or synergistically from small follicles (Fenwick et al., 2013) and the growth-promoting actions of oocytes are mediated, at least in part, by these factors (Su et al., 2004; Hussein et al., 2006), although the signals exchanged between the oocyte and the surrounding cells are far from being fully understood. Studies in mice and sheep, as well as evidences from in vitro studies in other species, have demonstrated that cooperative interactions between GDF-9 and BMP-15 occur in many functions (McNatty et al., 2005; Mottershead et al., 2012). GDF9 promotes the expansion of cumulus cells by induction of expression of Has2, Tnfaip6, Ptx3, and Ptgs2 (Varani et al., 2002); BMP-15 also is involved in this process (Gueripel et al., 2006), and both factors promote proliferation of granulosa cells (Kidder and Vanderhyden, 2010).

It has been reported that BMP-15 and GDF-9 are expressed only in oocytes in rodents (Gilchrist et al., 2008), however, these factors are expressed also in cumulus and mural granulosa cells in many other mammals (Hosoe et al., 2011; Lim et al., 2014), including canine (De los Reyes et al., 2013; Maupeu et al., 2015), demonstrating that the expression patterns and biological functions may differ among species. Therefore, many species differences have already emerged (Galloway et al., 2000; Hussein et al., 2006; Sun et al., 2008). In previous studies we suggested that oocyte-produced GDF-9 in vitro may be insufficient for promoting cumulus cell expansion in canine (De los Reyes et al., 2013), which could be related to delay for resumption of meiosis. However, the levels of GDF-9 and BMP-15 expression in canine granulosa cells during follicular development are largely unknown. As GDF-9 and BMP-15 are both possibly present in follicles throughout most stages of follicular growth, it is important to evaluate whether these paracrine factors are differentially expressed in these follicles. The knowledge of the physiology of GDF-9 and BMP-15 factors in canine could contribute to understanding the special reproductive characteristics of this species and also potentially helps to improve protocols that may to overcome the low success of IVM in canine oocytes.

Flow cytometry is a powerful analytical instrument for rapid evaluation of high numbers of cells; it detects labeling by multiple fluorochromes associated with individual cells flowing in a flowing stream (Hirshfield et al., 1988). This technique has been successfully used for granulosa cell analysis (Rao et al., 1991; De Neubourg et al., 1996; Douville and Sirard, 2014; Regan et al., 2015), providing an alternative method for assessing protein expression in follicular cells. Therefore, the experiments reported in the present study were undertaken to evaluate by flow cytometric methods the expression of GDF-9 and BMP-15 in canine follicular cells during follicular development.

2. Material and methods

This study was approved by the Bioethics Committee, Faculty of Veterinary Sciences, University of Chile and the Research Ethics Committee of the Chilean National Commission for Scientific and Technological Research (FONDECYT).

2.1. Processing of ovaries

Ovaries were collected from adult non-pregnant bitches undergoing routine ovariohysterectomy at the Veterinary Hospital El Roble, University of Chile. The selected bitches were clinically healthy of various breeds, and were 1–6 years of age. Immediately after removal, the ovaries were placed in physiological saline solution (pH 7.4, 0.9% NaCl) at 4 °C and then transported to the laboratory.

Only healthy ovaries, with no visual abnormalities, were used for experiments. The stage of estrous cycle of each donor was assessed by evaluating the presence or absence of follicles and corpus luteum (Songsasen and Wild, 2005), and by progesterone analysis from blood samples obtained during the surgery, as previously described (De los Reyes et al., 2013). Briefly, blood of each animal was collected without anticoagulant and then centrifuged at 3000 rpm for 10 min. Plasma was stored at −20 °C until use. Plasma progesterone concentrations was assessed by enzyme-linked immunosorbent assay (ELISA) (Ververidis et al., 2002), (PHomo Microplate Reader®; Autobio Labtec Instruments, Zhenghaidong, China) with a progesterone (P4) canine kit (Prog ELISA Kit, MyBioSource®; San Diego, CA, USA). Duplicate wells were used for each sample. Sen-