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Expression of growth differentiation factor 9 (GDF-9) during *in vitro* maturation in canine oocytes

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ABSTRACT

The aim of this study was to characterize in canine oocytes and cumulus cells the dynamic expression of growth differentiation factor 9 (GDF-9) in relation to meiotic development and cumulus expansion throughout *in vitro* maturation (IVM). Cumulus oocytes complexes (COCs) from ovaries of adult bitches were cultured intact for IVM during 0, 48, 72, and 96 hours. At 0 hours or after IVM, COCs were divided into two groups: one group remained with their cumulus cells and in the other group the cumulus cells were extracted. The expression levels of GDF-9 were determined in both groups using indirect immunofluorescence and Western blot analysis. For immunofluorescence assay, *in vivo*-matured oocytes collected from oviducts were also used as a positive control. The nuclear stage was analyzed in parallel with 4′-6-diamidino-2-phenylindole staining in denuded oocytes from all maturing groups. The intensity of fluorescence, indicative of GDF-9 expression level, decreased with time ($P < 0.05$). High expression was observed only in germinal vesicle nonmature oocytes; in contrast, second metaphase oocytes showed only low expression. Western blot analysis showed bands of approximately 56 kd and a split band of approximately 20 kd representing the proprotein and possibly two mature protein forms of GDF-9, respectively. The proprotein was detected in all samples, and it was highly expressed before IVM and in a lesser degree, during the first 48 hours, declining thereafter in coincidence with the expansion of the cumulus cell ($P < 0.05$). There was a negative correlation ($r = -0.97$; $P < 0.05$) between the expression level of GDF-9 and mucification. Mature forms were evident only in COCs, before culture and up to 48 hours of IVM. It was concluded that GDF-9 is expressed in canine oocytes and cumulus cells, mainly in the early developmental states, with low levels in mature oocytes *in vitro* and *in vivo*, representing the first approach of GDF-9 dynamic in dog oocyte maturation.

1. Introduction

Impaired oocyte meiotic maturation in dog has been the major obstacle to fertilization and embryo development in culture. Different *in vitro* maturation (IVM) protocols in canine have been established to study oocyte development; although significant progress has been achieved in recent years, the current IVM systems are far from optimal in terms of the percentage of mature oocytes obtained, and fertilization and *in vitro* embryo development.

A well-coordinated maturation between cytoplasm and nucleus depends on a complex interplay of endocrine and paracrine events, and direct communication between oocytes and neighboring somatic cells [1,2]. In our recent studies, we have long observed that during culture some parameters of cytoplasmic maturation in canine oocytes, including cortical granules migration [3], mitochondria distribution [4], and the ability of the ovum cytoplasm to

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induce sperm nuclear decondensation [5], can be completed in a manner similar to that *in vivo*; however, nuclear maturation is not assured at high values and is not coordinated with cytoplasmic changes. Many IVM dog oocytes that can support cytoplasmic changes are only partially competent to undergo nuclear maturation [3–5].

Meiotic competence is gained gradually during folliculogenesis and oocyte growth [1,6]. Several studies have demonstrated the importance of paracrine signals from oocytes via receptors located in the gap junction of neighboring granulosa cells. This cooperativity between these cells has been described to be under the control of the oocytes and it is mediated by factors, such as growth differentiation factor 9 (GDF-9), a member of the transforming growth factor beta superfamily [7–9]. This factor can activate the signaling pathways in cumulus cells to mediate the development of their neighboring oocytes through gap junctions channels [10,11] or connexins (CXs) [12], with CX37 and CX43 being important in this regard [13]. In fact, granulosa cells must be coupled via the CX43 gap junction in order to respond optimally to GDF-9 [14].

In rodents, GDF-9 is involved in proliferation of granulosa cells from small follicles [15], and the growth-promoting actions of oocytes are mediated, at least in part, by GDF-9 [2,16]. This protein also regulates cumulus expansion [17,18] through induction of expression of Has2, Tnfaip6, Ptx3, and Ptgs2 [19,20]. Hence, it is able to regulate oocyte meiotic resumption in preovulatory oocytes, activating the mitogen-activated protein kinase pathway by modification of cumulus cell function after the preovulatory LH surge [14]. In addition, the concentration of GDF-9 in follicular fluid has been significantly correlated with the nuclear maturation of human oocytes [21].

Cumulus cells control mitogen-activated protein kinase activation and phosphorylation in blue fox oocytes [22], confirming the influence of cumulus cells and oocyte interaction in regulating meiotic development in canine. In fact, the loss of cumulus gap junctions and thus, cumulus expansion has been associated with resumption of meiosis (germinal vesicle breakdown stage) in dog [23]. In the bitch, the mechanisms responsible for the delay of meiotic resumption after ovulation are not well known, but it has been suggested that the persistence of close relations between the oocyte and the cumulus cells might contribute to this delay [24].

It has been demonstrated that GDF-9 also inhibits granulosa cell LH receptor expression [1] and thus regulates progesterone production in murine [25], porcine [26], and bovine cumulus cells [27,28], both indicative of a role in suppression of luteinization. In the bitch, follicular luteinization before ovulation is a typical feature [29,30] and in contrast to other mammalian species, with increasing concentration of progesterone at the moment of ovulation [31]. Therefore, these progesterone-inhibiting actions of GDF-9 might be insufficient in dogs to prevent lutenization.

The relationship between cumulus cells, GDF-9, and other growing factors might have different functions depending on the species. The different phenotypes exhibited by mice and sheep with inactivating mutations in GDF-9 [32] are evidence of species differences in the actions of this factor, especially considering the peculiar aspects of dog reproductive physiology. To our knowledge, the presence of GDF-9 in canine oocytes or cumulus oocytes complexes (COCs) has not been described, and its influence on the canine oocyte and the surrounding cumulus cells could be relevant throughout maturation, because the oocyte-cumulus communication in the bitch is longer than in other animals because the cumulus cells remain strongly attached to the oocyte for several days after the LH peak and ovulation [33]. Therefore, this uncommon pattern of cumulus expansion in canine oocytes might be related to a different expression pattern of GDF-9, which would modulate among other proteins, granulosa cell functions involved in oocyte developmental ability. Therefore, in the present study we evaluated the dynamic expression of GDF-9 in relation to nuclear maturation and cumulus expansion throughout culture time in canine oocytes using indirect immunofluorescence and Western blot analysis.

2. Materials and methods

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All animals used in the present study were treated according the Chilean Bioethics Committee of the National Commission for Scientific and Technological Research (FONDECYT).

2.1. Oocyte collection and maturation

Oocytes were collected from pure and crossbred bitches aged 1 to 6 years. For *in vitro* experiments, oocytes were obtained from ovaries of bitches at different stages of the estrus cycle after neutering (N = 85), and for *in vivo* analysis, oocytes were collected from oviducts of bitches at the estrous stage, 50 to 70 hours after ovulation (N = 7), according previous reports [5]. The time of ovulation was estimated before surgery according to vaginal smears [34] and blood serum progesterone concentrations [30], assessed using enzyme-linked immunosorbent assay (ELISA) [35] (PHomo Microplate Reader; Autobio Labtec Instruments, Zhenghaidong, China), with a progesterone canine kit (Prog ELISA Kit; MyBioSource, San Diego, CA, USA). Follicular growth and ovulation were also monitored by color Doppler ultrasonography (Sonosite M-turbo; Sonosite Inc., Bothell, WA, USA) [36], from the beginning of proestrual bleeding.

At the laboratory, COCs were released by slicing the ovaries. Mainly large oocytes (with an estimated size $\geq$100 μm) with uniform covering of compacted cumulus cells and the presence of a dark and homogeneous cytoplasm were selected for the experiments, according previous reports [4,37].

In each experimental replicate, selected COCs were washed three times with TCM-199 Earle's salt, and 25 mM HEPES (Invitrogen, Carlsbad, CA, USA), and then they were cultured intact in 100-μL drops of maturation medium (TCM-199 Earle's salt, and 25 mM HEPES supplemented with 10% fetal calf serum, 0.25 mM pyruvate, 10 IU/mL of hCG, 100 IU/mL of penicillin, and 0.2 mg/mL of

streptomycin) overlaid with mineral oil and incubated for 0, 48, 72, and 96 hours at 38.5 °C in an atmosphere of 5% CO_2 and saturated humidity [4]. Each experiment was repeated four times and at least 20 to 25 oocytes were examined at each maturation time.

Ovulated oocytes (*in vivo*-matured) were obtained by flushing the oviducts with 15 to 20 mL of TCM-199 supplemented with 10% fetal calf serum [5]. These oocytes were used for cumulus evaluation and for immunofluorescence assays.

Because we wanted to evaluate GDF-9 in COCs and oocytes isolated from their cumulus cells, the samples were divided into two groups after each time point. The first group included denuded oocytes, obtained by removing cumulus cells passing the cells through a narrow pipette, and the second group included COCs.

2.2. Evaluation of cumulus cell expansion

The degree of cumulus expansion was analyzed in ovulated, nonmatured, and IVM oocytes with cumulus cells (COCs) after different culture times, as described by Prochazka et al. [38]. The scoring system was: 0, no expansion; 1, minimum expansion; 2, expansion of outer cumulus layers; 3, expansion of all cumulus layers except the corona radiata; and 4, expansion of all layers. The degree of cumulus cells expansion was evaluated at magnification ×200 with an inverted microscope (Nikon TMS 301953, Tokyo, Japan).

2.3. Immunofluorescence and oocyte nuclear evaluation

All oocyte maturity groups (nonmatured, ovulated, and IVM at different time periods) were processed using indirect immunofluorescence. Oocytes from each group were fixed in 4% paraformaldehyde in PBS at 4 °C for 20 minutes, followed by permeabilization in 0.1% Triton X-100-PBS (pH 8.2) for 10 minutes. Nonspecific binding sites were blocked for 1 hour with PBS-BSA 0.2% buffer (pH 8.2), supplemented with 5% goat blood serum then washed in 1× PBS and incubated with anti-human GDF-9 polyclonal antibody (C-18) (SC-12244, 1/100 dilution) overnight at 4 °C. After three 10-minute washings with 0.05% Tween-20 in PBS (pH 8.2), oocytes were transferred to a 1:500 dilution of fluorescein-conjugated goat anti-mouse second antibody for 1 hour at room temperature (20 °C–21 °C) in darkness. For all instances, replacing the primary antibody with PBS buffer and adding only secondary antibody was used as a negative control to confirm the specificity of labeling. To examine the meiotic status in parallel, only denuded oocytes were incubated for 15 minutes with 1 μg/mL 4′-6-diamidino-2-phenylindole staining (Thermo Fisher Scientific Inc., Rockford, IL, USA) at room temperature.

Finally, all morphologically normal oocytes were mounted on a glass slide with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and examined at magnification ×200 using an Olympus IX71 inverted epifluorescence microscope, equipped with an IX2-RFA lamp and a ProgRes-Capture Pro camera (Olympus, Tokyo, Japan). The same exposure time was applied to all oocytes. During evaluation, digital photos were taken and subsequently analyzed. The intensity of fluorescence was evaluated in each denuded oocyte or COC, using Image J version 1.45s software (National Institutes of Health, Bethesda, MD, USA). The images were transformed to gray scale and the corrected total cell fluorescence (CTCF) was calculated by subtracting the mean fluorescence of background reading to the integrated density of each cell.

Chromatin configurations were classified as germinal vesicle (GV), GVBD, first metaphase (MI), and second metaphase (MII), as previously described [4].

2.4. Western blot analysis

Expression levels of GDF-9 protein were also determined using Western blot assay, from 80 denuded oocytes and 80 COCs (oocytes with its cumulus cells) before (0 hours) or after each culture time (48, 72, and 96 hours) in each of the four replicates (total, 1280 oocytes and 1280 COCs). Oocytes and COCs were suspended in Laemmli loading sample buffer (Winkler BM 0300) with protease inhibitor mixture and boiled for 5 minutes before storage at −80 °C. Protein extracts were resolved in 12% SDS-polyacrylamide gel, at 120 V, in 1% running solution (TRIS 25 mM, glycine 250 mM, SDS 0.1 % in distilled water). Electrophoretically separated peptides were transferred to polyvinylidene-fluoride membrane (Immobilon-P membrane, Millipore Corp., Bedford, MA, USA) using the Mini-Vertical Slab Gel/Blotting Electrophoresis System (DCX-700, C.B.S. Scientific Company, Inc., Del Mar, CA, USA) and blocked with 5% skimmed milk in 0.1% PBS Tween-20 (PBST) at room temperature for 1 hour, followed by incubation with anti-human GDF-9 polyclonal primary antibody (1:250 dilution) (C-18) and mouse anti–β-actin polyclonal antibody (1:250 dilution) (C-1615) at 4 °C overnight. Blots were washed with PBST and then incubated with anti-goat secondary antibody (1:500 dilution) with alkaline phosphatase (C-2771) for 1 hour at room temperature. Membranes were washed extensively with PBST and when the signal was visible the reaction was stopped by rinsing with distilled water. Protein bands were visualized with nitro blue tetrazolium, 300 pg per mL/bromo-chloro-indolyl phosphate-p-toluidine salt, 150 pg per mL substrate in 0.1 M TRIS, pH 9.5). Molecular masses of separated proteins were estimated by comparison with positions of a molecular-mass standard run in parallel.

Densitometry of band intensities in Western blot analyses were evaluated using the GEL-PRO Analyzer V4 program (Media Cybernetics Inc., Silver Springs, MD, USA). The amount of dark pixels in the same area for each band was determined and expressed as integrated optical density.

2.5. Statistical analysis

In immunofluorescence assays, approximately 200 oocytes and 200 COCs distributed in each group (nonmatured and IVM for 48, 72, or 96 hours) and 16 ovulated oocytes and COCs, were studied throughout experiments which were repeated independently a minimum of four times. The data were analyzed using a longitudinal model (dependent on time) using logistic regression. In Western blot analysis, density values were compared using ANOVA

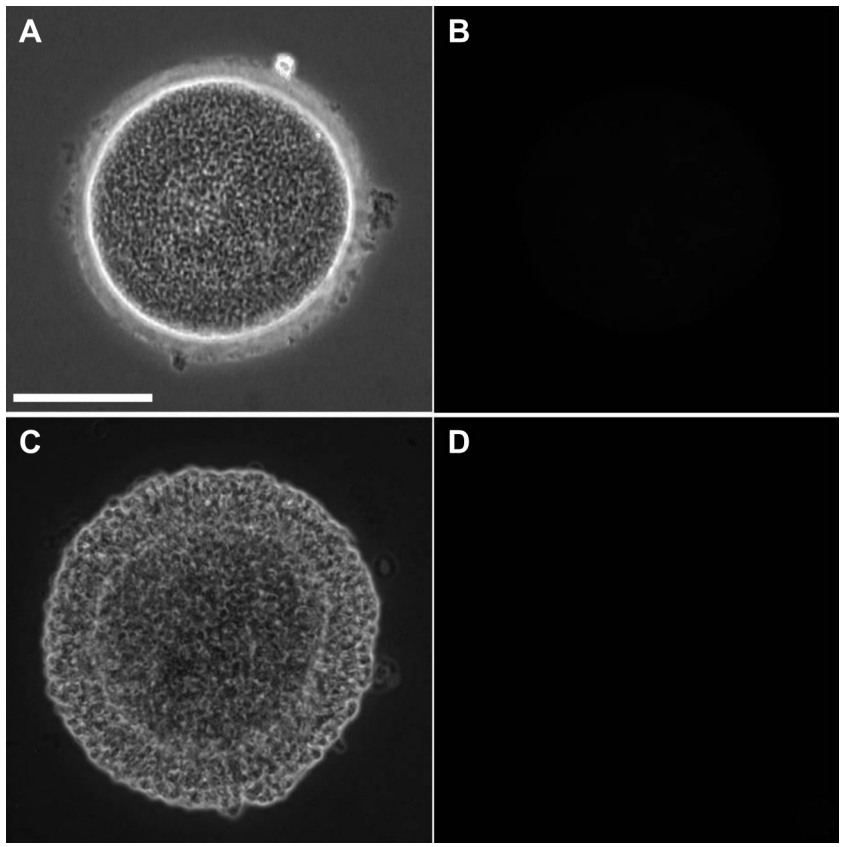


Fig. 1. Negative control. Photomicrographs of representative nonmature canine oocyte (A and B) and cumulus oocyte complexes (C and D), analyzed using phase-contrast (A and C) and epifluorescence (B and D); no signal was obtained when the C-18 human anti-human growth differentiation factor 9 antibody was not included in the procedure. Scale bar, 50 μm.

(InfoStat Professional Program, Version 2004; National University of Córdoba, Argentina), including the effects of oocyte maturity, duration of culture, and their interaction. Significant differences among means were evaluated using Tukey tests. Correlations between expression of GDF-9, determined using the Western blot technique, and cumulus expansion of *in vitro* mature canine COCs were done using Pearson's correlation coefficient.

Values were considered significantly different when $P < 0.05$.

3. Results

The use of the indirect immunofluorescence technique with the polyclonal antibody C-18 raised against human GDF-9 allowed us to immunolocalize this factor in permeabilized canine oocytes and also in cumulus cells, indicating the presence of this protein in both types of cells. This label appears to be specific because the negative controls, without the first antibody, did not show any label (Fig. 1).

A varying degree of GDF-9 immunosignal was visible in denuded oocytes and COCs treated using the immunofluorescence technique, determining three distinct patterns of fluorescence intensity according to the number of pixels in the same oocyte, as shown in Figure 2 (oocytes) and Figure 3 (COCs). Low fluorescence level (L-pattern) was assigned to oocytes or COCs with CTCF values less than 5×10^6. Medium fluorescence emission (M-pattern) oocytes or COCs had CTCF values between 5 and 10×10^6. Finally, oocytes or COCs with high fluorescence (H-pattern) had CTCF values greater than 10×10^6.

The proportions of oocytes with different fluorescence intensities were different ($P < 0.05$) through the incubation period in maturation medium. The presence of GDF-9 during the IVM of oocytes and COCs decreased during the IVM process, because there was a decrease of immunofluorescence labeled from the onset of incubation to after 96 hours (Fig. 4; Table 1; $P < 0.05$).

The simultaneous evaluation of oocytes for protein immunodetection and nucleus analysis using 4′-6-diamidino-2-phenylindole stain, enabled evaluation of GDF-9 presence with respect to meiotic development in all maturing groups (Table 1). In this regard, the intensity of fluorescence was high in nonmatured GV oocytes and decreased ($P < 0.05$) with time of culture in MI–MII oocytes. Almost all oocytes with the high level of fluorescence (H-pattern) were at the GV stage; however, most oocytes at the GV stage displayed medium fluorescence emission (M-pattern). Oocytes at the GV stage were only nonmatured oocytes; this stage of meiosis was not present during *in vitro* culture. Medium (M) and low (L) immunolabel pattern were observed in the middle stage of meiotic

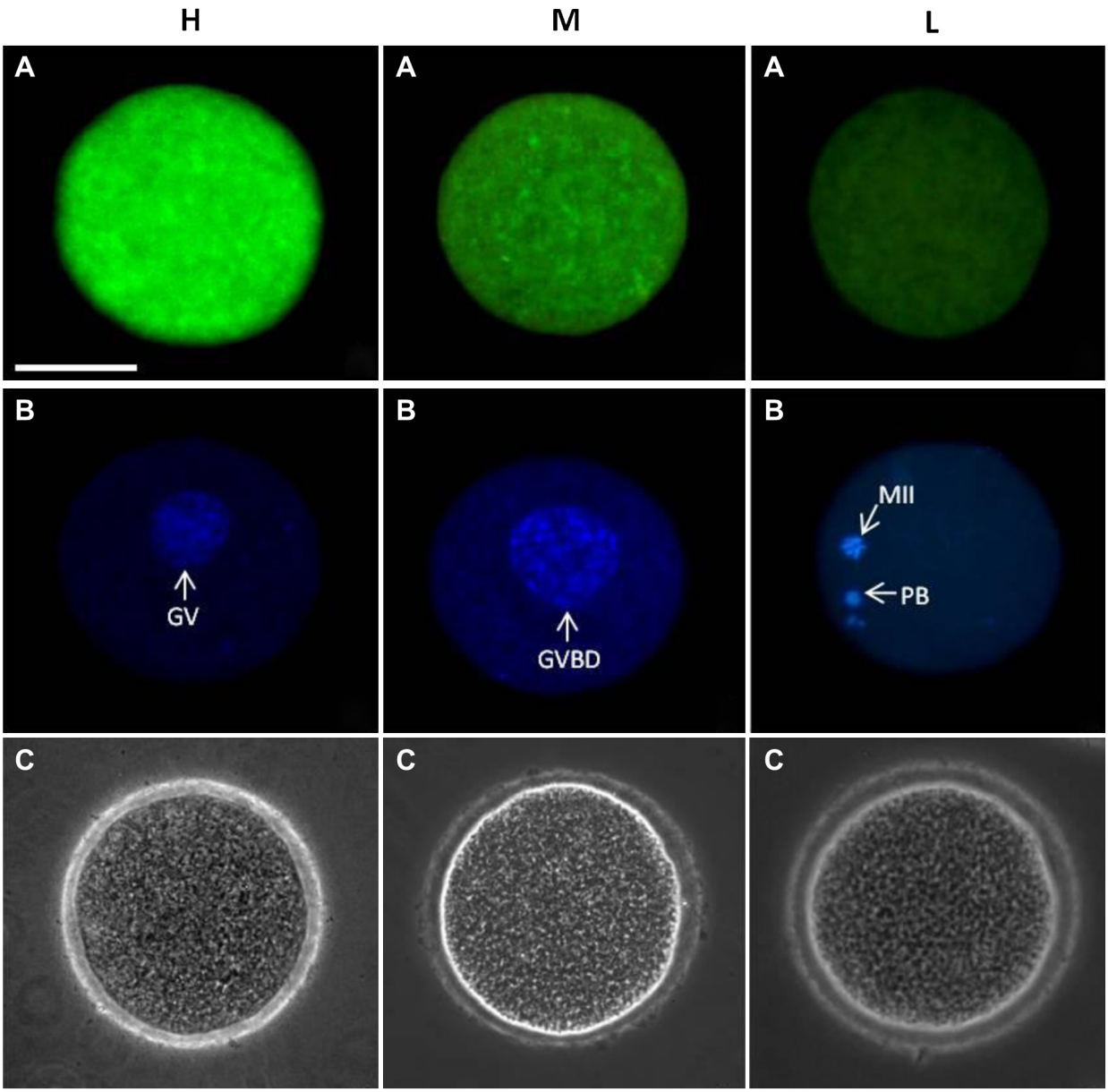


Fig. 2. Photomicrographs of detection of growth differentiation factor 9 expression using indirect immunofluorescence in canine oocytes. Images show representative patterns of three canine oocytes with (A) fluorescein-conjugated with C-18 human anti-human growth differentiation factor 9 polyclonal antibody (green); H, high (nonmature); M, medium (IVM 48 hours); and L, low fluorescence emission (IVM 96 hours). (B) 4′-6-diamidino-2-phenylindole (blue) staining for nucleus evaluation: HB, germinal vesicle (GV) stage; MB, GV breakdown (GVBD) stage; LB, second metaphase (MII) stage. (C) Contrast phase. Observations were made with an epifluorescence inverted microscope. Scale bar, 50 μm. PB, first polar body extrusion.

maturation (GVBD–MI) after 48 hours of culture. The nuclear stages of MI and MII were seen more frequently in oocytes at 72 to 96 hours of incubation, displaying mostly a low fluorescence pattern ($P < 0.05$), highlighting the fact that all MII stage oocytes, including all ovulated, displayed only low fluorescence emission.

The evaluation of cumulus expansion showed that all nonmature oocytes had cumulus cells firmly attached to the oocyte, without signs of expansion (level 0). Cumulus expansion began to increase 48 hours after IVM and increased continuously up to 72 hours ($P < 0.05$) as shown in Table 2. Ovulated oocytes displayed levels 3 and 4 of cumulus oophorus expansion.

The levels of GDF-9 expression determined using Western blot analysis in denuded oocytes and COCs are shown in Figure 5A and B, respectively. Different bands were detected, at approximately 56 kd which represent the proprotein, and two bands very similar in molecular weight, approximately 20 kd, suggesting the possibility of two mature forms of GDF-9 protein. The optical density

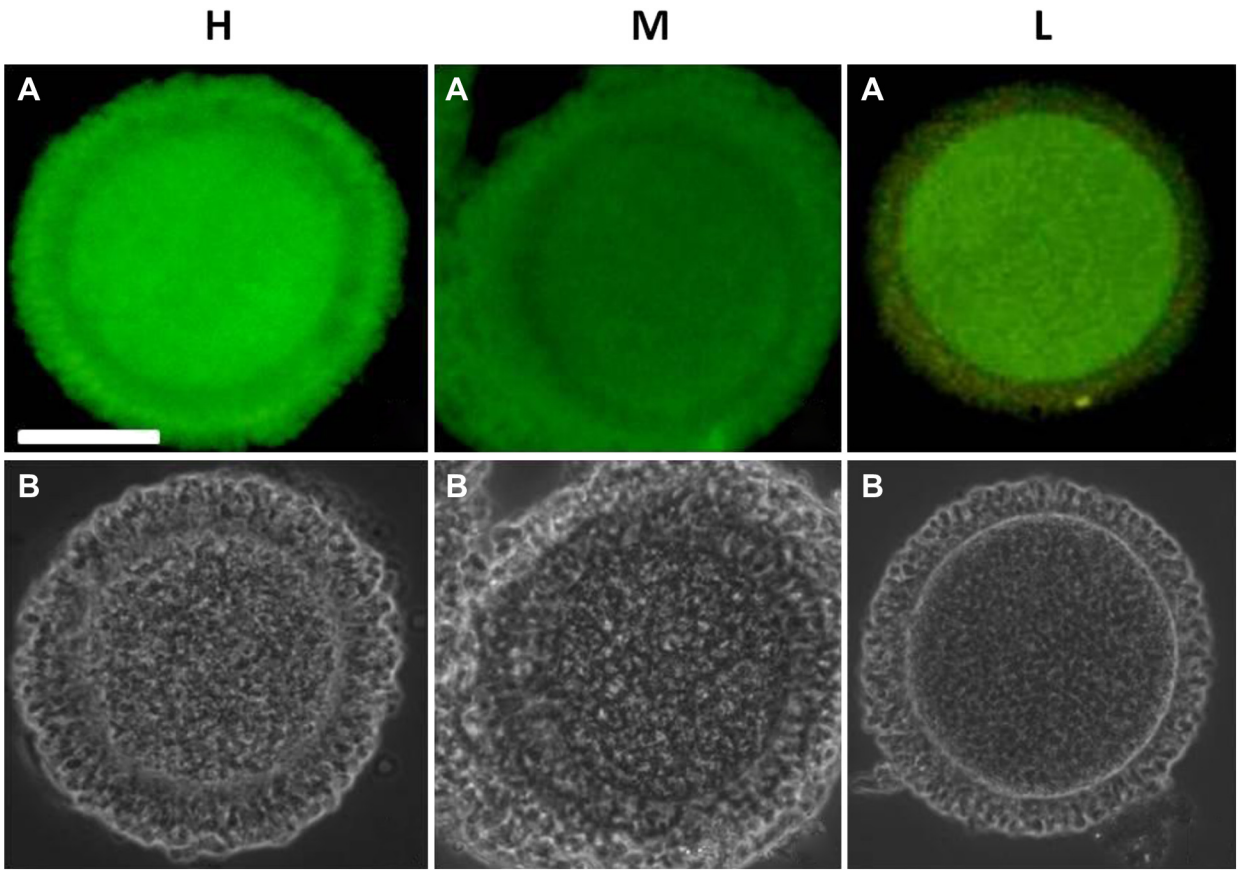


Fig. 3. Photomicrographs of expression level of growth differentiation factor 9 in representative bitch cumulus oocyte complexes (COCs) analyzed using (A) epifluorescence and (B) phase-contrast microscopy. Rows show COCs displaying different levels of fluorescence emission: H, high (nonmature COC); M, medium (COC IVM for 48 hours); and L, low immunofluorescence intensity (COC IVM for 72 hours). C-18 human anti-human growth differentiation factor 9 antibody was used and observed with an epifluorescence inverted microscope. Scale bar, 50 μm.

resulting from the proprotein GDF-9 band was higher ($P < 0.05$) than those resulting from the mature GDF-9 bands. The proprotein forms of GDF-9 were detected in all samples, in denuded oocytes and COCs. However, their mature forms were present only in COCs, mainly in those nonmatured *in vitro*. The expression of GDF-9 mature proteins in canine IVM oocytes was not observed in the other maturing groups after 48 hours.

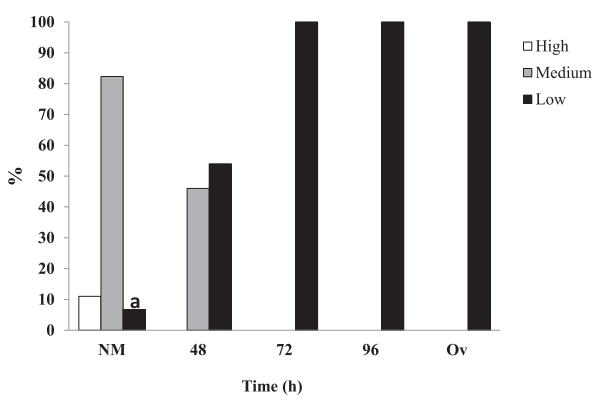


Fig. 4. Expression levels of growth differentiation factor 9, assessed using fluorescence emission, in nonmatured (NM), IVM, and ovulated (Ov) canine COCs: percentage of COCs displaying different fluorescence emission (high, medium, or low). Maturation time indicated as 48, 72, or 96 hours. COC, cumulus oocyte complex.

The expression levels of proproteins determined using densitometry analysis of the bands, were higher in COCs than in denuded oocytes ($P < 0.05$), and either with or without cumulus cells, a significantly higher expression was noticed in nonmatured oocytes or in those at the beginning of culture in comparison with other culture periods. The proprotein expression profile decreased ($P < 0.05$) from 48 hours, coincidentally with the beginning of cumulus expansion, as shown in Table 2.

The expression level of GDF-9 in COCs, determined using Western blot analysis, had a significantly negative correlation ($r = -0.97$; $P < 0.05$) with cumulus expansion throughout cultures according Pearson correlation coefficient.

4. Discussion

Given the fundamental role of GDF-9 in oocyte development and in the function of cumulus cells in particular

Table 1
Growth differentiation factor 9 expression pattern (assessed by fluorescence emission) in nonmatured, IVM, and ovulated canine oocytes.

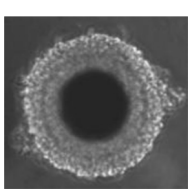
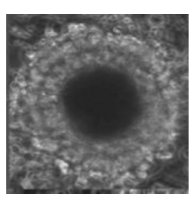
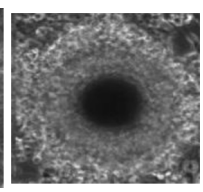
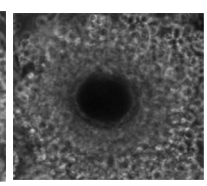
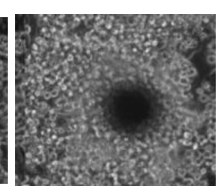
Oocyte groups	GDF-9 expression pattern	Nuclear development				Total, N = 209
		GV, N = 39	GVBD, N = 25	MI, N = 109	MII, N = 36	
Nonmatured, N = 47	H	13 (27.7)[a,A]	1 (2.1)[a,B]	—	—	14 (29.8)
	M	26 (55.3)[b,A]	7 (14.9)[b,B,x]	—	—	33 (70.2)
	L	—	—	—	—	—
IVM for 48 h, N = 46	H	—	—	—	—	—
	M	—	10 (21.7)[a,x]	9 (19.6)[a,x]	—	19 (41.3)
	L	—	4 (8.7)[b,A,x]	21 (45.7)[b,B,x]	2 (4.3)[A,x]	27 (58.7)
IVM for 72 h, N = 48	H	—	—	—	—	—
	M	—	1 (2.1)[A,y]	5 (10.6)[a,B,y]	—	6 (12.8)
	L	—	1 (2.1)[A,y]	33 (68.8)[b,B,y]	8 (16.7)[C,y]	42 (87.5)
IVM for 96 h, N = 52	H	—	—	—	—	—
	M	—	—	4 (7.7)[a,y]	—	4 (7.7)
	L	—	1 (1.9)[A,y]	37 (71.2)[b,B,y]	10 (19.2)[C,y]	48 (92.3)
Ovulated, N = 16	H	—	—	—	—	—
	M	—	—	—	—	—
	L	—	—	—	16 (100)[z]	16 (100)

Data are presented as N (%) in each maturing group.
Abbreviations: GDF-9, growth differentiation factor 9; GV, germinal vesicle; GVBD, germinal vesicle breakdown; H, high fluorescence emission; L, low fluorescence emission; M, medium fluorescence emission; MI, first metaphase; MII, second metaphase.
[a,b] Within a column and sampling time, values without a common superscript differed ($P < 0.05$).
[A,B,C] Within a row, values without a common superscript differed ($P < 0.05$).
[x,y,z] Within a column and fluorescence pattern, values without a common superscript differed ($P < 0.05$).

[39,40], in this study we intended to identify the expression patterns of this factor in dog oocytes in the time course of *in vitro* maturation. This preliminary evidence indicates for the first time the presence of GDF-9 in canine oocytes and cumulus cells before and during culture, and in ovulated oocytes matured in the oviducts.

The immunofluorescence analysis showed different fluorescence emission and therefore, GDF-9 expression according state of maturity. The highest fluorescence emission (pattern H), was observed only in oocytes before culture, but the oocytes that displayed this pattern were approximately one-third of this group, because most nonmatured oocytes displayed medium emission instead (pattern M). This demonstrates that oocytes obtained from the ovaries had distinct levels of GDF-9, and thus different states of maturity, although at chromatin evaluation most of them were at the GV stage. We infer that oocytes that expressed high levels of GDF-9 (pattern H) were those in the earliest state of development among the nonmatured group, because such a high emission was not observed during IVM, or in oocytes at more advanced stages of meiosis. In our study, mainly large oocytes were selected for experiments, because they are more likely to have acquired meiotic competence; however, it is possible that among the large oocytes, there were oocytes somewhat smaller than 100 μm, which represented the low rate of oocytes displaying pattern H in the nonmatured group. In the bitch, oocytes that have reached 100 μm come from small follicles that have already developed an antrum [41]. Therefore, because florescence intensity was shown to

Table 2
Cumulus expansion during IVM culture and in ovulated canine oocytes.

Oocyte Group	Cumulus expansion					Total, N = 1170
	0	1	2	3	4	
						
	N = 285	N = 12	N = 168	N = 373	N = 332	
0 h	285 (100)	—	—	—	—	285
IVM for 48 h	—	12 (4.1)[A]	137 (47.2)[a,B]	100 (34.5)[a,C]	41 (14.1)[a,D]	290
IVM for 72 h	—	—	21 (7.3)[b,A]	158 (54.7)[b,B]	110 (38.1)[b,C]	289
IVM for 96 h	—	—	10 (3.4)[c,A]	110 (37.9)[c,B]	170 (58.6)[c,C]	290
Ovulated	—	—	—	5 (31.3)[d,A]	11 (68.8)[d,B]	16

Scoring was according to the grading system described by Prochazka et al. [38].
[a,b,c,d] Within a column, values without a common superscript differed ($P < 0.05$).
[A,B,C,D] Within a row, values without a common superscript differed ($P < 0.05$).

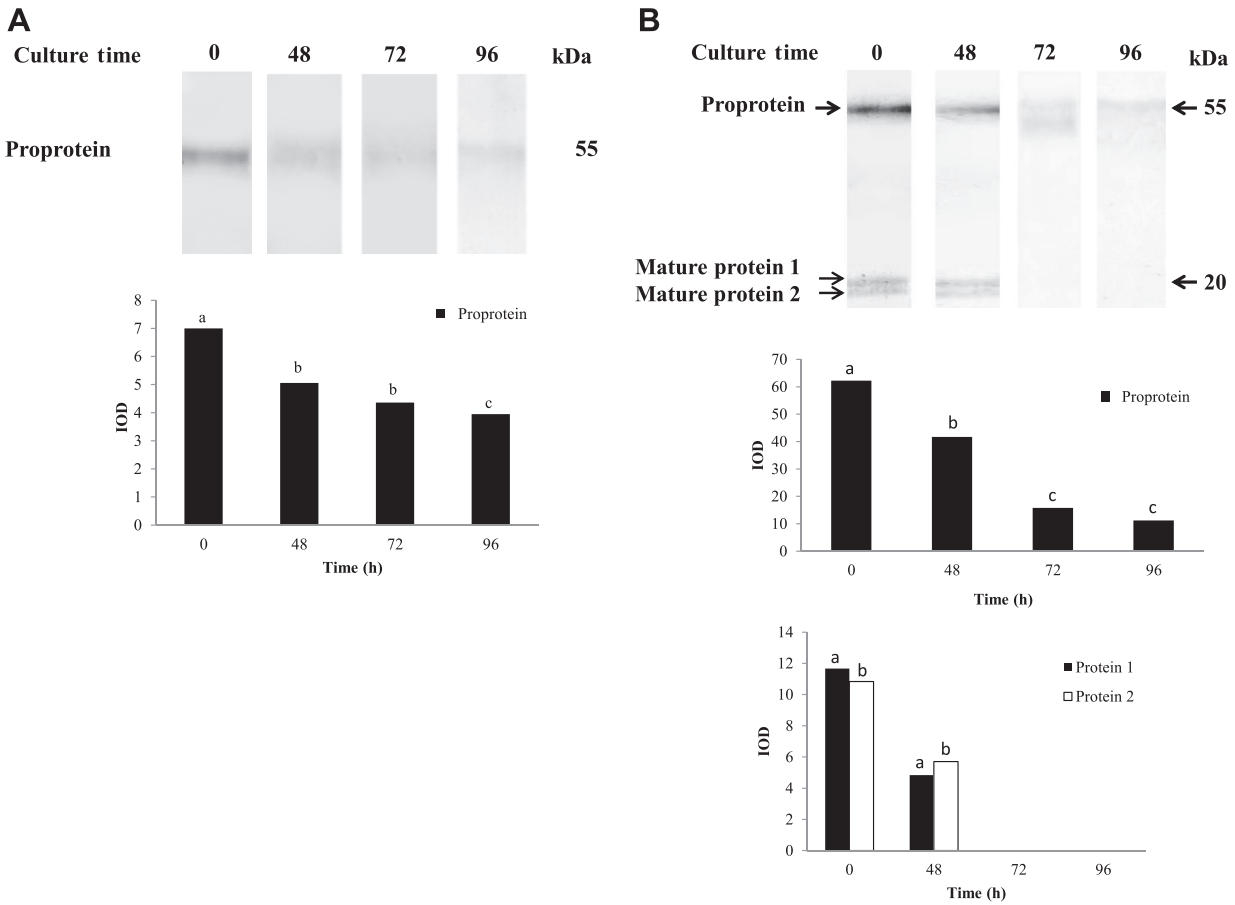


Fig. 5. Western blot analysis of growth differentiation factor 9 (GDF-9) using C-18 human anti–GDF-9 polyclonal antibody, before (0 hours) or after *in vitro* maturation of 48, 72, and 96 hours. Bands were analyzed densitometrically using the Gel-Pro Analyzer V4 program, and compared using ANOVA. The results are expressed as IODs of each band throughout culture time and appear in graphical format below the bands. (A) Denuded oocytes showed bands representing the GDF-9 proprotein after all culture periods. The expression level decreased throughout time; (B) oocytes with cumulus cells. Western blot analysis revealed distinct bands of approximately 55 kDa representing levels of GDF-9 proprotein that declined with time and at approximately 20 kDa representing the mature GDF-9. Specific mature protein was only detected at 0 and 48 hours and in all replicates the mature protein band was split, suggesting the possibility of two mature forms of GDF-9. IOD, integrated optical density.

reflect the level of GDF-9, presumably in dogs this factor is expressed mostly in oocytes from preantral follicles. These observations remain to be confirmed, but would agree with previous reports in mice where it has been suggested that the primary action of GDF-9 is to promote progression of primary follicles to later preantral stages, thus indirectly stimulating the primordial population to increase to primary transition [15].

During culture, the oocytes were able to resume meiosis and their chromatin was observed mainly at the GVBD or MI stage; along with this, fluorescence intensity decreased throughout time and appeared as oocytes displaying low fluorescence emission (pattern L). All oocytes incubated for IVM that reached the MII stage and those MII-ovulated oocytes, showed only a low fluorescence level, indicating a modest GDF-9 expression *in vitro* and *in vivo* in meiotically mature oocytes. It is known that protein synthesis patterns change significantly during the transition from an immature oocyte to a mature one [42], because at approximately the time it becomes competent to resume meiosis, transcription is inhibited [43]. Therefore, it appears that this change probably begins at approximately 48 hours of culture for GDF-9 in dog oocytes.

Similar to other members of the transforming growth factor beta super family, GDF-9 is produced as a proprotein, including a signal peptide, a large proregion, and a smaller mature region [44,45]. This proprotein must undergo proteolytic cleavage before the secretion of the mature form of the molecules [46,47]. In this study, the Western blot data of the expression profile of GDF-9 was consistent with that fact, because we detected bands of GDF-9 at approximately 56 kd, representing proprotein and mature protein of approximately 20 kd.

Western blot analysis was used to compare levels of GDF-9 (proprotein and mature protein) in canine oocyte and cumulus cells throughout different intervals of maturation *in vitro*. During all culture periods the predominant form of GDF-9 was the unprocessed precursor protein. The level of proprotein signal, measured using densitometry, exhibited decreased expression from 0 to 96 hours of culture, for denuded oocytes and COCs. The total expression level was higher in oocytes processed with their cumulus

mass than those denuded, suggesting expression in the cumulus cells. In this respect, although GDF-9 is expressed exclusively by the oocyte in rodent [7,32], in bovine, the transcripts encoding GDF-9 are detected in cumulus cells, providing evidence that the oocyte is not the sole source of this factor [44]. This is consistent with the finding of GDF-9 protein and mRNA in porcine [38,48], bovine [49], caprine [50], human [51], and nonhuman primate [52] cumulus cells.

The banding pattern of the mature form found herein, might suggest the presence of two protein bands with similar molecular weights (approximately 19 and 21 kd). Although it is not completely clear, this could be related to the two splicing variant forms of GDF-9 cDNAs, described recently in canine ovarian tissue [45]. Interestingly, this was observed only in oocytes nonmatured or *in vitro*-cultured up to 48 hours that were processed with their cumulus cells for Western blot analysis. Therefore, the presence of the mature forms of GDF-9 would be restricted to the cumulus cells before expansion or at the time that it starts. Ongoing studies at our laboratory show that protein expression profiles detected herein *in vitro* would be similar to that of GDF-9 mRNA in canine COCs according to the results of reverse transcription quantitative polymerase chain reaction (unpublished data).

In porcine oocytes, the expression level of the GDF-9 gene also declines during the *in vitro* maturation process and is not elevated at the time of cumulus expansion [38,48,53]. On the contrary, in buffalo oocytes, GDF-9 proprotein and mature GDF-9 protein was increased during maturation [54]. In the same way in caprine, GDF-9 mRNAs were expressed at low levels in immature oocytes and increased to the highest level at 12 hours of IVM, which coincides with the time of cumulus cell expansion [50]. Similarly, a significant relationship between nuclear maturation and mature GDF-9 levels was found in human oocytes [21]. These discrepancies among studies could be because of species differences.

The negative correlation between the expression level of GDF-9 and cumulus expansion in canine, raise the possibility that other factors play a role in this function at this time or maybe a more delayed effect of GDF-9 exists in this regard. It has been described that canine GDF-9 has an extra N-glycosylation site in the mature protein in contrast to other mammals, which could be related to a corresponding reduction of mature protein secretion [45], because N-linked glycans play a role in regulating post-translational processing to produce the mature protein [55]. This fact agrees with the lack of mature forms after 48 hours of IVM, supporting the notion that this factor could be more limited in dogs compared with in other species which might be related, at least in part, with the delay in mucification, because a low concentration of GDF-9 does not stimulate enzymes regulating cumulus expansion (hyaluronon synthetase 2 and cyclooxygenase-2) [38], affecting therefore the meiotic resumption.

4.1. Conclusions

So far, the exact mechanism for decreased expression of GDF-9 during IVM in canine is not clear. The signaling pathway for GDF-9 is only beginning to be understood in other species and is largely unknown in dogs; but in summary, the present data support the idea that in dogs the major expression of GDF-9 in oocytes and cumulus cells is present at an early stage of development, possibly in a species-specific manner, and IVM does not seem to be different to *in vivo* in the minor levels of GDF-9 production in meiotically mature oocytes.

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