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PERFIL PROTEICO DE OÓCITOS CANINOS

(*Protein profile of canine oocytes*)

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RESUMO

O presente estudo foi conduzido para caracterizar o proteoma de oócitos caninos. Oócitos foram coletados de 120 cadelas e apenas os COCs grau 1 foram selecionados para o cultivo *in vitro*. Após o cultivo, os oócitos foram submetidos à extração de proteínas. As proteínas foram digeridas com tripsina e analisadas por espectrometria de massa. Trinta e quatro proteínas foram identificadas nos oócitos caninos. Estas proteínas foram agrupadas em três categorias de acordo com a sua função biológica, molecular e localização celular. Quanto ao processo biológico, foram encontradas diversas proteínas envolvidas no ciclo celular, fertilização, regulação da transcrição e via de sinalização. A análise da ontologia do gene revelou alta porcentagem de proteínas envolvidas na atividade de ligação. Com base na análise da rede proteína-proteína usando a plataforma STRING, observou-se que a vimentina apresentou interações com as CASP3, CASP6, CASP7 e CASP8, envolvidos na apoptose. O componente de complemento C3, interagiu com receptores do complemento, como CR1 e CR2. A proteína de ligação retinol 4 interagiu com precursores de retinol. Actina esteve intimamente relacionada com as proteínas cofilinas 1 e 2. A queratina 10 interagiu com a proteína CDK9 relacionada ao processo de sinalização celular. Essas proteínas são essenciais para o desenvolvimento completo de oócitos e fertilização. O presente estudo contém a primeira descrição da composição proteica dos oócitos caninos. A construção de bibliotecas de proteínas de oócitos, para cada espécie, estabelecerá as bases para a compreensão e o mapeamento dos eventos cruciais que definem a competência dos oócitos.

Palavras-chave: Maturação *in vitro*, ciclo celular, proteômica, cadelas.

ABSTRACT

The present study was conducted to characterize the major proteome of canine oocytes. Ovaries were collected from 120 bitches and only Grade 1 COCs were selected for *in vitro* culture. After *in vitro* maturation, oocytes were subjected to protein extraction. Proteins were then trypsin-digested and analyzed by tandem mass spectrometry. Thirty-four proteins were identified in the canine oocytes. These proteins have been grouped into three different categories according to their biological, molecular function and cellular localization. With regard to biological process, we found many proteins involved in cell

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cycle, fertilization, transcription regulation and signaling pathway. The gene ontology analysis also revealed a high percentage of proteins involved in binding activity. Based on protein–protein network analysis using STRING platform, we found that vimentin presents links with CASP3, CASP6, CASP7 and CASP8, which are involved in apoptosis. Complement component C3, interacted with complement receptors, such as CR1 and CR2. Retinol-binding protein 4 interacted with retinol precursors. Actin potentially interacted with cofilin protein 1 and 2. Keratin 10, in turn, had interacted with CDK9, which are involved in pathway signaling. These proteins are essentials for the complete oocyte development and fertilization. In summary, the present study contains the first description of the main protein composition of canine oocytes. Construction of libraries of oocyte proteins, for each especies, will set the foundations for understanding and mapping the crucial events that define oocyte competence.
Key words: *In vitro* maturation, cell cycle, proteomic, bitches.

INTRODUCTION

In mammals, when oocytes are removed from the follicular environment, they no longer have the signal that keeps the meiosis block and restart meiosis. In bitches, although oocytes can spontaneously resume meiosis *in vitro*, maturation rates are as low as 20% reach metaphase II (M-II) (PEREIRA *et al*., 2014). Canids have reproductive characteristics that differentiate them from other species. In most mammalian species, oocytes in germinal vesicle stage (prophase I) undergo meiosis resumption in the final stages of follicular maturation, being ovulated in metaphase II. However, in canids, oocytes are released in an immature stage early in the first meiotic division, while still in the germinal vesicle stage (OTOI *et al*., 2007). The mechanisms that control induction and coordination of these morphological events are still unclear in canids.

The control of cell cycle is regulated by proteins related to meiosis resumption through phosphorylation and dephosphorylation processes. Any changes in synthesis and consequently stock of these proteins influence oocyte development. Although some proteins have already been identified in the oocyte of species such as bovine (MEMILI *et al*., 2007), porcine (GUPTA *et al*., 2009), murine (VITALE *et al*., 2007) and bovine, the precise roles played by the major oocyte proteins as well as the signaling pathways for initiating intracellular events have not yet been fully elucidated. Proteomics has emerged as a powerful technology for evaluation of the protein profiles of *in vivo* and *in vitro* maturated oocytes. These efforts will definitely help to better understand the molecular processes that occur during oocyte maturation. Thus, the present study was conducted to characterize the first major protein composition of canine oocytes.

MATERIAL AND METHODS

Selection of ovaries and oocytes

Ovaries were collected from 120 adult bitches. These ovaries were obtained from spay/neuter programs developed at Sorocaba, Sao Paulo, Brazil. The use of animals for this research was approved by Ethics Committee from São Paulo State University (reference number 84/2015). Right after ovariohysterectomy, ovaries were immersed in saline solution (0.9% NaCl) and transported at 4 °C to the Laboratory of Small Animal Reproduction at Sao Paulo State University (UNESP), Botucatu, São Paulo, Brazil. Ovaries were cut into thin slices for the release of cumulus-oocyte complexes (COCs; Fig. 1C). COCs were quantified under stereomicroscope (Leica® MZ 12.5) and only grade 1 COCs were selected.

In vitro oocyte maturation (IVM)

Grade I COCs were cultured in four-well plates containing TCM-199 medium supplemented with 25 mM HEPES, 50 µg/mL penicillin/streptomycin, 26 mM sodium bicarbonate, 1.5 mM sodium pyruvate, 2.9 mM sodium lactate pentahydrate, 0.6 mM cysteine, 0.03UI/mL hCG, 0.5 µg/mL FSH, 20 µg/mL E_2 and 10ηg/mL epidermal growth factor (EGF) at 38.5 ºC in a humidified atmosphere of 5% CO_2 for 72 h.

Protein extraction from oocytes

After IVM, oocytes were transferred to culture plates with 0.2% hyaluronidase solution for removal of cumulus cells. They were then washed in PBS and transferred to a glass conical tube containing 20mM Tris, 150mM NaCl, 1.0 EGTA, 1.0mM EDTA, 2.5mM sodium phosphate, 1.0mM β-glicerophosphate, 1.0mM Na_3VO_4, 1.0mg/mL leupeptin and 1.0mM PMSF solution. Samples were frozen in liquid nitrogen and sonicated (five times during 25 seconds, 10% amplitude and 1 minute interval). Cell extracts were stores at -80 °C until use. Proteins present in the extract were quantified in triplicates by the Bradford method (Bradford, 1976) (Protein Assay, BioRad®, USA), with bovine serum albumin (BSA) as standard protein. Since the amount of total protein obtained per oocyte was very small, pool was made using oocytes after 72h of culture and immature oocytes (0h). A total of 500 oocytes was used in this study.

Protein digestion

Samples were digested in solution starting with reduction and alkylation steps using 10 mM dithiothreitol (DTT) and 45 mM iodol acetic acid (IAA), respectively. Then, samples were submitted to proteolytic digestion in the presence of trypsin at concentration of 1:50 (enzyme: substrate), solubilized in 50 mM ammonium bicarbonate buffer, pH 7.8. Hydrolysis took place for 18 hours, being interrupted with the addition of 1% formic acid (v/v) over the sample volume. These were then desalted using Sep-Pak Vac C18 cartridges (Waters, Milford, MA, USA). Digested samples were desalted and freeze-dried in SpeedVac™ (Thermo Scientific, USA) and kept under refrigeration until the moment of analysis by mass spectrometry.

Mass spectrometry analysis

Samples were solubilized in 60 uL in 0.1% formic acid solution (v/v) and then an aliquot of 15 µL of tryptic digests. Each sample was individually injected into a C18 analytical column, 1.7µm BEH 130 (100µm x 100mm) on a reverse phase liquid chromatography system (RP-UPLC – Nano ACQUITY UPLC Waters - Milford, USA) coupled to a mass spectrometry device Q-Tof PREMIER (MicroMass/Waters, Milford, USA). Analyses were run in triplicates. The linear gradient used was from 2 to 90% (v/v) of acetonitrile in 0.1% formic acid (v/v) for 60 min and flow of 600 nL/min.

Database searching and protein identification

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02). Mascot was set up to search the Canis_sp_ncbi_032015 database (60.405 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.100 Da and aparent ion tolerance of 0.100 Da. Cysteine carbamidomethyl was specified in Mascot as a fixed modification. Methionine oxidation was specified in Mascot as a variable modification. Scaffold (version Scaffold 4.4.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications.

Gene ontology

Proteins were annotated with GO terms from NCBI. Data from the canine oocyte protein list obtained after MASCOT search were analyzed using the software for researching annotations of proteins (STRAP), an open-source application. Gene ontology terms for

biological process and molecular function were obtained from UniProtKB and EBI GOA databases (REGO *et al*., 2014).

In silico analysis of protein-protein interactions

Protein-protein interaction (PPI) networks were retrieved from STRING (http://string-db.org) version 9.0 database (Snel *et al*., 2009). This database consists of known and predicted protein interactions collected from direct (physical) and indirect (functional) associations. Network analysis was evaluated for six proteins: vimentin, complement C3, Retinol-binding protein 4, Actin, cytoplasmic 1, Keratin 10, Poly(A)-specific ribonuclease.

RESULTS AND DISCUSSION

In the present study, we used a gel-free proteomics approach to describe, for the first time, the major protein composition of canine oocytes. In the canine species, reproduction has unique aspects as oocytes are released while still immature and, therefore, they are not ready to be fertilized (OTOI *et al*., 2007). Several of these processes are surely regulated by proteins and their interactions (CURCIO *et al*., 2006). Thus, identification of the main oocyte proteome will help the understanding of the mechanisms regulating some aspects of canine oocyte growth and signaling pathways for initiation of intracellular events.

Thirty-four proteins were identified in the canine oocytes. Protein identifications, access codes and their functions were established using genebank and Uniprot (Tab.1).

These proteins have been grouped into three different categories according to their biological, molecular function and cellular localization (Fig. 1A, 1B and 1C).

Based on protein–protein network analysis using STRING platform, we evaluated interactions associated with proteins: Vimentin (Fig. 2A), Complement component C3 (Fig. 2B), Retinol-binding protein 4 (RBP4) (Fig. 2C), Actin (Fig 2D), Keratin 10 (Fig 2E) and Poly(A)-specific RNase (PARN) (Fig 2F).

Table 1: Identification by mass spectrometry of canine oocyte proteins.

	GENE	PROTEIN NAME	SCORE	CODE	TAXONOMY
1.	ACTB	Actin, cytoplasmic 1	210	GI:160332358	*Canis l. familiaris*

2.	AHSG	Alpha-2-HS-glycoprotein	36	GI|545553759	*Canis l. familiaris*
3.	ASTL	Astacin-like metalloendopeptidase isoform X1	34	GI|545527731	*Canis l. familiaris*
4.	ATP5B	ATP synthase subunit beta, mitochondrial isoform 1	140	GI|73968432	*Canis l. familiaris*
5.	C3	Complement C3	53	GI|545535669	*Canis l. familiaris*
6.	DYNLT3	Dynein light chain 2, cytoplasmic isoform X9	55	GI|928144846	*Canis l. familiaris*
7.	EEF1A1	Elongation factor 1-alpha 1	59	GI|308199425	*Canis l. familiaris*
8.	KRT1	Epithelial keratin 1	64	GI|34979907	*Canis l. familiaris*
9.	KRT10	Epithelial keratin 10	15	GI|34979905	*Canis l. familiaris*
10.	FGB	Fibrinogen beta chain isoform X3	55	GI| 555979020	*Bos mutus*
11.	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase-like	135	GI|925115133	*Canis l. familiaris*
12.	HSP90AB1	Heat shock protein HSP 90-beta isoformX1	05	GI|60592792	*Bos taurus*
13.	HIST3H2A	Histone H2A type 1-E-like	19	GI|74004170	*Canis l. familiaris*
14.	KRT3	Keratin, type II cytoskeletal 3	08	GI| 345791833	*Canis l. familiaris*
15.	KRT6A	Keratin, type II cytoskeletal 6A	06	GI| 545545388	*Canis l. familiaris*
16.	KHDRBS1	KH domain-containing protein 3-like	38	GI| 928152308	*Canis l. familiaris*
17.	MDH1	Malate dehydrogenase, mitochondrial isoform X1	51	GI| 57092971	*Canis l. familiaris*
18.	MVP	Major vault protein	397	GI| 560879431	*Canis l. familiaris*
19.	PARN	Poly(A)-specific ribonuclease (PARN)	65	GI| 73958883	*Canis l. familiaris*
20.	PADI6	Protein-arginine deiminase type-6	40	GI| 924183491	*Canis l. familiaris*
21.	F2	Prothrombin	104	GI| 359321961	*Canis l. familiaris*
22.	RBP4	Retinol-binding protein 4	186	GI| 928175781	*Canis l. familiaris*
23.	ALB	Serum albumin	129	GI| 22531688	*Canis l. familiaris*
24.	HSPA9	Stress-70 protein, mitochondrial	57	GI| 73970888	*Canis l. familiaris*
25.	HADHB	Trifunctional enzyme subunit alpha, mitochondrial	31	GI| 359321587	*Canis l. familiaris*
26.	TUBA1C	Tubulin alpha-1C chain	55	GI| 359323129	*Canis l. familiaris*
27.	TUBB2B	Tubulin beta-2B chain	72	GI| 345796951	*Canis l. familiaris*
28.	TDRKH	Tudor and KH domain-containing protein	50	GI| 545528950	*Canis l. familiaris*
29.	VIM	Vimentin	198	GI| 559098393	*Canis l. familiaris*
30.	YBX2	Y-box-binding protein 2	32	GI| 57086603	*Canis l. familiaris*
31.	ZP2	Zona pellucida sperm-binding protein 2	98	GI| 50979132	*Canis l. familiaris*
32.	ZP3	Zona pellucida sperm-binding protein 3	86	GI| 50979000	*Canis l. familiaris*
33.	ZP4	Zona pellucida sperm-binding protein 4	112	GI| 345798853	*Canis l. familiaris*
34.	ZP2	Zona pellucida 2 glycoprotein	1172	GI| 633050	*Canis l. familiaris*

Code: Genebank Access Code at, *Canis l. familiaris: Canis lupus familiaris.*

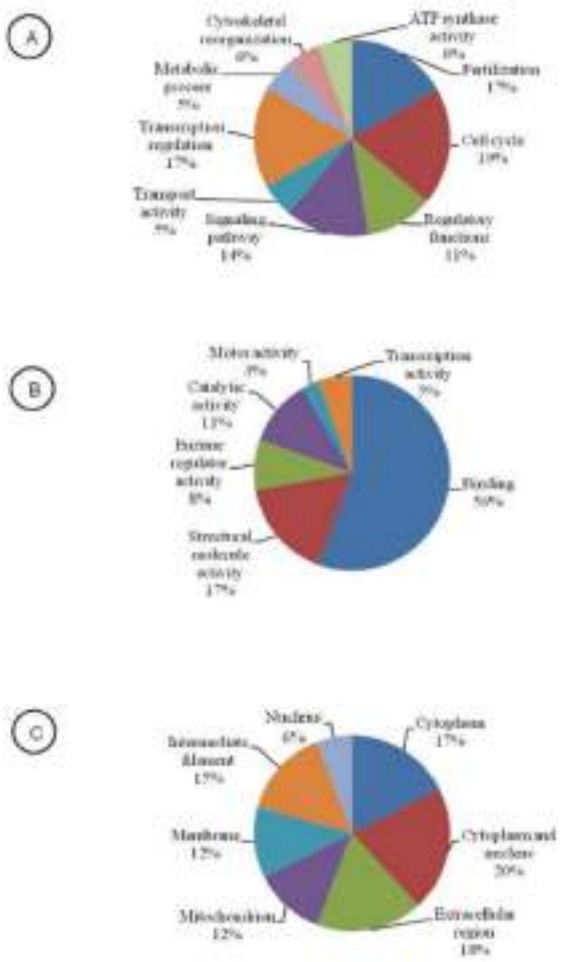

Fig.1. Gene ontology (GO) of canine oocytes proteins. These proteins were grouped into three different categories according to their biological (1A), molecular function (1B) and cellular localization (1C).

Most proteins identified in the oocytes of bitches used in our study are functionally related to cell cycle, transcription and fertilization. Keratin 1 (K1) and 10 (K10) are among the proteins identified in the present study. Keratins are intermediate filament proteins of epithelia. K10 is co-expressed with K1 and research conducted by Paramio *et al.* (2001) showed that K10 protein inhibits cell proliferation by reducing the expression of cyclin D1. These effects are the result of interaction of K10 with protein kinase B (Akt) and protein kinase C (PKC), which are key molecules in cell signaling through phosphatidylinositol 3-kinase. Still according to the same authors (PARAMIO *et al*., 2001), the connection of Akt and PKC associated with K10 prevents translocation of these proteins to the membrane and their respective activation. This inhibition would be responsible for the induction of meiosis resumption, playing a role in cell proliferation, differentiation and apoptosis. The interactome (Fig. 2E) reveled that Keratin 10 has links with cyclin D1 and cyclin-dependent

kinase 9, which are involved in the regulation of transcription. Cyclin-dependent kinases are required only at specific times during mitotic progression. Although levels of cdks remain constant, the levels of cyclins usually fluctuate during the cell cycle due to periodic synthesis and degradation, resulting in transient kinase activities (VORONINA *et al.*, 2003). Thus, their levels are essentials for cell signaling and any alteration could cause severe consequences during oocyte maturation.

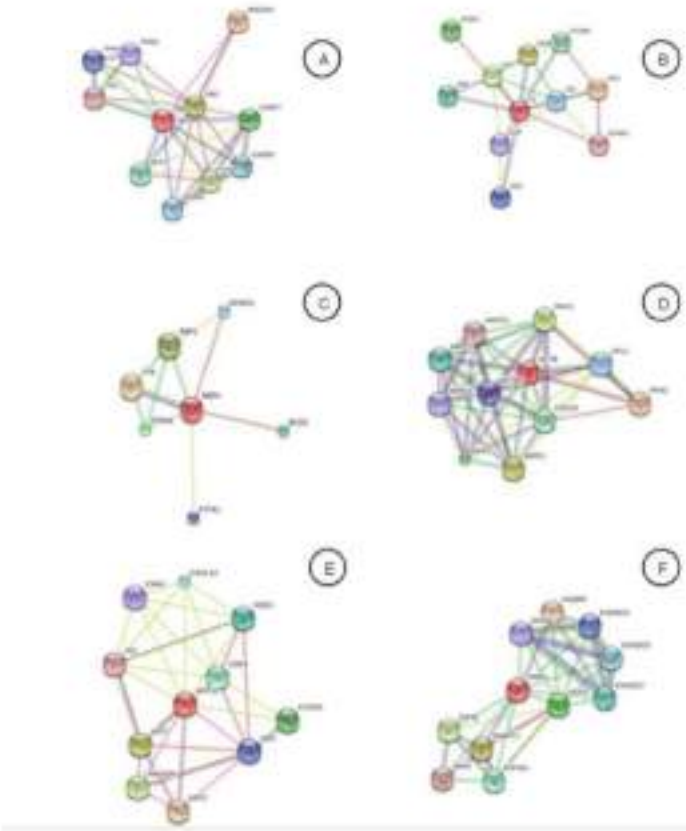

Fig. 2. (**A**) Protein- protein interaction (PPI) networks of vimentin protein; (**B**) PPI of complement C3 protein; (**C**) PPI of retinol-binding protein 4; (**D**) PPI of actin protein; (**E**) PPI of keratin 10 protein; (**F**) PPI of Poly(A)-specific Rnase.

Vimentin, also identified in our study, has been found in mouse oocytes (LEHTONEN *et al.*, 1983) and porcine oocytes (KONG *et al.*, 2014). In the initial stage of folliculogenesis, the primordial follicle consists of the oocyte surrounded by a layer of granulosa cells forming a stock of cells that will differentiate and others that will remain quiescent. After differentiation, these granulosa cells will change resulting in a single layer of cells with round nucleus and cytoplasm containing vimentin intermediate filaments. Such filaments are associated with changes in the shape of granulosa cells (flat to cubical), demonstrating a high mitotic activity and differentiation of granulosa cells and oocyte (VAN

DEN HURK and SANTOS, 2009). Kong *et al.* (2014) observed that maternal vimentin protects DNA from damage during reprogramming of somatic nucleus in oocyte. This way, vimentin can be an important factor for meiosis progression. The interactome (Fig. 2A) revelead that vimentin is associated with caspases 3, 6, 7 and 8. In this regard, Morishima (1999) observed that vimentin is an apoptotic substrate of caspases during apoptosis and caspase-8, shown in the interactome, functions as an inducer of apoptosis and activates other caspases as well. Cleavage of vimetin in apoptotic cells facilitates apoptotic destruction of cells. Knowledge of the appropriate concentrations of these proteins and their respective changes during meiosis will help in understanding the reasons for high degeneration rate observed in oocytes during *in vitro* culture.

Actin and tubulin were also identified in the canine oocytes. These proteins are major structural components of eukaryotic cells and involved in oocyte maturation and competence aquisition. According to Nolasco *et al.* (2005), the alpha-tubulin/beta-tubulin ratios are strictly regulated in mammalian cells due to the signaling process. According to these authors, the reduction in the amount of soluble tubulin is correlated with cell death. Yu *et al.* (2014) suggested that actin and microtubules promote the spindle organization and the oocyte's ability to activate after fertilization. The interactome (Fig.2D) showed actin associated with cofilin protein 1 and 2. The cofilin family of protein is crucial for splitting and depolymerization actin filaments to increase the rate of actin filament turnover and to promote formation of microtubule structure (YU *et al.*, 2014). So, the presence of cofilin protein 1 and 2 in canine oocytes demonstrate that actin may be associated with meiosis progression and oocyte development.

Proteins such as zona pellucida proteins (ZP2, ZP3 and ZP4), tudor and KH domain, astacin, alpha-2-HS-glycoprotein are important for fertilization and have been identified as part of the proteome of canine oocytes. Formation of zona pellucida is associated with the onset of oocyte growth and components of the zona are synthesized and secreted by the developing oocyte. The zona pellucida consists of a family of glycoproteins represented by ZP1, ZP2, ZP3 and ZP4. ZP performs various functions such as the blocking of polyspermy, sperm-oocyte binding, and maintenance of embryo integrity during its early development. Alpha-2-HS-glycoprotein belongs to a family associated with protease inhibitors promoting zona pellucida hardening (WU *et al.*, 2004).

C3 complement protein has activity associated with reproduction and our *in silico* network analyses demonstrated that C3 protein is related to other complement factors. Studies showed that complement factors are secreted in human follicular fluid by granulosa cells and participate in many process, such as synapse maturation, tissue regeneration,

angiogenesis, lipid metabolism and early embryogenesis. The complement CR1 observed in our interactome (Fig.2B) and CR3 were expressed in human oocyte Dimeric C3b could serve as a bridge between sperm and oocyte complement receptors (CR1 and CR3), promoting fertilization (ANDERSON *et al.*, 1993). The presence of the complement protein in oocytes of different species suggests it plays an important physiological function in the oocyte development.

Retinol binding protein 4, identified in our study, was also observed in the porcine (SCHWEIGERT and SIEGLING, 2001) and it can play an essential role during early embryo transport. The retinol transported by retinol-binding protein is associated with follicular development, oocyte maturation and early embryonic development. A study carried out with bovine follicular fluid demonstrated that retinol concentration is an indicator of follicular quality and its presence is high in healthy follicles. Retinol may also act as a regulator of redox signaling pathways and can protect against oxidative damage by maintaining adequate rates of antioxidant compound and enzymes (LIVINGSTON *et al.*, 2004). Retinol precursors (carotene) observed in the interactome associated with retinol-binding protein (Fig. 2C) are potent antioxidants. Low production of retinol obtained from carotenoids can cause problems on events associated with ovulation, migration of the oocyte through the oviduct and implantation of the blastocyst into the uterus (SCHWEIGERT and SIEGLING, 2001). Thus, the transport and metabolism of retinol by retinol-binding protein is essential for the complete oocyte development and fertilization.

The poly(A) specific ribonuclease protein found in our study is involved in the regulation of gene expression in mammals. PARN is associated with the regulation of meiosis during maturation of *Xenopus* oocytes (KÖRNER *et al.*, 1998). PARN plays a specific function, targeting different subsets of mRNA at specific stages of development and also has an additional exonuclease activity and this activity was observed through association with exosomes components 2, 3, 4 and 5, as shown in the PARN interactome presently demonstrated (Fig. 2F). Many exosomes have catalytic functions and others make structural contributions in cell biology.

Based on gene ontology analysis, oocyte proteins that are located in the nucleus play activities related to gene expression, transcription and translation. In the mitochondria, we found proteins responsible for production of energy for cell activities. Some proteins localized in cytoplasm are essentials to cell structure and events related to mitosis and meiosis. Also, results of gene ontology showed that most proteins of canine oocytes were related with binding. Many proteins were observed involved in biological process like cell cycle, transcription regulation, signaling pathway, regulatory functions and fertilization. All

these functions are linked to meiosis and essential to oocyte development and early embryogenesis (THRATHUM and SROYRAYA, 2017).

In summary, the present study contains the first description of the main protein composition of canine oocytes. Among such proteins, several are related to the cell cycle, according to the literature. The supplementation of culture medium for maturation of canine oocyte is usually based on protocols developed for others species. So, the knowledge about the biological and molecular differences among species are crucial for the development of a *in vitro* culture system that promotes complete meiosis in bitches. Construction of libraries of oocyte proteins, for each species, will set the foundations for understanding and mapping the crucial events that define oocyte competence.

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