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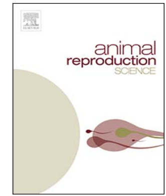

Proteomic analysis of follicular fluid from tropically-adapted goats

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ABSTRACT

The present study was conducted to characterize the major proteome of ovarian follicular fluid from locally-adapted, “Canindé” goats in the northeast of Brazil. Eight estrous cycling goats received a hormonal treatment consisting of medroxyprogesterone acetate, D-cloprostenol and FSH. Fluid was collected by laparoscopy from small (< 3 mm), medium (3–4 mm) and large (> 4 mm) follicles and then, proteins were analyzed by 2-D SDS-PAGE and tandem mass spectrometry. Thirty-six proteins were identified in the goat follicular fluid, including albumin, immunoglobulins, ceruloplasmin, complement factor B, alpha-1B-glycoprotein precursor, serotransferrin, complement C3 and serpins, among others. Albumin and immunoglobulins were the most abundant proteins. Protein concentrations were similar in the fluid from small (45.3 ± 3.1 mg/mL), medium (44.2 ± 3.3 mg/mL) and large follicles (45.1 ± 2.3 mg/mL). The intensities of spots identified in 2-D gels as serotransferrin, zinc-alpha-2-glycoprotein-like, complement factor B and complement protein C3 differed ($P < 0.05$) among follicle categories. The amount of serotransferrin was greater in the medium than small follicles ($P < 0.05$). Content of zinc-alpha-2-glycoprotein-like, complement factor B and complement C3 was greater ($P < 0.05$) in the fluid of large follicles than in medium follicles. Based on gene ontology, the major molecular functions associated with goat follicular fluid proteins were binding and catalytic activity, while the main biological processes were related to regulation, cellular processing, location and the immune system. In conclusion, the major proteome of the follicular fluid from goats subjected to hormonal stimulation was elucidated in the present study. Also, molecules associated with follicle development are potential biomarkers of oocyte competence were prevalent.

1. Introduction

In mammals, the follicular fluid provides a biological microenvironment for the development of oocytes. Follicular fluid accumulates during the formation of antral cavity and it is mainly formed by the secretory activity of theca and granulosa cells and by

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diffusion of components from capillaries to the antrum (Gosden et al., 1988). Follicular secretion has an important role during nuclear and cytoplasmic maturation of the oocyte as well. For *in vitro* fertilization (IVF) of several species, ovaries are hormonally stimulated to enable the collection of as many oocytes as possible. Currently, morphology is the only criteria used for oocyte selection as there is limited information about the molecular components of female gametes (Virant-Klun and Krijgsveld, 2014). Studies about the proteome of ovarian follicular fluid become important as these will provide information about the follicular microenvironment and knowledge about factors that potentially influence oocyte growth and maturation. Furthermore, follicular fluid is a useful biological entity for the assessment of potential markers of fertility in all animals including humans (Powell et al., 2010).

When Europeans discovered Brazil more than 500 years ago, the settlers brought to the new land the first ruminant species of farm animals. As natural selection occurred over the years, these animals developed morphological and physiological characteristics adapted to environmental conditions of the different regions of Brazil, forming the "criollo", "local" or "naturalized" breeds (Mariante and Cavalcante, 2006). In the semi-arid region of Brazil, the majority of naturalized goats are endangered, including breeds known as "Canindé", "Moxotó", "Marota" and "Repartida". Such breeds are well adapted to semi-arid climate and need to be preserved because of their unique attributes and to favor biodiversity. The animals of the Canindé breed have a remarkable adaptation to heat stress and dairy performance in low-input production systems in comparison to highly demanding European goats (Mariante et al., 2009). A study reported that the number of fertilized oocytes was greater ($P < 0.0001$) in Canindé than in Saanen females (98.9 compared with 36.2 oocytes, respectively) after hormonal treatment and natural service (Moura et al., 2010). Such results indicate that local breeds have some unique reproductive capacity as compared with non-adapted breeds in a tropical environment. Efforts are currently being made to prevent the extinction of locally-adapted breeds, using reproductive biotechnologies such as IVF. Efficiency of *in vitro* production of goat embryos is, however, still low due to, among other factors, limited knowledge of the specific processes and molecular pathways that regulate oocyte development (Souza-Fabjan et al., 2014). Studies with different approaches in proteomics have been conducted to identify proteins in the ovarian follicular fluid of several species, such as cattle (Maniwa et al., 2005), swine (Bijttebier et al., 2009), dogs (Fahiminiya et al., 2010), horses (Fahiminiya et al., 2011) and humans (Ambekar et al., 2013). The present study was, therefore, conducted to characterize the major proteome of follicular fluid as related to follicle development in locally-adapted Canindé goats subjected to hormonal stimulation.

2. Material and methods

2.1. Locality of study and experimental animals

The experiment was conducted at the Ceará State University, located in Fortaleza, Brazil (3°47′38″S, 38°33′29″W) and used eight estrous cycling Canindé goats that were 1.9 ± 0.3 years of age and weighing 23.1 ± 1.5 kg. Animals were kept in shaded pens with free access to unshaded areas and received diets containing Tifton (*Cynodon dactylon*) hay and commercial concentrate (0.2 kg/day; 20% crude protein), with free access to water and mineralized salt. Experimental protocols and animal handling were approved by the Ethics Committee of the Ceará State University (3246402/2014). In addition, this study was conducted in accordance with the guidelines for animal care (Association for the Study of Animal Behaviour, 2006).

2.2. Hormonal treatment and collection of follicular fluid

As part of the hormonal treatment, goats initially received vaginal sponges with 60 mg medroxyprogesterone acetate (Progespon, Syntex, Buenos Aires, Argentina) for 10 days. On Day 7, 75 μg D-cloprostenol (Prolise, ARSA S.R.L., Buenos Aires, Argentina) were given to all animals and ovarian stimulation was achieved with five pFSH injections (30, 30, 20, 20 and 20 mg/goat; Folltropin-V, Bioniche, Belleville, Canada). The pFSH injections were given at 12-h intervals, starting in the morning of the day D-cloprostenol was injected (Sanchez et al., 2014). Laparoscopy was performed 24 h after goats received the last pFSH injection.

For collection of follicular fluid, goats were subjected to food (36 h) and water (24 h) deprivation, followed by anesthesia with 20 mg/kg thiopental (Tiopentax 2.5%, Cristália, São Paulo, Brazil) and 3% isoflurane (Isoforine, Cristália, São Paulo, Brazil). Follicles were punctured and fluid was collected using a 5-mm Hopkins laparoscope (Karl Storz, Tuttlingen, Germany), and a 22-G needle and a vacuum pump (WTA, Cravinhos, Brazil) adjusted to 35 mmHg. Follicles selected for fluid collection were determined as small (< 3 mm), medium (3–4 mm) and large (> 4 mm). Definition of these groups was based on a study previously conducted for evaluation of follicle categories in locally-adapted goats (Sousa et al., 2011). Based on such study, follicles larger than 4 mm in Canindé goats are considered mature, preovulatory follicles (Sousa et al., 2011). Each follicle was punctured separately to prevent mixing of follicular fluid from follicles with different sizes. Considering all females used in the present study, 222 follicles were punctured and follicles were defined as large ($n = 95$), medium ($n = 85$) and small ($n = 42$). The average number of follicles punctured per goat was 6.3 ± 4.5, 5.7 ± 2.4 and 6.0 ± 1.4 for large, medium and small follicles, respectively. Fluid from the same follicle sizes and same animal were pooled and centrifuged at 3000*g* for 20 min at 4 °C. The supernatant was subsequently pipetted into 1.5-mL tubes and stored at −80 °C until further analysis.

2.3. Two-dimensional gel electrophoresis

Reagents for electrophoresis were purchased from GE Life Sciences (Piscataway, NJ, USA) and Sigma-Aldrich (St. Louis, MO, USA). Soluble protein content in follicular fluid samples was determined according to Bradford method (Bradford, 1976). Aliquots containing 500 μg protein were mixed with re-hydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% immobilized pH gradient

(IPG) buffer, pH 3–10, 16 mM dithiothreitol (DTT), and traces of bromophenol blue. The mixture was subsequently incubated with 13 cm linear IPG strips in the pH range of 3–10 (GE Life Sciences) for 17 h. Isoelectric focusing was performed in an Ettan IPGphor 3 apparatus (GE Life Sciences), at 250 V for 2 h, 500 V for 1 h, 800 V for 1 h, 1000 V for 1 h, 10,000 V for 22,000 Vh and 10,000 V for 40,000 Vh, in a total of 67,200 Vh. After focusing, proteins were equilibrated, for two times, in equilibration buffer I (6 M urea, 75 mM Tris–HCl, pH 8.8, 29.3% glycerol, 2% SDS, traces of bromophenol blue and ultrapure water *qsp* 250 mL). Samples were re-equilibrated for an additional 20 min in equilibration buffer II (similar to equilibration buffer I, but containing 2% DTT) and for more 20 min in equilibration buffer using 2.5% iodoacetamide instead of DTT). After equilibration, IPG strips were placed on the top of SDS–PAGE gels, containing a homogeneous concentration of acrylamide (10%). Standards from 12 to 225 kDa were used and gels were run in an SE 600 Ruby apparatus (12.5% T/2.6% C, 250 V, and 25 mA/gel) for 5 h, approximately (O'Farrel, 1975; van Tilburg et al., 2013). Gels were stained in colloidal Coomassie blue with modifications (Candiano et al., 2004; van Tilburg et al., 2013). Briefly, gels were placed in a fixation solution containing phosphoric acid (2%) and ethanol (30%) for 12 h. After, gels were washed three times (20 min each) in a solution with phosphoric acid (2%) in water, and then put in a solution with phosphoric acid (2%), ethanol (18%), and ammonium sulfate (15%) added with 2 mL of a Coomassie Blue G-250 solution (2%) for five days. Images from 24 gels, eight of each follicular group, were assessed at 300 dpi (ImageScanner II; GE Life Sciences, USA), and analyzed using PDQuest Software, version 8.0.1 (Bio-Rad Laboratories, Rockville, MD, USA) according to a strategy that was previously described in detail (Moura et al., 2006).

2.4. Statistical analyses

Data referring to spot intensities calculated from protein gels were assessed for normality, using the Kolmogorov-Smirnov's test, and for asymmetry and kurtosis, using the UNIVARIATE procedure with NORMAL and PLOT options of SAS 9.1 (SAS Institute, Cary, NC, USA). Variables without a normal distribution were log ($\log(x + 1)$) transformed. Means were compared by ANOVA with Tukey's test ($P < 0.05$) using GraphPad Prim 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

2.5. Protein identification

The major follicular fluid proteins identified by 2-D SDS-PAGE as well as those in different ($P < 0.05$) amounts among small, medium and large follicles were subjected to in-gel trypsin digestion (Moura et al., 2006; van Tilburg et al., 2013). The selected spots were cut from three different gels (from the same follicle category). Excised gel pieces were washed three times with 400 µL of a solution containing ammonium bicarbonate (25 mM) and acetonitrile (50%), pH 8.0 to remove SDS and dye. Then, gel pieces were dehydrated after two washes with 200 µL of absolute acetonitrile, and completely dried using vacuum pressure (Concentrator Plus, Eppendorf, Hamburg, Germany). Gel pieces were then incubated for approximately 20 h at 37 °C with trypsin (166 ng/spot; Promega, Madison, WI, USA). Peptides were then extracted from gel pieces by washing two times with 25 mL of trifluoroacetic (5%) in ammonium bicarbonate (50 mM) and acetonitrile (50%) for 30 min. The extracts were dried under vacuum again.

2.6. Protein identification by tandem mass spectrometry

Initially, peptides were separated using reversed-phase chromatography on a Shimadzu Prominence nano LC system (Shimadzu, Kyoto, Japan) coupled to a mass spectrometry Synapt HDMS (Waters Corp., Milford, MA, USA), as described before (van Tilburg et al., 2013; Santos et al., 2014). In summary, a Waters Symmetry 300TM column (C-18, 5 µm; 0.3 mm × 5 mm) was used for desalination and pre-concentration. After three washes, peptides were eluted in an Acquity HSS T3 column (C-18, 5 µm, 0.075 mm × 150 mm). Eluted peptides were directly analyzed on an ESI-Q-Tof 5600 instrument (AB Sciex, Ontario, Canada) using a Nanospray III interface. An MS TOF using *m/z* 400–1500 was performed for 1.5 s. Data were converted to Mascot Generic Format using Protein Lynx Global Server 2.1 prior to database search in MASCOT (www.matrixscience.com) using the NCBInr databank in MS/MS ion search mode.

2.7. Protein interaction analysis

In silico analyses of protein–protein interactions were evaluated using STRING 10.0 platform (http://string-db.org), which was set to show no more than 10 interactions and medium confidence (Snel et al., 2000). Pathways not described for *Capra hircus* were analyzed for *Bos taurus*.

2.8. Gene ontology

Data from the follicular fluid protein list defined after MASCOT search were analyzed using the Software for Researching Annotations of Proteins (STRAP). Gene ontology terms for cellular components, molecular functions and biological processes were obtained from UniProtKB database (Bhatia et al., 2009; Santos et al., 2014).

3. Results

On average, 155.4 ± 4.5 spots were detected per 2-D gel of goat follicular fluid, based on the analysis of 24 biological samples,

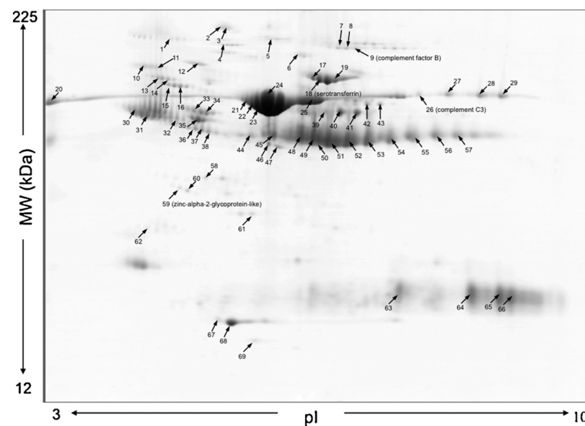

Fig. 1. Two-dimensional map of follicular fluid proteins from hormonally-treated Canindé goats. Proteins were stained with Commassie blue and identified by tandem mass spectrometry.

eight for each category of follicles (Fig. 1). Tandem mass spectrometry allowed the identification of 69 spots, associated with 36 different proteins (Table 1; Supplemental Table 1). The intensity of these 69 spots represented 66.7% of the intensity of all spots detected in the 2-D gel of goat follicular fluid (Fig. 1). Albumin and immunoglobulins were the most abundant proteins of the goat follicular fluid, as spots matching such molecules accounted for 11.8% and 35.0% of the total intensity of all spots defined in the 2-D gels. Other proteins identified in follicular fluid include ceruloplasmin, complement factor B, alpha-1B-glycoprotein precursor, serotransferrin, complement C3, serpins, alpha-1-antiproteinasis, fibrinogen and hemopexin (Fig. 1; Table 1; Supplemental Table 1).

Total protein concentrations were similar ($P > 0.05$) in the follicular fluid obtained from small (45.3 ± 3.1 mg/mL), medium (44.2 ± 3.3 mg/mL) and large follicles (45.1 ± 2.3 mg/mL). In addition, no spots were exclusively detected in any follicle category but the intensities of five spots were different ($P < 0.05$) among follicle size categories. Such spots corresponded to four proteins: serotransferrin, zinc-alpha-2-glycoprotein-like, complement factor B and complement protein C3 (Figs. 1 and 2; Table 1 and Supplemental Table 1). The intensity of a protein spot identified as serotransferrin was greater in medium than small follicles ($P < 0.05$). Another three proteins (zinc-alpha-2-glycoprotein-like, complement factor B and complement C3) were in greater amounts ($P < 0.05$) in large compared with medium sized follicles (Fig. 2A and B).

According to *in silico* analysis of protein-protein networks, amount of serotransferrin (TF) was associated with a set of 10 other proteins (Fig. 3A). Zinc-alpha-2-glycoprotein-like (AZGP1) protein was associated with beta-2-microglobulin and with prolactin-inducible protein homolog precursor (Fig. 3B). Complement factor B (CFB) was associated with 10 different molecules, including protein complement C3 (C3) (Fig. 3C), which is linked to another set of molecules (Fig. 3D).

Based on gene ontology, the main cellular components associated with proteins of the goat follicular fluid were defined as extracellular, cytoplasm, nucleus and other intracellular organelles proteins. The most prominent molecular functions related to follicular fluid proteins were binding and catalytic activity, whereas the main biological processes were described as regulation, cellular process, location and immune system process (Fig. 4).

4. Discussion

Based on a platform consisting of 2-D gel electrophoresis and tandem mass spectrometry, the present study describes the major proteome of the follicular fluid from estrous cycling goats subjected to hormonal stimulation. There was also differences in specific proteins of the follicular fluid according to follicle size. To our knowledge, this is the first characterization of the ovarian follicular fluid proteome in goats. The present study was with a locally-adapted Canindé breed. These goats have been subjected to natural selection for centuries in the Brazilian semi-arid region and are more adapted to tropical conditions when compared with purebred European breeds (Ahmed et al., 1997). The Canindé breed is considered an endangered breed in Brazil (Mariante et al., 2009) and is a unique source for genetic diversity for breeding programs.

Albumin and immunoglobulins were the main follicular fluid proteins in the ovarian follicles of the goats, regardless of follicle size. This pattern is similar to the composition of follicular fluid from other mammalian species, such as cattle (Maniwa et al., 2005), dogs (Fahiminiya et al., 2010) and horses (Fahiminiya et al., 2011). Studies indicate the blood-follicle barrier is permeable to proteins with mass up to 500 kDa (Gosden et al., 1988) and this explains the significant amount of albumin in the follicular fluid. Albumin is a major component of blood serum and a multifunction molecule, functioning as transport of proteins, steroids and other lipophilic molecules, prevention of membrane lipid peroxidation and control of osmotic pressure (Shamay et al., 2005; Otsuki et al., 2012).

According to gene ontology analysis, the majority of follicular fluid proteins of Canindé goats are extracellular, which is consistent with findings of previous investigations conducted with horses and humans (Fahiminiya et al., 2011; Zamah et al., 2015). Other follicular fluid proteins are of cellular origin, indicating that some follicular cells undergo apoptosis and release the components into the antrum during follicle development (Ambekar et al., 2013). Functions of follicular fluid proteins related mostly to binding and

Table 1
Proteins of follicular fluid from hormonally-treated Canindé goats and identified by mass spectrometry (ESI-Q-TOF). Spot numbers refer to those shown in Fig. 1.

Protein	Experimental[a] kDa/pI	NCBInr accession number	MS/MS protein score	Sequence covered (%)
Ceruloplasmin-like [*Capra hircus*]				
Spot 02	150.0/5.1	548451476	575	13
Spot 60	31.0/4.7	548451476	628	11
Ceruloplasmin precursor [*Ovis aries*]				
Spot 03	150.5/5.1	57619174	343	6
Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [*Bos taurus*]				
Spot 04	108.7/5.1	59857769	113	3
Gelsolin isoform b [*Bos taurus*]				
Spot 06	76.6/6.0	77736201	159	6
Complement factor B [*Ovis aries*]				
Spot 07	102.0/6.7	148645283	292	7
Complement factor B, partial [*Bos taurus*]				
Spot 08	101.0/6.8	66866253	71	16
Complement factor B [*Capra hircus*]				
Spot 09	100.04/6.94	548517907	304	10
Vitronectin [*Capra hircus*]				
Spot 10	74.5/4.2	75756542	66	4
Factor XIIa inhibitor precursor [*Bos taurus*]				
Spot 11	75.1/4.4	27807349	70	2
Prothrombin [*Capra hircus*]				
Spot 12	79.7/4.8	548496646	166	5
Alpha-1B-glycoprotein precursor [*Bos taurus*]				
Spot 13	58.9/4.3	114053019	262	9
Spot 14	57.1/4.4	114053019	482	15
Spot 15	51.7/4.4	114053019	335	11
Spot 16	51.5/4.6	114053019	339	10
Serotransferrin [*Ovis aries*]				
Spot 17	56.30/6.19	426218284	966	28
Serotransferrin [*Capra hircus*]				
Spot 18	53.67/6.29	548451659	1130	41
Spot 19	54.40/6.60	548451659	148	7
Spot 05	123.79/5.85	548451659	754	27
Serum albumin precursor [*Ovis aries*]				
Spot 20	48.86/3.16	57164373	739	25
Spot 21	49.82/5.35	57164373	138	6
Spot 22	49.42/5.39	57164373	353	12
Albumin precursor [*Capra hircus*]				
Spot 23	48.43/5.44	193085052	998	30
Spot 24	49.70/5.68	193085052	1489	41
Spot 25	50.3/6.3	193085052	1099	33
Spot 61	25.94/5.37	193085052	258	11
Complement C3 [*Capra hircus*]				
Spot 26	48.36/7.70	548472985	432	11
Spot 27	48.06/8.17	548472985	125	2
Spot 28	48.94/8.63	548472985	311	5
Spot 29	48.56/8.90	548472985	401	8
Serpin A3-1-like, partial [*Capra hircus*]				
Spot 30	47.9/4.0	548535008	173	18
Spot 31	47.2/4.2	548535008	138	14
Serpin A3-5, partial [*Ovis aries*]				
Spot 32	44.2/4.5	426248890	159	10
Spot 01	132.8/4.5	426248890	152	7
Alpha-1-antiproteinase-like [*Capra hircus*]				
Spot 33	46.0/4.8	548514091	94	7
Spot 34	45.6/4.9	548514091	126	12
Vitamin D-binding protein [*Ovis aries*]				
Spot 35	43.8/4.8	426231846	140	8
Serpin A3-7-like isoform X1 [*Capra hircus*]				
Spot 36	42.1/4.8	548533537	155	13
Spot 37	41.8/4.9	548533537	103	7
Spot 38	41.3/5.0	548533537	167	12
Fibrinogen beta chain [*Ovis aries*]				
Spot 40	43.3/6.7	426246917	570	27
Spot 41	43.6/6.9	426246917	639	30
Fibrinogen alpha chain [*Capra hircus*]				
Spot 42	46.8/7.0	548499843	143	4
Spot 43	46.7/7.2	548499843	387	9
Fibrinogen gamma chain isoform X2 [*Ovis aries*]				

(*continued on next page*)

Table 1 (*continued*)

Protein	Experimental[a] kDa/pI	NCBInr accession number	MS/MS protein score	Sequence covered (%)
Spot 46	38.0/5.7	426246913	341	23
Spot 47	37.4/5.8	426246913	226	11
Ig heavy chain C region − sheep (fragment)				
Spot 44	38.8/5.5	109029	74	10
Spot 45	39.71/5.68	109029	271	25
Spot 48	38.0/6.10	109029	142	19
Spot 49	38.1/6.28	109029	243	23
Spot 50	38.3/6.4	109029	98	10
Spot 52	39.22/6.85	109029	115	10
Ig gamma-2 chain C region (clone 32.2) − bovine (fragment)				
Spot 51	38.1/6.5	89611	60	4
Immunoglobulin gamma 2 heavy chain constant region [*Capra hircus*]				
Spot 53	39.22/6.85	147744654	328	43
Spot 54	39.30/7.05	147744654	162	17
Spot 55	39.59/7.17	147744654	69	17
Spot 56	39.58/7.28	147744654	120	17
Spot 57	39.61/7.54	147744654	71	12
Alpha-1-antiproteinase precursor [*Ovis aries*]				
Spot 58		57526646	106	4
Zinc-alpha-2-glycoprotein-like [*Capra hircus*]				
Spot 59	31.53/4.60	548521029	248	21
Clusterin preproprotein [*Bos taurus*]				
Spot 62	24.4/4.2	27806907	79	4
Immunoglobulin lambda light chain F7-299 [*Capra hircus*]				
Spot 63	17.0/7.3	61378762	127	14
Spot 65	16.0/9.1	61378762	264	29
Immunoglobulin lambda-like polypeptide 1-like isoform X1 [*Capra hircus*]				
Spot 64	16.2/8.9	548502304	166	21
Spot 66	15.6/9.3	548502304	167	25
Apolipoprotein A-I [*Ovis aries musimon*]				
Spot 67	14.5/5.1	803225470	423	33
Apolipoprotein A-I precursor [*Bos taurus*]				
Spot 68	14.10/5.30	162678	830	56
Retinol-binding protein 4 [*Capra hircus*]				
Spot 69	12.0/5.5	548522469	69	10
Hemopexin [*Capra hircus*]				
Spot 39	44.3/6.4	548495399	389	18

[a] Experimental values were deduced from the respective 2D map by the PDQuest software.

catalytic activity which is consistent with the functions of proteins such as albumin, retinol-binding protein 4, serotransferrin, ceruloplasmin, prothrombin and complement protein C3 (Fu et al., 2016). Biological processes of follicular fluid proteins were mainly defined as regulation, cellular process, location and immune system processes. Various proteins associated with inflammatory response were, therefore, identified in the goat follicular fluid, such as complement and coagulation proteins. This is expected because activation of inflammatory events is necessary for ovulation (Zamah et al., 2015). Also, studies describe the participation of proteins from the complement cascade during innate immune function in human follicular fluid, suggesting a possible association with angiogenesis and follicular development (Jarkovska et al., 2010). Functions of some goat follicular fluid proteins relate to follicle vascularization and permeability of blood vessels as well (Fortune, 1994; Hanrieder et al., 2008).

In the current study, there were no variations in total protein concentration among different follicle sizes and no differences in amounts of protein in any follicle category (small, medium or large). The amount of serotransferrin, zinc-alpha-2-glycoprotein-like, complement factor B and complement protein C3, however, varied in the fluid collected from follicles of different sizes. Transferrins are responsible for iron transport and have an additional role in the stimulation of cell proliferation. Synthesis of transferrin occurs mainly in the liver (Schaeffer et al., 1987) but it can also be secreted by the granulosa cells and transported to the oocyte by endocytosis (Briggs et al., 1999). Transferrin stimulates follicle development, reducing the generation of reactive oxygen species (ROS) that causes follicular atresia (de Jong et al., 1990; Tilly, 1998). Based on *in silico* analysis, serotransferrin interacts with a diverse array of proteins, such as apolipoprotein-A1 (APOA1). The APOA1 protein was detected in the goat follicular fluid and participates in cholesterol and triglyceride transport, also having an important association with follicular development due its mitogenic and angiogenic functions (von Otte et al., 2006). The APOA1 facilitates in protection of the oocyte because of its antioxidant properties as well (von Wald et al., 2010). In human and mouse ovaries, transferrin gradually increases in the granulosa cells, cumulus and oocytes in more advanced stages of follicular development and antrum expansion (Briggs et al., 1999). In the present study, there was an increase in follicular fluid serotransferrin when comparing small and medium follicles of the Canindé goats, probably as the result of increased proliferation of the granulosa cells during follicular growth.

Zinc-alpha-2-glycoprotein-like (AZPG1) is a glycoprotein originally purified from human plasma (Burgi and Schmid, 1961). The lipolytic effect of AZPG1 has been associated with activation of β3-adrenoceptors, resulting in an intracellular cAMP increase (Russell

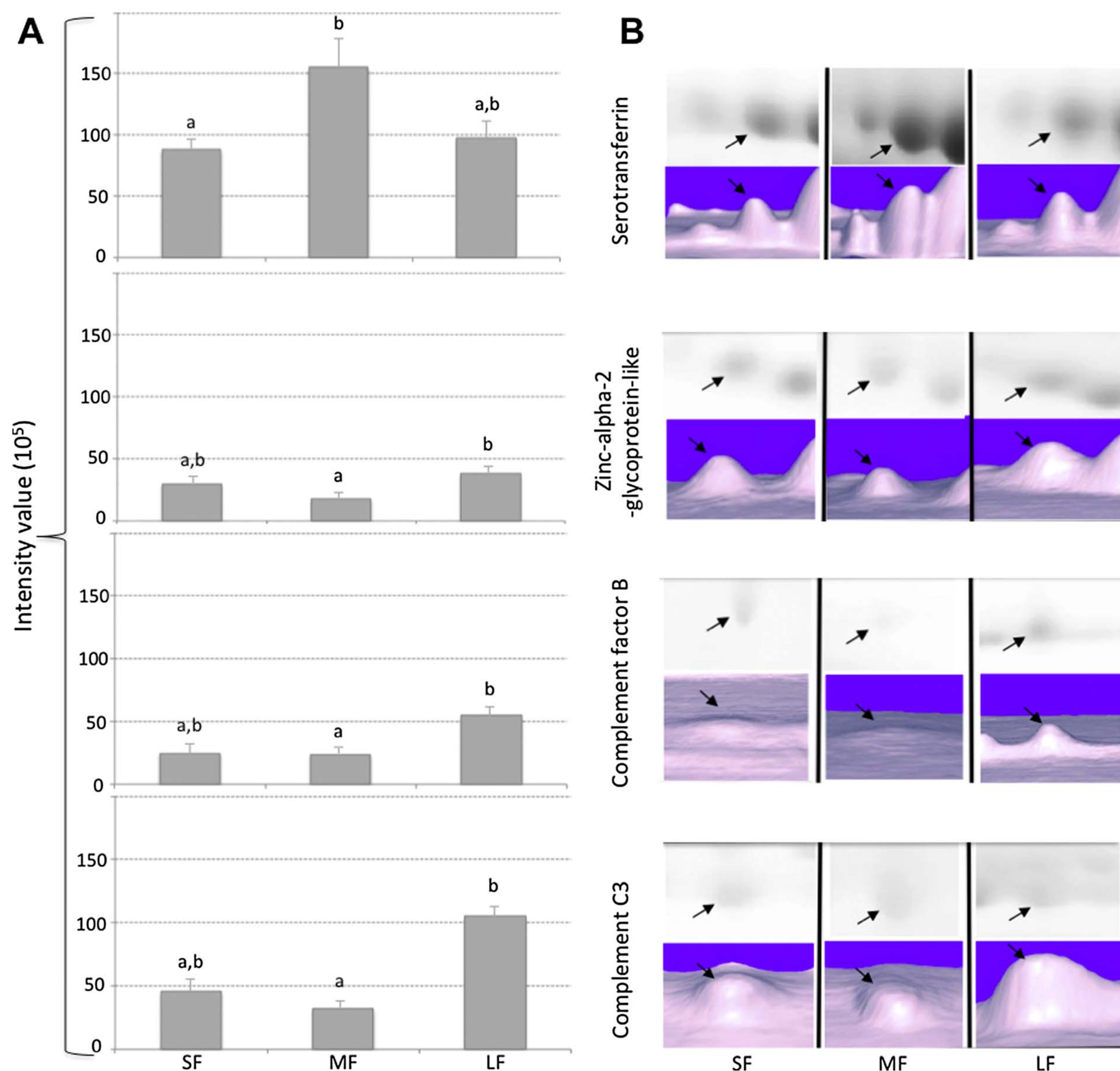

Fig. 2. Intensity values (mean ± SEM) of protein spots in small (SF), medium (MF) and large follicles (LF) from hormonally-treated Canindé goats (A). Three-dimensional image of spots (shown in arrows) differentially expressed among follicle categories (B). Gel and spots images were generated by PDQuest software.

et al., 2004). This effect, in turn, is important during sperm capacitation and may be involved in calcium signaling pathway (Qu et al., 2007). In the present study, the amount of complement factor B and complement protein C3 increased as follicles developed from medium to large size. Such proteins obviously belong to the complement system, which consists of over 30 proteins present either in plasma or on cell surfaces (Jarkovska et al., 2010). The C3 proteins have an important role during fertilization. Proteases from the sperm acrosome cleave C3 to C3b in the medium, which in turn binds to the membrane cofactor protein (LOC616002) present in sperm. Sperm bound C3 protein will then mediate sperm-oocyte recognition through CR1 and CR3 complement receptors (Anderson et al., 1993). In women, cases of IVF failure are related to alterations in specific follicular fluid proteins, including complement protein C3 (Estes et al., 2009). Thus, all this evidence supports the concept that complement proteins of the follicle fluid have important roles during fertilization.

In vitro production of embryos depends on both the quality of immature oocytes and conditions provided for maturation. In goats, oocytes undergoing *in vitro* maturation (IVM) and subsequent IVF have lesser development when compared with those matured *in vivo* (Cognié et al., 2003). It, therefore, appears to be clear that the oocyte competence is potentially modulated by different substances present in the follicular fluid, including proteins. In the present study, the amount of follicular fluid serotransferrin, zinc-alpha-2-glycoprotein-like, complement factor B and complement C3 increased with follicle size, and such proteins regulate important

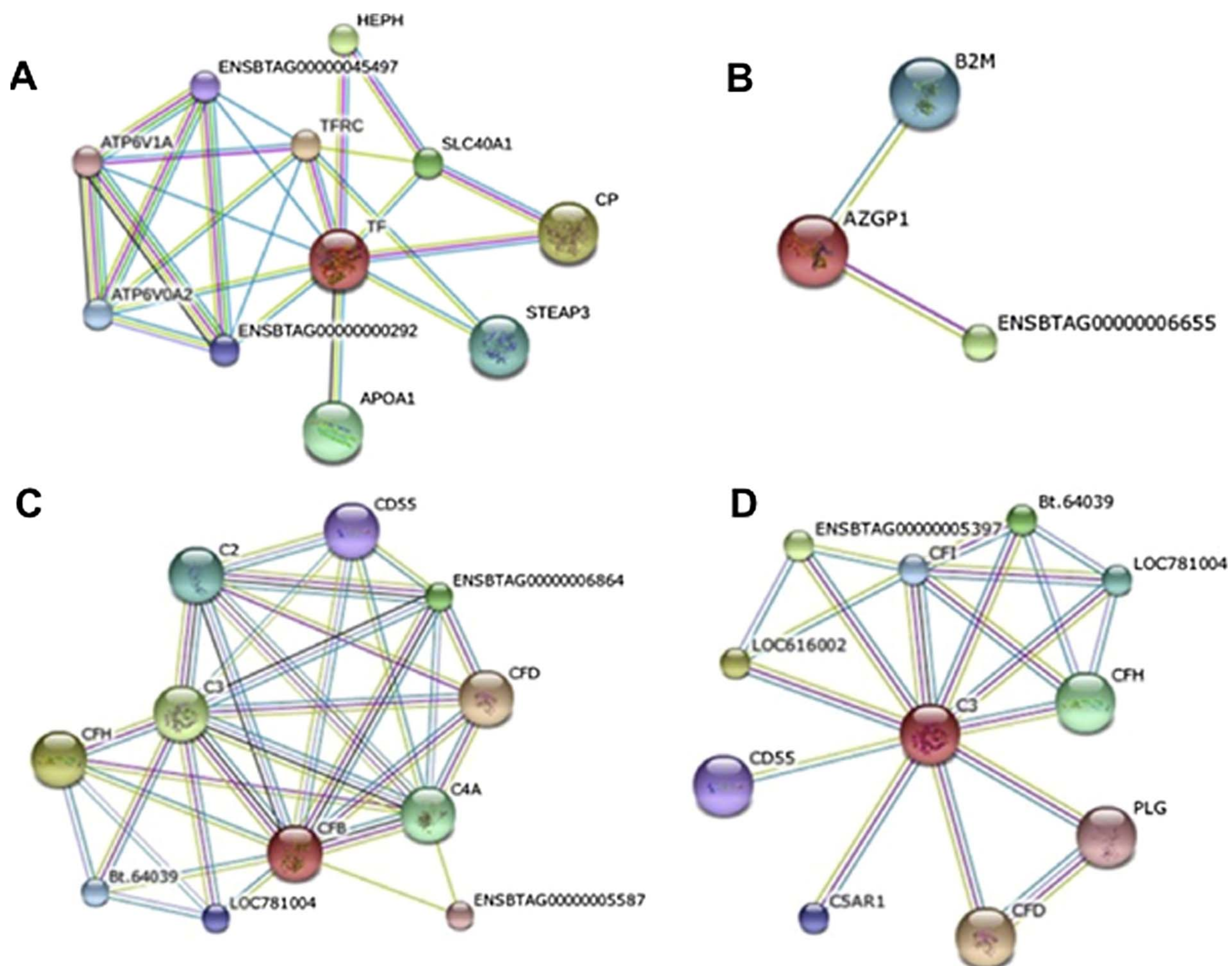

Fig. 3. *In-silico* interaction analysis of goat follicular fluid proteins identified as serotransferrin – TF (A), zinc-alpha-2-glycoprotein-like – AZGP1 (B), complement factor B – CFB (C) and complement C3–C3 (D). Analysis was conducted with the web-based STRING software (http://string-db.org). Pathways not described for *Capra hircus* were analyzed for *Bos taurus*. Line colors represent the type of evidence to support associations among proteins (green – textming; black – coexpression; blue – databases; and pink – experiments). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

events such as iron and lipid transport, control of ROS, inflammation and innate immunity. After recruitment and as follicles develop, granulosa and other somatic cells synthesize proteins required for oocyte maturation. Thus, synthesis of proteins into the follicular milieu is the result of activation of intracellular signaling and activation of steroid receptors, among other factors.

In conclusion, molecules that we presently describe as associated with follicle development are potential biomarkers of oocyte competence. Moreover, such key proteins can be used in IVM and/or IVF media to optimize the efficiency of *in vitro* embryo production and improve programs for preservation of locally-adapted breeds.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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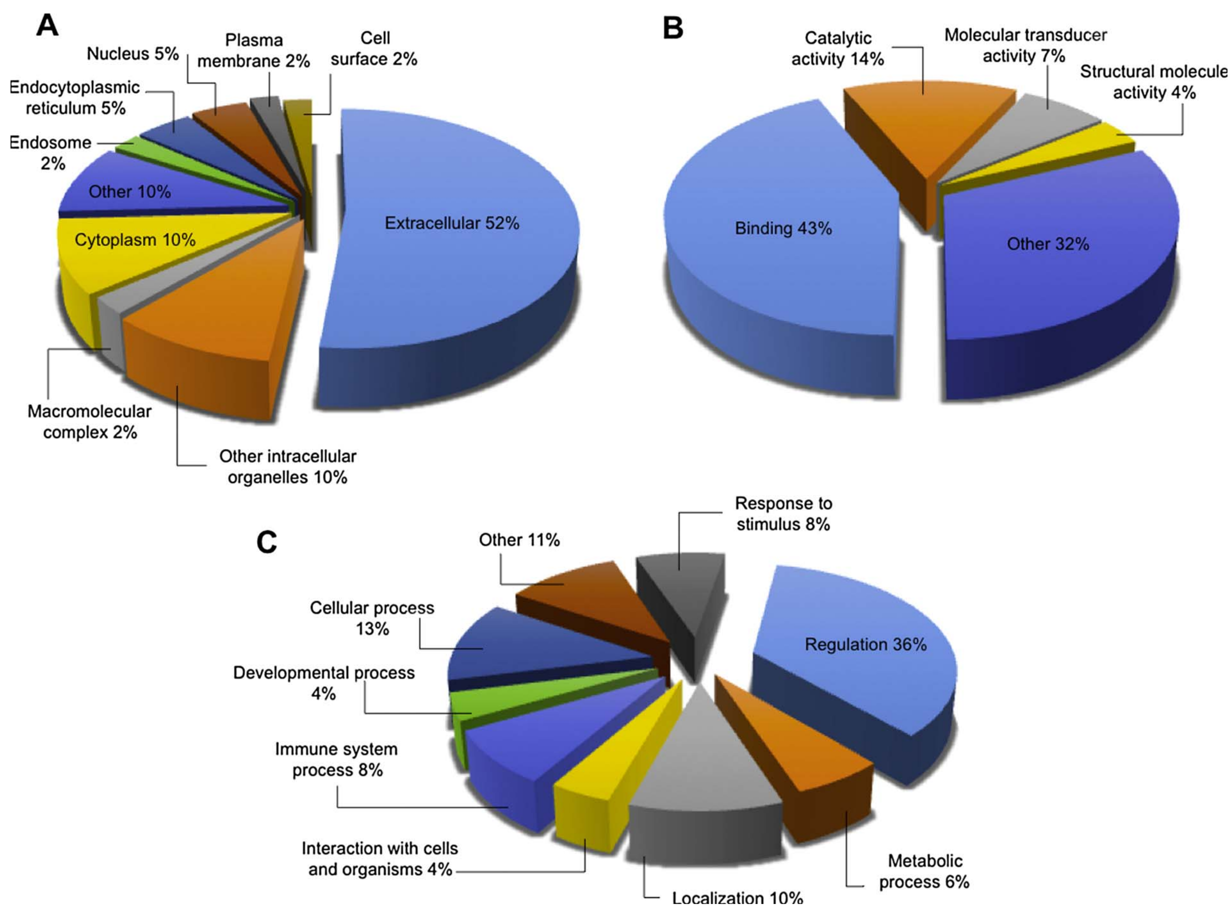

Fig. 4. Gene ontology annotations of proteins identified in follicular fluid from hormonally-treated Canindé goats. Proteins were classified based on cellular component (A), molecular function (B), and biological process (C).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anireprosci.2017.11.005.

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