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FULL PAPER

Theriogenology

Proteome profiling of bovine follicular fluid-specific proteins and their effect on *in vitro* embryo development

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ABSTRACT. The objective of this study was to determine the effect of bovine follicular fluid proteins (bFF) and their differently charged groups as maturation media supplements for *in vitro* embryo development. bFF was obtained by aspiration from large healthy follicles (4–10 mm in diameter) and was precipitated by 30–50% $(NH_4)_2SO_4$. The precipitated protein was fractionated into basic and acidic fractions by ion-exchanger columns. In experiment 1, the oocytes were matured in TCM-199 with 1) FBS+hormones (control) and 2) 10% bFF. The oocyte maturation rate, the development to the blastocyst stage rate and blastocyst cell number were not significantly different between the groups. However, the INFα and IGF-2r expression levels in the 10% bFF were higher than in the control ($P<0.05$). In experiment 2, the specific charge proteins of bFF (basic and acidic) were also used as media supplements in the maturation medium. The basic fraction had higher oocyte maturation rate and blastocyst cell number when compared with addition of acidic fraction ($P<0.05$). The expression levels for almost all developmentally important genes in the basic fraction were greater than those in the acidic fraction, particularly INFα ($P<0.05$). Most of the protein in the basic fraction was associated with the immune response and mRNA processing. In conclusion, supplementation of 10% bFF alone in maturation medium can support oocyte maturation and embryo development. The basic fraction in bFF seemed to have effect on oocyte maturation rate and blastocyst cell number.

KEY WORDS: bovine, embryo development, follicular fluid, specific protein

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In vitro production (IVP) of bovine embryos provides an excellent opportunity to generate abundant embryos for basic research and for application in emerging biotechnologies. *In vitro* maturation (IVM) of oocytes is an important process for the successful development of IVP embryos, as developmental failure of an embryo may result from incomplete oocyte maturation [11]. Protein supplementation during IVM can profoundly affect both the rate and overall efficiency of the maturation procedure [1]. Fetal bovine serum (FBS) is widely used as protein source in embryo culture media. In the bovine system, 10–20% (v/v) FBS is recommended as an oocyte IVM medium supplement, and this is an expensive component [13]. However, oocyte IVM requires culture media that imitate natural physiological conditions, which thus replace the expensive components of culture media with a less expensive material.

Follicular fluid (FF) comprises the preconception microenvironment in which the oocyte develops [12]. This biologic fluid provides a unique window into the processes occurring during follicular maturation, because of the intimate proximity of the follicular fluid to the maturing oocyte [13]. In the past few years, the impact of FF on the IVM of oocytes, fertilization and embryonic development has been studied extensively in domestic animals, including cattle [4], pigs [5] and buffalo [7]. FF has the potential to be used as a natural and inexpensive serum source and for IVM medium.

Nevertheless, FF has a vast protein complexity and very broad dynamic range of protein abundances that hinder its analysis. Thus, the identification of the proteins in bFF might explain their roles in oocyte maturation and the mechanisms by which they

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aid embryonic development to the blastocyst stage. Moreover, insights into the follicular fluid composition provide a useful indication of the requirements for *in vitro* oocyte maturation and may be used as a guide for the inexpensive formulation of cell culture conditions. Recently, limited data have been obtained on the fractionated FF proteins and their effects on *in vitro* embryo development. This study reports not only the fractionation of specific proteins from bFF and their biological effects on *in vitro* embryo development but also the subsequent effect on developmentally important genes at the blastocyst stage.

MATERIALS AND METHODS

Follicular fluid collection

The ten bovine ovaries from 10 cows were collected at a local slaughterhouse and carried to the laboratory within 1 hr in Dulbecco's phosphate-buffered saline (PBS) at 35–37°C. The bFF was obtained by aspiration from large healthy follicles (a bright appearance, extensive and very fine vascularization and contained an unexpanded COC, and no loose floating particles in FF) that were 4–10 mm in diameter. Fluid samples from two or three follicles were pooled to prepare one batch of bFF. Phenyl methyl sulfonyl fluoride (PMSF) was added (20 μg/m*l*) to the FF. Then, the FF was made cell-free by centrifugation at 4°C at 1,500×g for 30 min and the supernatant was filtered through a 0.45-μm membrane filter. The supernatant containing the proteins was precipitated with $(NH_4)_2SO_4$ (30, 40, 50 and 60%). After precipitation, the protein samples were dialyzed in 50 mM Tris-HCl buffer (pH 7.0) to remove $(NH_4)_2SO_4$.

Ion exchange chromatography of the bFF proteins

To fractionate the basic proteins, the dialyzed protein samples were loaded into a Macro-Prep High S support in a 5-m*l* column (Bio-Rad, Hercules, CA, U.S.A.) equilibrated in 10 mM Tris buffer (pH 7.0). The bound proteins were extensively washed using equilibration buffer until the optical density of the washed buffer was less than 0.05 at 280 nm. The bound proteins were eluted from the Macro-Prep High S column in 10 mM Tris-HCl (pH 7.0) containing 350 mM NaCl. To purify the acidic proteins, unbound proteins from the Macro-Prep High S column were loaded into the Macro-Prep High Q support in a 5-m*l* column (Bio-Rad) equilibrated in 10 mM Tris buffer pH 7.0. The bound proteins were also extensively washed in the same buffer until the O.D. of the buffer at 280 nm was less than 0.05. The bound proteins were eluted from the column in 10 mM Tris-HCl (pH 7.0) containing 350 mM NaCl. The bound and unbound proteins were collected separately and dialyzed in phosphate buffer saline, quantified and used to see the effects on the *in vitro* maturation.

Proteomic approach

The protein bands were slices from Coomassie Brilliant Blue SDS gel.Then, each resulting gel lane was washed twice with 50% acetonitrile (ACN)/25 mM ammonium bicarbonate (NH_4HCO_3) at room temperature for 30 min. Next, diluted trypsin was added to each gel slice at 37°C for 16–24 hr. Peptides were extracted twice by adding 50% acetonitrile and 5% trifluoroacetic acid to a gel slice for 15 min. Finally, the extracted peptides were dried in a vacuum centrifuge until dry. For sample analysis by LC-ESI-MS/MS, the peptides were reconstituted in 0.1% formic acid.

The LC-MS/MS system consisted of a liquid chromatography part (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, MA, U.S.A.) in combination with an electrospray ionization function (ESI)/ Quadrupole ion trap mass spectrometer (Model amazon SL, Bruker, Germany). LC separation was performed on a reversed phase column (Hypersil GOLD 50 × 0.5 mm, 5 μm C18) protected by a guard column (Hypersil GOLD 30 × 0.5 mm, 5 μm C18) and eluted at a flow rate of 100 μl/min under gradient conditions of 5–80% B over 50 min. The mobile phase A consisted of water/formic acid (99.9:0.1, v/v), while phase B consisted of acetonitrile (100, v). Mass spectral data from 300 to 1,500 m/z were collected in the positive ionization mode. The raw data were processed into a text file format (mgf) with Proteome Discoverer Version 1.3.

Protein identification

For protein identification, the mgf files generated from the MS/MS spectra were uploaded to the MASCOT search engine v2.2 (Matrix Science, London, U.K.) (http://www.matrixscience.com). Functional bioinformatics analysis was completed with publicly available Gene Ontology (GO) annotations from UNIPROT to identify the differentially affected processes, pathways, interactions and cellular distribution of the proteins.

Oocyte collection and IVM

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in a 0.9% physiological saline solution with 40 μg/m*l* gentamycin (A.N.B. Laboratories Co., Ltd., Bangkok, Thailand) at 29 to 32°C in a thermo container. Cumulus–oocyte complexes (COCs) were collected by the aspiration of follicles from the ovaries using an 18-gauge needle connected to a 10-m*l* syringe. Oocytes surrounded by a compact cumulus mass with an evenly granulated cytoplasm were selected. COCs were washed 3–4 times in TCM–HEPES containing 5 mM sodium pyruvate, 100 IU/m*l* penicillin and 100 μg/m*l* streptomycin (antibiotic, Gibco®, NY, U.S.A.) and washed three times with the following maturation medium: TCM-199 supplemented with 10% (v/v) fetal bovine serum (FBS), 0.2 mM Na-pyruvate, 5 μg/m*l* Luteinizing Hormone (LH), 0.5 μg/m*l* porcine Follicle Stimulating Hormone (pFSH), 1 μg/m*l* estradiol 17β and 50 μg/m*l* gentamycin. COCs were placed in the maturation medium under sterile mineral oil at 39°C in an atmosphere of 5% CO_2 in air for 24 hr.

Table 1. Primers used for real-time PCR amplification of embryonic genes

Genes	Primer sequence (5′→3′)	Accession no.
INFτ	F 5′ TGTTGGAGCCCAGTGCAGA 3′	X65539
	R 5′ TCCATGAGATGCTCCAGCAGT 3′	
PLAC8	F 5′ GACTGGCAGACTGGCATCTT 3′	NM 1076987
	R 5′ CTCATGGCGACACTTGATCC 3′	
IGF-2r	F 5′ CAGGTCTTGCAACTGGTGTATGA 3′	J03527
	R 5′ TTGTCCAGGGAGATCAGCATG 3′	
Hsp70	F 5′ GACAAGTGCCAGGAGGTGATTT 3′	U09861
	R 5′ CAGTCTGCTGATGATGGGGTTA 3′	
BAX	F 5′ TGACGAGATCATGAAGACAG 3′	XM010823819
	R 5′ GCTCCATGTTACTGTCCAAT 3′	
BCL2	F 5′ ATTTGCTGCTTATTCTGCTC 3′	XM006058115
	R 5′ ATCCACTGTACTGCCATCTC 3′	
GAPDH	F 5′ GTCTGTTGTGGATCTGACCT 3′	XM001252511
	R 5′ AGAAGAGTGAGTGTCGCTGT 3′	

F: forward primer, R: reverse primer.

In vitro fertilization (IVF)

Frozen semen was thawed in a water bath at 38°C for 30 sec. Sperm samples were prepared for IVF through the swim-up separation method in heaprin (10 μg/m*l*) and BSA (8 mg/m*l*) supplemented HEPES buffered IVF medium. Matured oocytes were washed and placed into 30 μl drops (10 oocytes/drop) of heparin and BSA supplemented IVF under mineral oil. A prepared sperm sample was added into each drop and concentration of 1×10^6 sperm/m*l*. The oocytes and spermatozoa were co-cultured under mineral oil at 39°C in an atmosphere of 5% CO_2 in air for 20 hr.

In vitro culture (IVC)

Following IVF, the presumptive zygotes were washed three times in TCM-199 supplemented HEPES, cultured in the drops of SOF-IVC medium supplemented with BSA (4 mg/m*l*), 7.27 mM sodium pyruvate and 50 μg/m*l* gentamycin and incubated at 39°C in a humidified atmosphere of 5% CO_2 in air. The cleavage rate was assessed at 2 days of culture, and the blastocyst rate and number of cells in the blastocyst were determined at day 8. The blastocyst rate and number of cells in the blastocyst were determined at day 8 as described [12].

Assessment of oocyte maturation status

The oocyte maturation rate was determined by meiotic progression. For the assessment of nuclear progression to metaphase II stage, the oocytes were stripped off cumulus cells with 0.2% hyaluronidase (Hyalozima®, Aspen, CO, U.S.A.) in phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS Ca^{2+} and Mg^{2+}free) and stained with 10 μg/m*l* Hoechst 33342, using the method described by Cherr *et al*. [3], with modifications.

Embryonic gene expression

Total blastocyst RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was completed with a SensiFAST™ cDNA synthesis kit (Bioline Inc., Taunton, MA, U.S.A.). The mRNA levels were quantified on a Mastercycler® ep realplex^4 (Eppendorf AG, 22331 Hambrug, Germany). The reaction mixture contained SensiFAST™ SYBR No-ROX Master Mix (Bioline Inc., Taunton, MA, U.S.A.). The DNA sequences of the oligonucleotides used for the qRT-PCR analysis are given in Table 1. The relative expression levels of each gene were represented as the ratio to that of GAPDH gene expression. Relative transcript abundance calculations were performed using the comparative CT (Δ_{CT}) method.

Experimental design and statistical analysis

This experiment was designed to test the effects of supplementing IVM medium with bFF on their *in vitro* embryonic development. Experiment 1 was designed to investigate the effects of bFF on bovine oocyte maturation and subsequent embryo development. The oocytes were sorted into the following 2 groups: (1) the positive control group, in which the oocytes were matured in IVM medium with 10% FBS + hormone and (2) the experimental group, in which the oocytes were matured in TCM199 with 10% bFF. Experiment 2 was designed to investigate the effects of a specific protein in the presence of bFF on oocyte maturation and subsequent embryo development. The oocytes were matured in TCM199 with two different fractions (basic and acidic) of bFF. Only one fraction of bFF was shown to promote a high rate of oocyte development and gene expression in this experiment when introduced to the analyzed protein. All data and data sets are presented as the means ± SE and were analyzed by Duncan's multiple range test using Statistical Analysis System ver. 8x (SAS, Cary, NC, U.S.A.). A value of $P<0.05$ was considered to be statistically significant.

Table 2. Effects of bFF in maturation medium on oocyte maturation and embryonic development

Treatment	No. of IVM oocytes	Oocyte maturation rate (%; n)	No. of IVM/IVF Oocytes	Cleavage rate (%; n)	Blastocyst rate (%; n)	Blastocyst cell number
Experiment 1						
Control	101	76.72 (78)[a)]	208	80.80 (168)[a)]	32.35 (46)[a)]	153.0[a)]
bFF	118	77.59 (94)[a)]	115	77.97 (90)[a)]	32.07 (25)[a)]	153.5[a)]
Experiment 2						
Acidic fraction	80	67.70 (53)[b)]	107	77.60 (83)[a)]	28.29 (20)[a)]	95.5[b)]
Basic fraction	82	80.11 (65)[a)]	117	78.35 (92)[a)]	27.66 (20)[a)]	139.5[a)]

Values with different superscripts in same column are different (P<0.05).

RESULTS

Biological effects of the bFF protein fractions on in vitro maturation and embryo development

Most of the follicular fluid proteins were precipitated at 30–50% $(NH_4)_2SO_4$. In experiment 1, the effects on *in vitro* embryo development of adding 10% bFF to the maturation medium (TCM-199 + 10% bFF) are presented in Table 2. In experiment-1, the oocyte maturation rates were not significantly (P>0.05) differing in the control medium (76.72%) and in TCM-199 containing 10% bFF (77.59%). The cleavage, blastocyst stage and blastocyst cell number rate were not significantly different between the treatments. Interestingly, INFτ and IGF-2r mRNA expressions were significantly higher in blastocysts from the 10% bFF treatment group than the control group (P<0.05), while the other genes did not lead to a significant different (Fig. 1A).

In experiment 2, the effects of a group of proteins belonging to a particular charge group on *in vitro* embryo development were assessed (Table 2). The oocyte maturation rate in TCM-199 containing basic fraction was significantly higher (80.11%) than acidic fraction (67.70%). The cleavage and blastocyst rates were not significantly different between the acidic and basic fraction treatments. However, addition of basic fraction to TCM-199 significantly higher the blastocyst cell number compared to the addition of acidic fraction to TCM-199 (P<0.05). INFτ and HSP70 mRNA expressions were significantly higher in the basic fraction treatment than in the acidic fraction treatment (P<0.05), while the other important blastocysts genes were not different (Fig. 1B). However, the basic fraction showed a higher expression value than the acidic fraction.

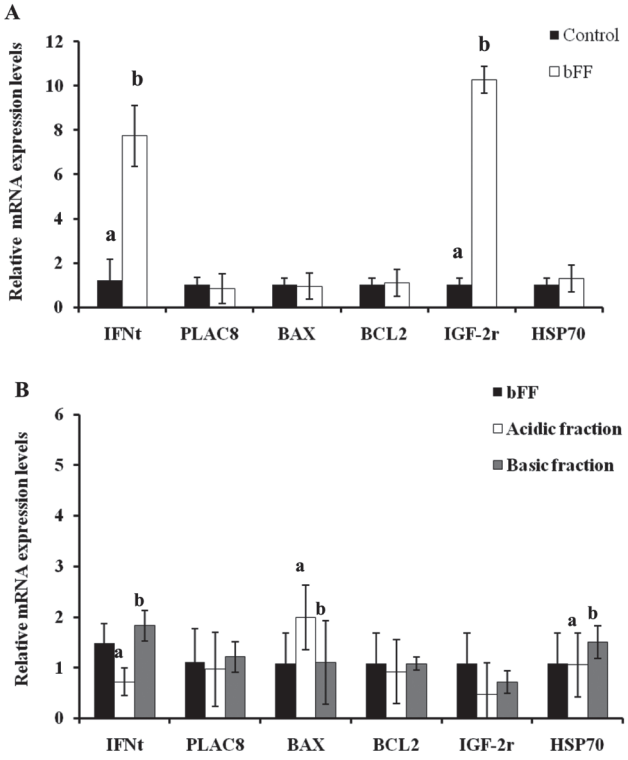

Fig. 1. Relative expression levels of various developmentally important genes in bovine blastocysts obtained from oocytes cultured in A) basic IVM medium (control) and TCM-199+10% bFF (bFF) and B) TCM-199+10% bFF (bFF), TCM-199+acidic fraction (Acidic fraction) and CM-199+basicfraction (Basic fraction). Each value represents the mean ± SEM of 3 samples. Values with different letters are significantly different (P<0.05).

Protein identification and quantification

Proteins from basic fraction were identified by ESI-LC MS/MS to determine their composition, which may affect embryo development. 1D-SDS-PAGE analysis of the proteins collected in the basic fraction detected 17 bands. These bands were selected for identification by mass spectrometry. Based on the GO analysis, the proteins were grouped according to their biological process, and 20 groups of proteins were classified (Fig. 2). The majority of the identified proteins were related to the immune system (20%), mRNA processing and transcription regulation (13%), apoptosis (10%), differentiation (8%) and gamete generation and oocyte maturation (7%).

DISCUSSION

The results of the present study demonstrate that the supplementation of the IVM medium with 10% bFF produced blastocyst yields comparable to those obtained after the addition of FBS and hormone treatment (control). In experiment 1, the development to the blastocyst stage rate were similar to those reported in previous studies (approximately 30–40%). It has been shown that embryo quality determined by blastocyst total cell number [6]. There was no significant difference among IVM medium with

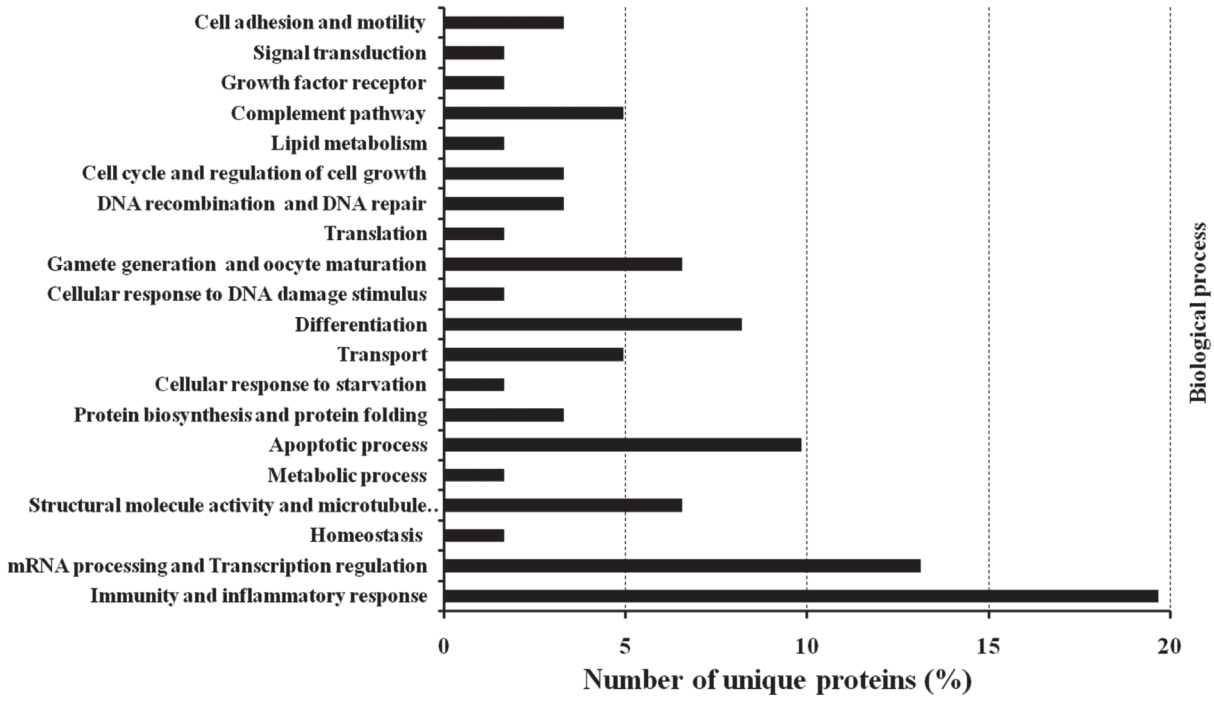

Fig. 2. GO analysis for the biological process of proteins identified in basic fraction protein of bFF.

10% bFF and control in terms of blastocyst cell number. These results indicate that the preimplantation stage embryos produced using 10% bFF supplement to IVM medium were not inferior to those from the control media. Moreover, the mRNA expression showed the bFF affects the relative abundance of the gene transcripts as shown by the increased expression of INFτ and IGF-2r. The preimplantation period of mammalian embryogenesis is notable for many critical events that affect the genome both before and after implantation [2]. It is known that embryonic culture conditions influence gene expression. INFτ is exclusively secreted by the tophectodermal cells of the blastocysts and the primary factor responsible for the maternal recognition of bovine pregnancy [10]. Result of present study suggested that the use of bFF substitute supplement in IVM medium could create more sensitivity in response to a cross-talking between embryo and the bovine endometrium in order to establishment of pregnancy. Another upregulated gene was IGF-2r, which was found to be significantly higher in the blastocysts in the bFF treatment group. IGF-2r is the receptor corresponding to the signal pathway for the growth factor regulation of fetal growth [9]. In the present study, bFF supplemented to the IVM medium (only TCM-199) was beneficial to embryonic development, improving the quality of the obtained embryos. The ability of FF to stimulate cytoplasmic maturation may be attributable to the presence of several factors, including proteins. FF contains several proteins derived from the blood plasma or that are secreted by the granulosa and thecal cells. This implies that the follicular fluid, which could be routinely harvested with the oocytes, could be used as an effective and inexpensive media supplement in IVM.

In the experiment 2, we wanted to explore any specific effect of a group of proteins belonging to particular charge group on *in vitro* embryo development. The oocyte maturation rate of medium containing basic fraction were higher than acidic fraction. In terms of blastocyst stage rate results of the present study did not differ significantly in both fractions. However, blastocyst cell numbers from basic fraction was significantly higher than the acidic fraction. In addition, the basic of protein was responsible for gene expression, especially of INFτ and HSP70. The mRNA patterns of experiment 2 showed that the different charge groups of the proteins in IVM affect the relative abundance of gene transcription. The results revealed that a particular protein in the basic fraction is responsible for embryo quality and enhancing developmentally important genes than in the acidic fraction. This increase in the mRNA expression might be caused by the greater availability of this protein in FF. The majority of basic proteins identified in the present study have been reported in the follicular fluid of mammals, including pig [5]. GO biological function analysis revealed that 20% of the identified basic proteins were related to immunity and the inflammatory response. This suggests that the ovulatory process may be divided into three phases: the inflammatory phase, the rupture phase and the repair phase [8]. The immunoglobulins were the most abundant protein in the immunity and inflammatory response group, and they have been reported in porcine follicular fluid protein [5]. An additional 13% were classified as the mRNA processing and transcription regulation group. Moreover, the high proportion of gamete generation and oocyte maturation (7%) may reflect the functional characteristics of the follicular biochemical environment, which may affect oocyte maturation and subsequent embryo development. Regarding, the functional effect on embryo development in bFF treatment group may be due to both basic and acidic fraction. Nevertheless, the blastocyst developmentally important genes were affected by basic fractions treatment. In conclusion, our results suggest that 10% bFF from the large healthy follicles has substances that support the *in vitro* maturation of oocytes and the subsequent embryo

development capacity, particularly IFNτ and IGF-2r. Thus, only bFF has the potential to be used as an IVM medium supplement for the production of *in vitro* bovine blastocysts. Additionally, we have been able to generate information on the effect of different charged groups of bFF proteins on *in vitro* embryo development. Based on our results, we concluded that the basic fraction has the effect on oocyte maturation, embryo quality and developmentally important genes at the blastocyst stage. This study contributes to our understanding of the follicular fluid proteins, providing a useful basis for applications in embryonic development and further establishment of pregnancy in bovine.

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