



## Effect of follicular fluid supplementation during *in vitro* maturation on total cell number in bovine blastocysts produced *in vitro*

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**ABSTRACT** - This study evaluated the influence of follicular fluid (FF) added to the maturation medium on the quality of bovine embryos produced *in vitro*. In the first experiment, oocytes were matured in media containing five different FF concentrations with different maturation times and classified according to meiotic progression and migration of cortical granules. In the second experiment, oocytes matured in the same media were fertilized at three different maturation times; thereafter, cleavage and blastocyst rates were evaluated. In the third experiment, oocytes were matured in media containing three different FF concentrations at two different maturation times, and embryo quality, inferred by the ratio of inner cell mass and trophectoderm cells compared with total cell number, was evaluated. Higher FF concentration (75 - 100% FF) slowed meiotic progression and CG migration (control - 78.13% vs. treated - 52.58% and control - 52.7% vs. treated - 11.59%, respectively, at 24 h of maturation). Also, FF at concentration of 75% or 100% had a negative influence on cleavage and blastocyst rates (control - 90.13% vs. treated - 82.64% and control - 35.73% vs. treated - 11.57%, respectively, at 24 h of maturation). The 50% FF resulted in embryos with increased inner cell mass numbers (control - 29.91 vs. treated - 35.49, at 24 h of maturation) and total cell numbers (control - 109.53 vs. treated - 120.67, at 26 h of maturation). Even though higher concentration of FF added to the maturation medium reduced embryonic development rates, in lower concentrations, FF slowed the meiotic progression and migration of CG and contributed to increases in inner cell mass number. Thus, FF added to the maturation medium enhances the number of cells in bovine embryos produced *in vitro*, especially for inner cell mass.

Key Words: embryo development, inner cell mass, *in vitro* fertilization, oocytes

### Introduction

The oocyte maturation process involves the activation and inhibition of enzymes, hormones and growth factors, which results in nuclear and cytoplasmic maturation (Gilchrist and Thompson, 2007). Nuclear maturation occurs spontaneously, and mechanical removal of the oocyte from the follicle is capable of triggering the process, but cytoplasmic maturation occurs more gradually (Brevini et al., 2007).

There are clear differences regarding embryonic development, particularly between oocytes matured *in vitro* and those matured *in vivo* (Choi et al., 1998). Numerous studies have shown that under *in vitro* conditions, the nuclear and cytoplasmic maturation occurs at different times (Sirard et al., 1995; Bevers et al., 1997), which might be the cause of blastocyst rates around 35% observed for *in vitro* production of bovine embryos (Rizos et al., 2002), and lower

embryo quality compared with embryos produced *in vivo* (Gonçalves et al., 2008).

The identification of substances capable of delaying the nuclear maturation time and thus allowing cytoplasmic and nuclear changes to occur synchronously has been the subject of several studies (Liu et al., 2003; Hussein et al., 2006; Sirard et al., 2007; Albuz et al., 2010). Follicular fluid (FF), consisting of electrolytes, hormones, amino acids, growth factors, among other components, has been used as a natural substance for blocking the meiosis (Aguilar et al., 2001). Some studies have documented that maturation medium supplemented with FF provides appropriate environment to bovine oocyte development (Romero-Arredondo and Seidel, 1996), since it increases the degree of cumulus cells expansion (Aguilar et al., 2001) and enhances the embryonic development (Algriany et al., 2004).

There is still controversy about the real contribution of FF to embryonic development. Follicular fluid as a maturation

medium is sufficient to promote nuclear maturation; however, it presents limitations to support appropriate embryo development (Avery et al., 2003; Coleman et al., 2007). Due to the inhibitory effect on germinal vesicle breakdown and on meiotic progression, FF leads to a delay on the nuclear maturation (Ducolomb et al., 2013). In this sense, the identification of the optimal time for fertilization of oocytes matured in FF might improve output and quality of blastocyst. Thus, to increase understanding about the action of this fluid on *in vitro* development of oocytes and embryos, the present study evaluated the influence of FF on the nuclear and cytoplasmic maturation and the quality of bovine embryos produced *in vitro*.

## Material and Methods

Reagents and culture media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Three experiments were designed. The first experiment evaluated oocytes subjected to *in vitro* culture media (TCM 199) containing five different FF concentrations (0, 25, 50, 75 and 100% FF) in four maturation times (22, 24, 26 or 28 h), with six replicates, to verify the influence of FF on oocyte maturation. In experiment II, we evaluated the same culture media from experiment I and three fertilization moments (24, 26 or 28 h of *in vitro* maturation - IVM), with six replicates, aiming to analyze the effect of FF on embryonic development.

Considering the lower rates for embryonic development when *in vitro* fertilization (IVF) was performed at 28 h of *in vitro* maturation (IVM), in Experiment III we evaluated the quality of embryos at two moments of fertilization (24 and 26 h), with three replicates. Ovaries obtained post-mortem from adult cattle, slaughtered at a local abattoir, were transported to the laboratory in 0.9% saline at 30-35 °C. The follicles between 8 to 15 mm in diameter, with a clear appearance and a good blood supply, were aspirated with a 20-gauge needle connected to a 20 mL syringe. The aspirated fluid was placed in a polystyrene tube containing 50 IU of sodium heparin and centrifuged at 2,840 g for 7 min. The supernatant was filtered through a 0.20 µm membrane, placed into microtubes (1.0 mL) and stored at -20 °C until use.

Follicles measuring 3 to 8 mm in diameter were aspirated and the cumulus-oocyte complexes (COC) recovered were washed in HEPES-buffered TCM-199 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Cripion, Andradina, Brazil), 16 µg/mL sodium pyruvate and 83.4 µg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil) and classified according to morphological appearance. Groups of 15 COC

were transferred to 100 µL drops of medium containing sodium bicarbonate-buffered TCM-199 supplemented with 10% FBS, 1.0 µg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada), 50 µg/mL hCG (Profasi™, Serono, Sao Paulo, Brazil), 1.0 µg/mL estradiol, 16 µg/mL sodium pyruvate and 83.4 µg/mL amikacin, covered with sterile mineral oil (Dow Corning Co., Midland, MI) and incubated at 38.5 °C in humidified air containing 5% CO<sub>2</sub> under saturated humidity, for various maturation times, based on the specific experiment.

The nuclear maturation rate (NMR) 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, USA) in phosphate buffered saline without Ca<sup>2+</sup> and Mg<sup>2+</sup> (PBS Ca<sup>2+</sup> and Mg<sup>2+</sup>-free) and stained with 10 µg/mL Hoechst 33342, using the method described by Cherr et al. (1988), with modifications. The cytoplasmic maturation rate (CMR) was determined by cortical granules (CG) migration. To evaluate the migration of CG to the periphery of the cytoplasm, the zona pellucida was removed from the denuded oocytes with Tyrode's solution (pH 2.5). For permeabilization, oocytes were treated for 3 min at 38 °C in blocking solution (SB) plus 0.1% Triton X-100 (USB, Cleveland, OH, USA). Subsequently, they were incubated for 15 min at 38 °C in 10 µg/mL fluorescein isothiocyanate-conjugated Lens culinaris agglutinin. This lectin specifically binds α-D-mannose present in CG. For assessment of cytoplasmic maturation, oocytes were classified according to the distribution of CG, following the classification of Hosoe and Shioya (1997).

The semen used for the study was obtained from a single bull. The frozen straws (0.25 mL) were thawed at the time of use at 37 °C for 30 s and semen was deposited on Percoll gradient 45-90%, and centrifuged at 3,600 g for 7 min. The pellet was resuspended in 500 µL IVF-TALP supplemented with 0.6% BSA, 10 mg/mL heparin, 18 µM penicillamine, 10 µM hypotaurine and 1.8 µM epinephrine, and covered with sterile mineral oil to form a new pellet after centrifugation at 520 g for 5 min. After centrifugation, the pellet was collected from the bottom of the tube, at a final concentration of approximately 10<sup>4</sup> spermatozoa for each oocyte.

The matured oocytes were transferred in groups of 20-25 per fertilization drop (IVF-TALP). These were co-incubated with spermatozoa for 18-20 h (38.5 °C, humidified air containing 5% CO<sub>2</sub>) for fertilization. Presumptive zygotes were denuded by vigorous pipetting for removal of cumulus cells and spermatozoa. Then the embryos were washed three times in SOF medium supplemented with 2.5% FCS and 5 mg/mL BSA, and transferred to the

*in vitro* culture (IVC) dish, with droplets containing 100  $\mu$ L development medium, and cultured (38.5 °C in humidified air containing 5% CO<sub>2</sub>) for six days. In the third and fifth days of culture, approximately 50% of the drop medium was removed and an equal volume of fresh medium was added.

The cleavage rate (CR) was determined 32-36 hours after insemination, using a stereomicroscope (50 X). Zygotes that had two or more cells were considered cleaved. The blastocyst rate (BR) was determined on the seventh day after IVF. The quality of blastocysts was evaluated by the inner cell mass (ICM) and trophectoderm (TE) cell number. The identification of ICM and TE, for subsequent quantification, was based on the modified technique for differential fluorochrome staining. The blastocysts obtained on day 7 after IVF were incubated in TCM199 with 1% Triton X-100 and 100  $\mu$ g/mL propidium iodide for 30 seconds. Subsequently, the blastocysts were incubated for 15 min in PBS containing 4% paraformaldehyde and 10  $\mu$ g/mL Hoechst 33342. Then, the embryos were washed in PBS and placed on glass slides with glycerol and covered with a coverslip, as described by Iwasaki et al. (1990). To determine all rates (NMR, CMR, CR and BR) the quantity of identified structures (mature/cleaved/blastocyst) was considered in relation to the total number of initial structures (immature oocytes).

The experimental design was randomized in a factorial arrangement and statistical model considered the fixed effects (culture medium and maturation time) and their interactions on variables NMR, CMR, CR, BR, cell number of ICM, TE and ICM + TE (total cell number). Data were analyzed by least squares method of GLM proc (SAS, version 9.0) and means were compared by Tukey's test ( $P < 0.05$ ).

## Results and Discussion

The meiotic progression was affected when FF was added to TCM199 medium, detected by a gradual reduction

in nuclear maturation rate as the concentration FF in TCM199 medium increased ( $P < 0.05$ ). The culture media without FF (TCM 199) and supplemented with 25% FF (TCM 199 + 25% FF) were superior compared with pure FF ( $P < 0.05$ ), except for the maturation time of 28 hours, in which all groups were similar. Conversely, the nuclear maturation rate obtained with TCM 199 + 25% FF and TCM 199 + 50% FF were similar to that observed for TCM199 at all maturation times analyzed. The lowest rate obtained at 22 h of maturation in pure FF ( $P < 0.05$ ) revealed the interaction between concentration and maturation time on nuclear maturation (Table 1).

Considering that the major nuclear changes occur during oocyte maturation, the assessment of nuclear maturation rate is a useful tool to evaluate oocyte competence (Pereira et al., 2010; Prentice et al., 2011). In horses, Dell'Aquila et al. (1997) and Bøgh et al. (2002), using 100% FF, found nuclear maturation rates of 68.5 and 79%, respectively. In the present study, pure FF provided lower rates, ranging from 43 to 65%, similar to the reports of Avery et al. (2003) and Coleman et al. (2007), who describe nuclear maturation rates of 41 and 44%, respectively, in cattle.

Yet, an inverse relationship occurred between FF concentration and NMR in all maturation times; the lowest rate obtained in short maturation time indicated an interaction between FF concentration and maturation time on the NMR. Avery et al. (2003) and Coleman et al. (2007) also observed interaction of maturation time with the concentration between FF on nuclear maturation rate.

The nuclear maturation rates achieved at 28 h of maturation in media containing high concentrations of FF (75 or 100%) were greater than those observed in other maturation times, possibly due to the delay of meiotic progression caused by FF, which provided additional time for the oocytes to acquire competence (Pereira et al., 2010; Prentice et al., 2011; Gottardi et al., 2012). According to Aguilar et al. (2001), a slower nuclear maturation provides

Table 1 - Nuclear maturation rate of bovine oocytes matured *in vitro* for four different maturation times in culture media containing different concentrations of follicular fluid

Medium	Nuclear maturation rate							
	22 h <sup>1</sup>		24 h <sup>1</sup>		26 h <sup>1</sup>		28 h <sup>1</sup>	
	N	NMR (%)	N	NMR (%)	N	NMR (%)	N	NMR (%)
TCM199	91	72.53Aa	84	78.13Aa	87	77.14Aa	102	82.35Aa
TCM199 + 25%FF	94	68.27Aba	79	77.42Aa	84	79.79Aa	91	75.70Aa
TCM199 + 50%FF	112	66.96Aba	77	70.11Aba	93	74.76Aa	83	69.39Aa
TCM199 + 75%FF	95	54.13BCc	88	61.32BCbc	81	69.39Aab	87	72.90Aa
100% FF	116	43.10Cb	77	52.58Cab	70	59.79Ba	106	65.89Aa

<sup>1</sup> *In vitro* maturation time.

NMR - nuclear maturation rate; FF - follicular fluid.

ABC Different capital letters, in the same column, indicate difference by Tukey's test ( $P < 0.05$ ).

abc Different small letters, in the same row, indicate difference by Tukey's test ( $P < 0.05$ ).

oocytes with more time to synthesize, modify and store new proteins and ribonucleoproteins, and therefore enhances their competence. Moreover, despite the higher nuclear maturation rate observed when the maturation time was extended, the rates obtained with pure FF remained lower than those with TCM199 medium.

The migration of CG was influenced by the FF concentration only at 24 h of maturation. At this maturation time, there was a gradual decrease in the cytoplasmic maturation rate (CMR) to the extent that FF concentration was rising (Table 2). The CMR reached with 25% FF was similar to those obtained in TCM199 medium at 22, 26 and 28 h of maturation. At 22 h of maturation, the CMR was lower than those observed in all other maturation times ( $P < 0.05$ ).

Mammalian CG first appears in the early stages of oocyte growth. However, the exact time window in which CG are synthesized differs among species. The CG migration is an important step in cytoplasmic maturation and has been used routinely as a criterion in assessing the maturity and organelle organization of developing oocytes (Liu, 2011). A recent study revealed that FF promotes oocyte cytoplasmic maturation during IVM and suggested that the major role of FF is to provide protection against oxidative stress (Gruppen and Armstrong, 2010). Several FF components, including amino acids (Hong and Lee, 2007) and plasminogen activators and plasmin (Papanikolaou et al., 2008) have been found to exert positive effects on porcine oocyte cytoplasmic maturation *in vitro*.

The results of the present study showed that FF interferes with oocyte maturation by slowing the migration of CG to the periphery of the oocyte, causing a delay in cytoplasmic maturation and possibly providing more adherence time to cells of COC. Even though the cytoplasmic maturation delay promoted by FF might be beneficial to oocytes, a previous study suggests that delayed cytoplasmic maturation is responsible for the abnormalities observed during fertilization and *in vitro* development,

which commonly involved the migration and dispersal of CG (Damiani et al., 1996).

It is likely that steroids, gonadotrophins and the maturation medium itself all play an important role, particularly if the oocytes are matured in defined or semi-defined culture media (Accardo et al., 2004). Follicle-stimulating hormone (FSH) maintained associations between growing oocytes and cumulus cells during *in vitro* growth culture, probably by supporting the oocytes (Cayo-Colca et al., 2011). The FSH:Luteinizing hormone (LH) ratio in IVM culture media is directly correlated with oocyte cytoplasmic maturation (Sha et al., 2010). Schoevers et al. (2003), using porcine oocytes, stated that FSH initially retards germinal vesicle breakdown, but overall has a stimulatory effect on nuclear and cytoplasmic maturation. Considering the strategic role of FF on *in vitro* cytoplasmic maturation (Gruppen and Armstrong, 2010), the delay in cytoplasmic maturation verified in the present study may have occurred due to insufficient concentration of FSH, since the FSH concentration was not adjusted when the FF concentration was increased.

The fertilization moment influenced cleavage (CR) and blastocyst rates (BR). Cleavage rate and BR obtained at 28 h were lower than those achieved at 24 h and 26 h, in all maturation media ( $P < 0.05$ ). On the other hand, when fertilization was performed at 24 or 26 h, the FF concentration influenced CR and BR, which were lower when higher concentrations ( $\geq 75\%$ ) were used ( $P < 0.05$ ). Follicular fluid concentration negatively affected BR when the fertilization was performed at 28 h; however, it had no influence on CR (Table 3).

Contrary to the present results, Avery et al. (2003) obtained unsatisfactory cleavage rates (around 9%) when using pure FF. These researchers attributed the low cleavage rates to the ability of FF to cause adhesion of solids to cumulus cells, forming a physical barrier that hinders the penetration of sperm. These findings suggest that FF at high concentrations might negatively influence adhesion to the

Table 2 - Cytoplasmic maturation rate of bovine oocytes matured *in vitro* for four different maturation times in culture media containing different concentrations of follicular fluid

Medium	Cytoplasmic maturation rate							
	22 h <sup>1</sup>		24 h <sup>1</sup>		26 h <sup>1</sup>		28 h <sup>1</sup>	
	N	CMR (%)	N	CMR (%)	N	CMR (%)	N	CMR (%)
TCM199	72	6.94Ab	74	52.70Aa	75	56.00Aa	76	61.84Aa
TCM199 + 25%FF	69	7.25Ac	70	31.43Bb	72	44.44Ab	73	68.49Aa
TCM199 + 50%FF	73	4.11Ac	73	9.59Cbc	73	31.51Ab	75	62.67Aa
TCM199 + 75%FF	72	5.56Ab	72	11.11BCb	75	30.67Ab	73	69.86Aa
100% FF	73	2.74Ac	69	11.59BCbc	75	29.33Ab	76	64.47Aa

<sup>1</sup> *In vitro* maturation time.

CMR - cytoplasmic maturation rate; FF - follicular fluid.

ABC Different capital letters, in the same column, indicate difference by Tukey's test ( $P < 0.05$ ).

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COC cells. In the present study, however, the use of heparin with subsequent FF filtering probably prevented the solid accumulation to COC, and allowed adequate fertilization.

The low blastocyst rates obtained when fertilization was performed at 28 h of IVM might be explained by the fact that fertilization does not occur immediately after insemination. According to Méo et al. (2007), the pronuclei first appear 12 h after insemination. Thus, oocytes inseminated later (28 h) would have missed the optimal moment for fertilization due to aging. Moreover, since aged oocytes (28 h of IVM) are more susceptible to spontaneous parthenogenetic activation (Shirazi et al., 2009), the cleavage rates obtained probably include parthenogenetically activated zygotes.

The FF concentration affected ICM cell number and the total cell number, when fertilization was performed at 24 and 26 h IVM, respectively ( $P < 0.05$ ); in both cases, the medium TCM199 + 50% FF was higher than others ( $P < 0.05$ ). The ICM cell number was similar for all culture media, when the fertilization was performed at 26 h IVM. The TE cell number was not affected by addition of the FF, but it was influenced by the moment of fertilization in TCM199 + 50% FF, given that cell numbers at 26 h IVM were higher than at 24 h IVM ( $P < 0.05$ ). The total cell number (ICM + TE) was influenced by the fertilization moment, regardless of maturation medium ( $P < 0.05$ ). The FF, in the 50% concentration, increased the total cell number when fertilization was performed at 26 h IVM (Table 4).

According to Dulcibella et al. (1990) and Fissore et al. (2002), during the aging process, progressive deterioration of the oocyte occurs, including the rupture of mitochondrial membranes and inability to generate ATP. The dramatic decrease in CG release affects cell proliferation and survival. It is important to point out that oocytes subjected to IVM have a higher energy demand, which contributes to greater damage due to aging (Prentice et al., 2011). The satisfactory blastocyst rates obtained with TCM199 medium + 25% FF in maturation times of 24 or 26 h may have been due to the ability of FF to create an environment for oocytes that made them suitable for further *in vitro* embryo development, by modulating the progression of CG.

Considering that follicular modifications occur gradually in the *in vivo* environment, the low blastocyst rates obtained using FF concentrations  $\geq 50\%$  might be explained by the sudden change of media in the *in vitro* environment. This abrupt environmental change probably caused veering in oocyte metabolic pathways, negatively interfering with the maturation process. These results agree with those of Elmileik et al. (1995) and Kim et al. (1996), who pointed out that FF at concentrations of 50 or 60% have an adverse effect on embryo development. Another explanation for the low blastocyst rate might be the negative action of a specific fatty acid present in FF. The linoleic, oleic, stearic and palmitic fatty acids are the most prevalent in bovine FF (Bender et al., 2010). However, the increase in palmitic

Table 3 - Cleavage and blastocyst rates of bovine oocytes fertilized at three different moments of *in vitro* maturation in culture media containing different concentrations of follicular fluid

Medium	Cleavage and blastocyst rates								
	24 h <sup>1</sup>			26 h <sup>1</sup>			28 h <sup>1</sup>		
	N	CR (%)	BR (%)	N	CR (%)	BR (%)	N	CR (%)	BR (%)
TCM199	375	90.13Aa	35.73Aa	379	90.77Aa	33.77Aa	187	55.61Ab	12.83ABb
TCM199 + 25%FF	384	89.32Aa	41.41Aa	395	90.63Aa	38.73Aa	191	53.40Ab	14.14Ab
TCM199 + 50%FF	408	87.75Aa	17.65Ba	401	88.28Aa	18.45Ba	189	49.74Ab	8.47Aba
TCM199 + 75%FF	243	81.89Ba	16.46Ca	241	81.74Ba	17.01Ca	186	43.01Ab	7.53Aba
FF	242	82.64Ba	11.57Ca	239	80.75Ba	13.39Ca	183	37.70Ab	3.83Bb

<sup>1</sup> *In vitro* fertilization moment.

CR - cleavage rate; BR - blastocyst rate; FF - follicular fluid.

ABC Different capital letters, in the same column, indicate difference by Tukey test ( $P < 0.05$ ).

abc Different small letters, in the same row, indicate difference by Tukey test ( $P < 0.05$ ).

Table 4 - Number of inner cell mass, trophectoderm cell and total cells in bovine embryos fertilized at two moments after *in vitro* maturation and cultured for seven days in culture media containing different concentrations of follicular fluid

Medium	Cell number of ICM, TE and Total					
	ICM		TE		ICM + TE	
	24 h <sup>1</sup>	26 h <sup>1</sup>	24 h <sup>1</sup>	26 h <sup>*</sup>	24 h <sup>1</sup>	26 h <sup>1</sup>
TCM199	29.91±3.83Ba	34.84±3.78Aa	67.93±3.78Aa	74.69±2.02Aa	97.84±5.70Ab	109.53±4.24 Ba
TCM199 + 25%FF	28.87±0.83Bb	36.29±1.46Aa	67.98±3.91Aa	75.73±3.32Aa	96.84±4.85Ab	112.02±3.98Aba
TCM199 + 50%FF	35.49±1.46Aa	40.04±2.80Aa	72.51±3.32Ab	80.62±5.87Aa	108.00±3.98Ab	120.67±7.96Aa

<sup>1</sup> *In vitro* fertilization moment.

ICM - inner cell mass; TE - trophectoderm; FF - follicular fluid.

ABC Different capital letters, in the same column, indicate difference by Tukey's test ( $P < 0.05$ ).

abc Different small letters, in the same row, indicate difference by Tukey's test ( $P < 0.05$ ).

acid during IVM has a detrimental effect on the blastocyst rate (LeRoy et al., 2005).

Similar to what happened with the cleavage rate, the low blastocyst rates observed when fertilization was performed at 28 h probably resulted from oocyte aging. According to Souza et al. (2009), although aged oocytes can be fertilized and initiate embryo development, they may not reach the blastocyst stage due to fertilization failure. At the moment of spermatozoa penetration into the aged oocyte, signaling can occur because of the influx of calcium ions, which acts inducing apoptosis, thus stopping embryo development. The results of the present study corroborate the oocyte aging theory, since, regardless of the FF concentration used, the blastocyst rates were unsatisfactory when fertilization occurred at the later time (28 h).

Romero-Arredondo and Seidel (1996) observed that supplementation of maturation medium with FF improves the developmental capacity in bovine oocytes and consequently enhances embryonic development. This is corroborated by the results found in the present study, in which the addition of FF to the maturation medium proved beneficial. We found that FF added to TCM199 medium at a concentration of 50% allowed the collection of embryos with higher ICM cell number than the other media. This increase in ICM cell number might be caused by the greater availability of nutrients in FF, including essential amino acids.

Initial approaches to metabolic profiling of FF indicate a relationship between FF amino acid concentrations and oocyte cleavage following IVF, particularly L-alanine and glycine, which are two of the most abundant amino acids in bovine FF (Sinclair et al., 2008; Bender et al., 2010). The supplementation of alanine and glycine in the culture medium synergistically improves development of *in vitro*-produced bovine embryos and increases blastocyst cell numbers (Lee and Fukui, 1996). Thus, the increase in total cell number verified in the present study might indicate that the addition of FF to the TCM199 medium improves embryonic quality.

## Conclusions

Although higher concentrations of follicular fluid added to the maturation medium reduce embryonic development, at lower concentrations follicular fluid slows meiotic progression and migration of cortical granules, and this contributes to increased number of cells in inner cell mass. Thus, follicular fluid added to the maturation medium at lower concentrations enhances the total cell numbers in bovine embryos produced *in vitro*, especially regarding inner cell mass cells.

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