Universiteit
Antwerpen

This item is the archived peer-reviewed author-version of:

Follicular fluid during individual oocyte maturation enhances cumulus expansion and improves embryo development and quality in a dose-specific manner

Reference:
Azari-Dolatabad Nima, Raes Annelies, Pavani Krishna Chaitanya, Asaadi Anise, Angel-Velez Daniel, Van Damme Petra, Leroy Jo, Van Soom Ann, Bogado Pascottini Osvaldo.- Follicular fluid during individual oocyte maturation enhances cumulus expansion and improves embryo development and quality in a dose-specific manner
Theriogenology: an international journal of animal reproduction - ISSN 0093-691X - 166(2021), p. 38-45
Full text (Publisher's DOI): https://doi.org/10.1016/J.THERIOGENOLOGY.2021.02.016
To cite this reference: https://hdl.handle.net/10067/1781560151162165141

uantwerpen.be
Institutional repository IRUA

1 **Revised non-highlighted**

2 **Follicular fluid during individual oocyte maturation enhances cumulus expansion and**
3 **improves embryo development and quality in a dose-specific manner**

4 Nima Azari Dolatabad[a]*, Annelies Raes[a], Krishna Chaitanya Pavani[a], Anise Asaadi[ab], Daniel
5 Angel-Velez[ac], Petra Van Damme[a], Jo L.M.R. Leroy[d], Ann Van Soom[a], Osvaldo Bogado
6 Pascottini[ad]*

7 [a]*Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine,*
8 *Ghent University, Merelbeke, Belgium*

9 [b]*Department of Animal Reproduction, School of Veterinary Medicine, Shiraz University,*
10 *Shiraz, Iran*

11 [c]*Research Group in Animal Sciences - INCA-CES, School of Veterinary Medicine and Animal*
12 *Production, Universidad CES, Medellin, Colombia*

13 [d]*Department of Veterinary Sciences, Gamete Research Center, Veterinary Physiology and*
14 *Biochemistry, University of Antwerp, Wilrijk, Belgium*

15 ***Corresponding authors**: Nima Azari Dolatabad nima.azaridolatabad@UGent.be; Osvaldo
16 Bogado Pascottini osvaldo.bogado@ugent.be

17

18 **ABSTRACT**

19 We evaluated the effect of supplementation of different concentrations of bovine follicular fluid
20 (FF) during *in vitro* maturation (IVM) on oocyte development and blastocyst quality in group
21 and individual culture conditions. To do so, *in vitro* maturation medium (TCM-199 with 20
22 ng/mL epidermal growth factor and 50 µg/mL gentamycin) was supplemented with 0 (control),
23 1, 5, or 10% of FF. Follicular fluid was collected from slaughterhouse-derived ovaries, selecting
24 follicles between 12 to 20 mm in diameter. Oocytes were either produced in groups or
25 individually matured, fertilized, and cultured to the blastocyst stage, allowing for separate
26 follow-up of each oocyte. Development (cleavage and blastocyst rates) among experimental
27 groups were fitted in mixed-effects models, and blastocyst quality parameters (assessed via
28 differential apoptotic staining) were evaluated in mixed linear regression models. We also
29 assessed the cumulus expansion (prior and after maturation) for individual culture conditions,
30 and their difference was fitted in mixed linear regression models. The FF was collected from
31 two batches, with an estradiol/progesterone ratio higher than 1. The FF batch did not affect the
32 development or blastocyst quality in group or individual culture conditions ($P > 0.05$). In group
33 culture, development was similar among experimental groups ($P > 0.05$). Five or 10% of FF
34 supplementation improved ($P < 0.05$) aspects of blastocyst quality such as total cell numbers
35 (TCN), trophectoderm (TE), inner cell mass (ICM), and ICM/TCN and apoptotic cells /TCN
36 ratio in comparison to control. In the individual culture system, 5% FF supplementation
37 increased ($P < 0.05$) day 8 blastocyst rate (33 ± 3.4% (LSM ± SE)) in comparison to control
38 (20 ± 2.7%) and 1% FF supplementation (19 ± 2.6%) but it was not different ($P > 0.05$) from
39 10% FF supplementation (28 ± 3.4%). Five percent of FF supplementation resulted in greater
40 TCN, ICM, and ICM/TCN than control ($P < 0.05$). It also resulted in a greater expansion of
41 cumulus cell investment than the other groups ($P < 0.05$), with a 3-fold increase compared to
42 control. In conclusion, 5% of FF supplementation during IVM improved the cumulus expansion

43 and the blastocyst development and quality in an individual culture system. However, FF
44 supplementation during maturation in a group culture system did not increase development, but
45 it modestly improved some embryo quality aspects when 5 or 10% of FF was added.

46 **Keywords:** individual culture; cumulus expansion; developmental competence; embryo
47 quality; follicular fluid.

48

1. Introduction

Substantial progress has been attained in recent decades for bovine *in vitro* embryo production (IVP). However, the quality of IVP embryos is lower than *in vivo*-derived embryos [1,2]. Optimized *in vitro* systems should mimic events that happen in the early stages of oocyte maturation and embryo development [3–5]. Hence, biofluids derived from the reproductive tract of healthy animals provide excellent starting material. However, they are composed of a complex matrix of growth factors, hormones, electrolytes, proteins, steroids, cytokines, metabolites, glycoproteins, antioxidants, extracellular vesicles, apoptotic factors, and other undefined components that are hardly possible to recreate in *in vitro* conditions [6,7].

The follicular fluid (FF) provides the ideal microenvironment to support the oocyte's growth and developmental competence [8,9]. To resemble *in vivo* conditions, studies have focused on the addition of growth factors, hormones, and protein sources, including serum and FF, to the *in vitro* maturation (IVM) medium [10–16]. It has been shown that supplementing specific components of FF to the IVM culture medium resulted in improved oocyte maturation rates, such as estradiol, which enhances the cytoplasmic maturation [17]. Also, it has been demonstrated that the estradiol/progesterone ratio in FF is crucial for the final stages of oocyte maturation [18,19]. Still, results are highly variable in terms of embryo development and quality. This high variability may be attributed to the maturation medium composition (serum vs. serum-free), percentage of FF supplementation, and type of culture system (group vs. individual). Moreover, factors associated with the FF composition vastly vary according to the age of animals [20,21], the size of follicles, metabolic state, and stage of the estrous cycle [22–24].

Currently, the best IVP results have been obtained through group culture [2]. This means that all steps of *in vitro* embryo production (oocyte maturation, fertilization, and embryo culture) are performed in groups of at least 25 oocytes or embryos at a density of 1:2 (1 zygote

74 in 2 μL medium per embryo culture drop). Typically, in our hands (under serum-free
75 conditions), 40 to 50% of cultured oocytes reach the blastocyst stage at 8 days post insemination
76 (dpi) [25]. When maturation and fertilization are performed in groups, and embryo culture is
77 done individually (density of 1:20; minimum droplet volume of 20 μL), blastocyst rates are
78 lowered to 30 to 40% [26]. Furthermore, day 8 blastocyst rate is more variable and, in some
79 cases, decreases to less than 30% [27] when also maturation and fertilization are performed
80 individually. Besides, blastocysts cultured individually have a lower chance of hatching,
81 displaying a lower total cell number (TCN) and increased apoptotic cell ratio when compared
82 to those cultured in groups [28,29]. One of the reasons for low blastocyst rate and quality during
83 individual culture conditions is the absence of autocrine/paracrine stimulation by neighboring
84 oocytes or embryos in contrast to the group culture system [28]. Nevertheless, it is of great
85 importance to achieve consistent, satisfactory results (under serum-free conditions) in a
86 complete single-oocyte culture system since it allows for the individual follow-up of markers
87 associated with embryo viability and quality [29].

88 The effect of FF supplementation on the maturation medium in an entirely individual
89 culture system has not been studied before. Furthermore, the dose-specific effect of FF
90 supplementation in a complete serum-free IVP procedure in a group system is still unclear. We
91 hypothesized that FF supplementation to the IVM medium would improve embryo
92 development and quality in single and group culture conditions. Thus, the objective of this study
93 was to assess the effect of 1, 5, and 10% of FF supplementation during oocyte maturation in
94 single and group culture systems, with serum-free conditions, on the subsequent embryo
95 development and quality.

96 2. Materials and methods

97 2.1. Media and reagents

Tissue culture media (TCM)-199-medium gentamycin and Phosphate-Buffered Saline (PBS) were purchased from Life Technologies Europe (Ghent, Belgium). All other chemicals not otherwise listed were obtained from Sigma-Aldrich (Overijse, Belgium). All media were filtered before use (0.22 µm; GE Healthcare-Whatman (Diegem, Belgium)).

2.2. Collection and steroid hormone analysis of the follicular fluid

Bovine ovaries were collected at the local slaughterhouse, and the FF was harvested within 2 h of collection. Briefly, the ovaries were washed three times in warm physiological saline supplemented with kanamycin (25 mg/mL), sterilized with 90% ethanol, and dried with clean towels. Two batches of FF were collected at two different, random days in a mix of Belgian Blue and Holstein cows' ovaries. The proportion of breed from the ovary mix, the origin, age, and metabolic status of the cows is unknown. Ovaries were selected based on the following criteria at the individual ovary level, 1) absence of corpora lutea, 2) absence of follicles > 25 mm in diameter, 3) and normal morphological characteristics (e.g., absence of adhesions, scars, etc.). Follicular fluid was aspirated using an 18-gauge needle attached to a 10 mL syringe, from follicles between 12 to 20 mm in diameter, and pooled into two 8 mL sterile Vacutainer tubes containing heparin (BD Vacutainer Precision Glide, Becton Dickinson, Franklin Lakes, NJ). Tubes were centrifuged at 4°C for 7 min at 2,460 × g [15], and the supernatant was filter-sterilized using a 0.22 µm filter (GE Healthcare-Whatman, Diegem, Belgium). Filtered FF supernatants were pooled into a 50 mL tube, gently mixed, aliquoted in 2 mL cryovial tubes, and stored at -80°C until usage.

The estradiol and progesterone concentrations were evaluated for the two FF batches. Their concentrations were measured with an auto-chemistry analyzer (Cobas 6000 c 501, Roche Diagnostics, Indianapolis, IN) using the Progesterone III and the Estradiol III kits (Roche Diagnostics, Indianapolis, IN). To not exceed the detection limits of the assays, FF samples were diluted 1:100 for the estradiol and 1:5 for the progesterone analyses. The intra-coefficient

123 of variation were <10%. The estradiol concentration was 238.4 and 255.8 ng/mL for the first
124 and second batch, respectively. The progesterone concentration was 223 ng/mL for the first and
125 61.3 ng/mL for the second batch. The estradiol/progesterone ratio was 1.06 and 4.17 for the
126 first and second batch, respectively. Both FF batches were considered to be obtained from active
127 follicles since their estradiol/progesterone ratio was > 1 [30-32].

128 **2.3. Study design**

129 The experimental design consisted of two biological replicates (two FF batches) from
130 which five technical replicates per batch were done for the group culture system and seven
131 replicates per batch for the individual culture system.

132 **2.4. Group and individual *in vitro* oocyte maturation and embryo production**

133 Bovine embryos were produced by routine *in vitro* methods as previously described by
134 Wydooghe et al. [33] with minor modifications. Briefly, cow ovaries were processed as for the
135 protocol described for the FF collection. The cumulus-oocyte complexes (COCs) were
136 aspirated from antral follicles between 4 and 8 mm in diameter using an 18-gauge needle
137 attached to a 10 mL syringe. Oocytes with uniformly granulated cytoplasm and surrounded by
138 more than three compact layers of cumulus cells were selected. *In vitro* maturation, fertilization
139 (IVF), and culture (IVC) were performed either in groups or in individual culture conditions.

140 For the group culture system, groups of 60 COCs were matured in 500 µL TCM-199 with
141 20 ng/mL epidermal growth factor (EGF) and 50 µg/mL gentamycin. For each replicate, the
142 IVM media were supplemented with 0 (control), 1, 5, or 10% v/v of FF and incubated for 22 h
143 at 38.5 °C in 5% CO_2 in humidified air. For IVF, frozen-thawed spermatozoa from a proven
144 fertile bull were passed over a Percoll gradient (45 and 90%; GE Healthcare Biosciences,
145 Uppsala, Sweden) and washed with fertilization medium. The final concentration of 1×10^6
146 sperm/mL was adjusted using IVF-Tyrode's Albumin Lactate Pyruvate (TALP), which

147 consisted of bicarbonate buffered Tyrode's solution, supplemented with BSA (Sigma A8806; 6
148 mg/ml) and heparin (20 µg/mL). Matured oocytes were incubated in 500 mL IVF-TALP with
149 spermatozoa for 21 h at 38.5°C in 5% CO_2 in humidified air. After fertilization, surplus
150 spermatozoa and cumulus cells were removed by vortexing. Randomly selected cumulus-free
151 presumed zygotes were transferred in groups of 25 to 50 µL droplets of synthetic oviductal fluid
152 (SOF), 0.4% BSA (Sigma A9647), and ITS (5 µg/mL insulin + 5 µg/mL transferrin + 5 ng/mL
153 selenium). Each droplet was covered with 900 µL paraffin oil (SAGE, CooperSurgical,
154 Trumbull, CT, USA) and incubated at 38.5°C for 8 days in 5% CO_2, 5% O_2, and 90% N_2.

155 For the individual culture system, the composition of all media was the same as for group
156 culture conditions, but all steps (IVM, IVF, and IVC) were performed individually. Briefly, 17
157 droplets of 20 µL medium each were prepared in Petri dishes (60 x 15 mm; Thermo Fisher
158 Scientific, Waltham, MA USA) and covered with 7.5 mL paraffin oil. Each droplet contained
159 a single COC or presumptive zygote. Individual COCs were matured in droplets of 20 µL of
160 maturation medium. The IVM medium was supplemented with 0 (Control), 1, 5, or 10% v/v of
161 FF and incubated for 22 h at 38.5°C in 5% CO_2 in humidified air. The Percoll gradient was
162 prepared as for the group culture system, but IVF was done in droplets of 20 µL IVF-TALP
163 with 1×10^6 spermatozoa/mL for 21 h at 38.5°C in 5% CO_2 in humidified air. After fertilization,
164 oocytes were vortexed, and the cumulus- free presumed zygotes were transferred individually
165 to 20 µL droplets of SOF and incubated at 38.5°C for 8 days in 5% CO_2, 5% O_2, and 90% N_2.
166 The 17 individual droplets from each treatment group (Control, 1, 5, or 10% of FF) were always
167 kept in the same Petri dish (different treatments were not mixed in the same dish at any step of
168 the IVP system).

169 **2.5. Cumulus oocyte complex expansion measurement and differential staining**

170 The COCs' expansion was only measured from individually-matured oocytes. The IVM
171 was performed as described above, and the maturation medium was supplemented with 0

172 (Control), 1, 5, or 10% v/v of FF. Pictures of COCs were taken using an Olympus
173 stereomicroscope, prior to and after 22 h incubation (Figure 1). Each COC picture was
174 measured at three places: shortest, medium, and the longest distance between the zona pellucida
175 and the extreme of the cumulus cells (Figure 1). The mean of the three distances was
176 determined, and the difference between the measurements prior and after maturation was
177 calculated (in µm) using the ImageJ software [34] (version 1.49 q; National Institutes of
178 Health).

179 Differential staining was performed to assess the blastocyst quality, as previously
180 described by Wydooghe et al. [35]. Briefly, day 8 blastocysts were fixed in 2%
181 paraformaldehyde for 20 min at room temperature and then stored in phosphate-buffered saline
182 containing 0.5% BSA at 4°C. Immunofluorescent staining was firstly done using CDX2, a
183 transcription factor uniquely expressed in trophectoderm (TE) cells (Figure 2). This was
184 followed by active caspase-3 staining, which plays a central role in all apoptotic pathways, and
185 further combined with 0.1% Hoechst 33342 staining (pan-nuclear fluorescent dye; Figure 2).
186 This differential staining allowed the assessment of the number of TE cells, inner cell mass
187 number (ICM), TCN (TE + ICM), ICM/TCN ratio, and the total number of apoptotic cells (AC)
188 as well as the ratio of apoptotic cells (ACR; AC/TCN). These assessments were done by
189 fluorescence microscopy (Leica DM 5500 B) using a triple bandpass filter by a single observer.

190 **2.6. Statistical analyses**

191 All statistical analyses were performed using R-core (version 3.6.1; R Core Team,
192 Vienna, Austria). Generalized mixed-effects models were used to test the effects of FF
193 supplementation during IVM (control vs. 1% FF vs. 5% FF vs. 10% FF) on developmental
194 parameters (cleavage and blastocyst rates). Mixed linear regression models were used to test
195 the effect of FF supplementation during IVM on COCs expansion and differential staining
196 parameters (TCN, TE, ICM, ICM/TCN, AC, and AC/TCN). Residuals for each model were

analyzed by Shapiro-Wilk's test. If the distribution of model residuals was not normal, log^{10}, ln, square root, square, or cubic transformations of the outcome were performed until visual inspection of the histogram and quantile plot of the residuals indicated the most-nearly normal distribution, and a scatter plot of model-predicted values vs. residuals indicated homoscedasticity. The FF batch (categorical variable; 1 or 2) was offered as a covariable, and the replicate nested with the FF batch was set as a random effect for all the models. The differences between treatment groups were assessed using Tukey's post hoc test. Results are expressed as least squares means and standard errors. The significance level was set at $P < 0.05$.

3. Results

Fourteen technical replicates for individual culture and ten technical replicates for group culture were performed with a total of 3,388 oocytes, including 2,207 oocytes for the group culture system (~60 oocytes per experimental group; ~240 oocytes per replicate) and 1,181 oocytes for the individual culture system (~17 oocytes per experimental group; ~68 oocytes per replicate. In some replicates, due to the higher availability of oocytes, we used two dishes for each treatment; ~34 oocytes per experimental group; ~136 oocytes per replicate). Half of the replicates for each system were performed with each batch of FF. Three hundred and thirty-eight blastocysts were stained and evaluated for the group culture and two hundred and forty-eight for the individual culture system. Six hundred and six COCs were used to measure their cumulus expansion. The FF batch did not affect the development or blastocyst quality in group or individual culture conditions ($P > 0.05$). The cumulus expansion measurement, which was performed only for the individual culture conditions, was not affected by the FF batch ($P > 0.05$).

3.1. Group culture: embryo development and quality

220 Cleavage, day 7, and day 8 blastocyst rates were similar among experimental groups (P > 0.05; Figure 3A). Differences in blastocyst quality parameters among FF supplemented oocytes are shown in Table 1. One percent of FF supplementation produced blastocysts with a lower TCN, ICM, and ICM/TCN ratio than control (P < 0.05). Five percent of FF supplementation increased the numbers of ICM and reduced the AC/TCN ratio compared to control (P < 0.05). Ten percent of FF supplementation increased the numbers of TCN, TE, and ICM/TCN ratio and reduced the AC/TCN ratio compared to control (P < 0.05).

227 **3.2. Individual culture: embryo development and quality, and cumulus expansion**

228 Five percent of FF supplementation increased (P < 0.05) the day 8 blastocyst rate (33 ± 3.4%) in comparison to control (20 ± 2.7%) and 1% FF supplementation (19 ± 2.6%) but it was not different (P > 0.05) than 10% of FF supplementation (28 ± 3.4%; Figure 3B). All the parameters of the differential-apoptotic staining among experimental groups are depicted in Table 1. Five percent of FF supplementation resulted in a higher TCN, ICM, and ICM/TCN ratio than control (P < 0.05). Five and 10% FF supplementation had similar TCN (P > 0.05), however, 5% FF supplemented oocytes had lower AC/TCN ratio than 1 or 10% FF supplementation (P < 0.05). Notably, 1% supplementation during IVM produced blastocysts with lesser TCN and TE than all the other groups (P < 0.05).

237 Five percent FF supplementation induced greater cumulus expansion (873 ± 24 µm; P < 0.05) than control (472 ± 22 µm), 1% FF (298 ± 21 µm), or 10% FF (787 ± 29 µm). One percent FF supplementation had the lowest cumulus expansion (P < 0.05), and the cumulus expansion of 10% FF was greater than the control (P < 0.05).

241 **4. Discussion**

242 The FF constitutes part of the natural environment for oocyte maturation. We hypothesized that FF supplementation to the IVM medium would improve the embryo

244 development and quality in group and individual culture systems. In this study, the group
245 culture system did not benefit from FF supplementation in aspects of embryo development, but
246 5 or 10% FF modestly improved some blastocyst quality parameters. For the individual culture
247 system, the addition of 5% of FF to the maturation medium significantly increased the day 8
248 blastocyst rate and improved the cumulus expansion, the TCN, and the AC/TCN ratio of
249 differentially stained blastocysts. Based on the results of this study, we recommend
250 supplementing 5% FF to the maturation medium to improve outcomes in individual culture
251 conditions.

252 It is important to notice that TCM-199 is commonly-used for bovine IVM experiments
253 with the supplementation of hormones and/or growth factors. The addition of bovine/calf serum
254 has recently become less common practice, and several research groups are now using simple
255 serum-free maturation medium (e.g., TCM-199 with EGF), with excellent results in group
256 culture systems [33,36]. However, serum supplementation is still being used in some
257 laboratories [37,38], mostly because it provides more consistent results. In a complete single-
258 oocyte culture system, serum supplementation had been shown to increase the blastocyst rate
259 up to 30% in two studies [39,40], but those results were difficult to reproduce and may have
260 been influenced by the batch of serum used. It has also been demonstrated that serum
261 supplementation has a substantial impact on the embryos' gene expression profile, diverting it
262 away from its *in vivo* counterpart, whereas the transcriptome of embryos produced in serum-
263 free conditions are showing a greater resemblance to that of *in vivo* derived embryos [41]. As
264 far as the FF is concerned, further research should demonstrate if there is a beneficial effect of
265 FF on the gene transcription pattern in resulting embryos, making them more *in vivo*-like or
266 not. Moreover, it would be interesting to direct future studies to compare the effects of
267 bovine/calf serum versus FF supplementaion to the maturation medium on embryo
268 development, quality, and transcriptomics.

Numerous studies attempted to prove the beneficial effect of the addition of FF to the maturation medium, but results remain highly variable [12-16]. A plethora of factors, including the composition of the maturation medium, the follicles' size, the percentage of FF, heat inactivation, and individual cow factors [12–16], may contribute to discrepancies among published results. Sena Lopes et al. [14] showed that heat inactivation of 10% FF has only subtle effects on the blastocyst rate. Still, the size of the follicles, the percentage of FF supplementation, and individual cow variations may play a major role. Folliculogenesis encompasses three stages of development: recruitment, selection, and dominance. Follicles in the recruitment (< 3mm) and selection (3 to 8 mm) stages are FSH dependent for their growth, and their intrafollicular concentration of estradiol is low. Moreover, during these stages, high intra-follicular cyclic adenosine monophosphate levels are vital for maintaining the oocyte in meiotic arrest (prophase I). Therefore, it is not surprising that the supplementation of FF derived from small follicles (2–5 mm) to the maturation medium resulted in a lower rate of maturation and blastocyst rates than the control [13]. On the other hand, the supplementation of FF derived from > 8 mm to the maturation medium substantially increases the day 8 blastocyst rate [13]. This is because the estradiol concentration, among other components, of dominant follicles (> 8 mm) is higher than in recruited and selected follicles. Interestingly, the addition of estradiol and FSH to the maturation medium improves the blastocyst development to over 50% [13]. Based on these results, we decided to collect FF derived from dominant follicles.

It is pivotal to mention that we used ovaries derived from the slaughterhouse. Therefore, we do not know the FF background at the individual cow level, which might be the main limitation of the present study. However, aging might not be considered a significant problem since the average lifespan of slaughtered cows, both Holstein and Belgian Blue, is around five years. Culling for uterine adhesions due to elective cesarean section in Belgian Blue cows will also happen at a relatively young age [42]. Negative energy balance and high-energy diet cause

294 hormonal and FF biochemical changes in high-yielding dairy cows [43,44]. However, the
295 incidence of high yielding dairy cows presented at the abattoir is rather low. Except for
296 uneconomical culling due to urgency (e.g., dystocia, severe mastitis, accidents, lameness), cows
297 are normally slaughtered toward the end of lactation [45]. Bias associated with the estrous cycle
298 stage was partially controlled by collecting follicles with > 12 mm diameter and the
299 measurement of the estradiol/progesterone ratio in the FF pools. The estradiol/progesterone
300 ratio was higher than 1 in both FF batches, indicating that on average, the FF originated from
301 active, dominant follicles. However, the progesterone concentration was greater in the second
302 than in the first batch. This is an crucial issue since it has been demonstrated that progesterone
303 may diffuse to the oil [46,47] and affect adjacent droplets (oocytes) in individual maturation
304 conditions. However, for this experiment, different FF concentration treatments were not mixed
305 in the same dish at any step of the IVP system; thus, progesterone diffusion may be discarded
306 as a cofounder among treatments. The difference in progesterone concentrations between
307 batches may be associated with the selection criteria of ovaries, which was done at the
308 individual ovarian level. It has been described that follicular (and luteal) dynamics of the two
309 ovaries act primarily as a single unit [48], and structures in either ovary may have affected the
310 FF steroid hormone concentration. This is a limitation of our study, and ideally, the selection
311 should have been done at both ovaries together. The presence of a cyst (e.g., follicular or luteal
312 cyst) may have also acted as a confounder, though the ovarian cyst incidence in beef cows or
313 Belgian Holstein cows is relatively low < 5% [49,50]. Moreover, in most cases, the presence of
314 follicular cyst inhibits the development of dominant follicles [51]. To somehow control these
315 limitations, we adopted two strategies: 1) pool the FF from a large number of ovaries, and 2)
316 perform the FF collection from 2 batches (or biological replicates). By pooling FF from a large
317 mix of ovaries (> 22 ovaries per batch), we diluted the chances of a potentially harmful effect
318 of specific individuals on our results. Additionally, by including two biological replicates and

319 using it as a random effect and offered as covariable to our models, we also corrected potential
320 individual variations. The lack of effect of the FF batch on the development or blastocyst quality
321 in group or individual culture conditions indicates robustness in our results.

322 We did not find an effect of 1% FF on embryo development, which confirmed the results
323 of other studies [13]. Regarding group culture, our blastocyst rates for 5% FF supplementation
324 are comparable to those achieved by Ali et al. [13]. However, the blastocyst rate in the control
325 group of Ali et al. [13] was only 30%, which is significantly lower than our control (~40% of
326 blastocyst rate). Carolan et al. [52] reported that 10% of FF supplementation resulted in a
327 similar blastocyst yield to control. Conversely, Ali et al. [13] showed that 10% of bovine FF
328 supplementation decreased the blastocyst rate. Our results resemble those obtained by Carolan
329 et al. [52], where the blastocyst rate in 10% FF was similar to control. We did not test
330 concentrations with >10% of FF because previous studies have demonstrated that it might be
331 detrimental rather than beneficial for further *in vitro* embryo development [15,16].

332 Embryos cultured in groups produce embryokines, which results in improved
333 developmental rates and embryo quality [53]. Such trophic stimulation results from autocrine
334 and paracrine signaling factors produced and released by embryos, which act upon the embryos
335 themselves or upon neighboring embryos [54-56]. However, individual culture systems lack
336 these factors [50]. To our knowledge, our study reports the highest blastocyst rate published to
337 date in a fully individual culture system in a complete serum-free setup (33% of blastocyst rate
338 in 5% FF supplemented oocytes). Importantly, these results were obtained among 14 replicates
339 from two FF batches, which further supports the reliability and robustness of our data. These
340 results might be considered as a first step to optimize the individual production of blastocysts.
341 The advantages of single embryo culture include the ability to track and monitor individual
342 oocytes and embryos. Under experimental conditions, this would allow specific data collection
343 of additional oocyte or embryo metabolism parameters. Moreover, the secretome of individual

344 oocytes or embryos could be monitored during development, which may lead to identifying
345 biomarkers that can be used routinely to assess embryo quality, increasing IVP efficiency and
346 pregnancy success after embryo transfer. Notwithstanding, it is essential to consider that FF is
347 an undefined supplement and may represent a health risk due to sanitary issues, mainly because
348 there was no FF heat inactivation in our study.

349 The proportion of ICM/TCN and AC/TCN is a crucial indicator of embryo quality [57].
350 A substantial percentage (approximately 32%) of *in vivo* produced blastocysts is occupied by
351 the ICM [58], and the viability of the future conceptus is a crucial embryo quality parameter.
352 We only obtained modest results in embryo quality aspects with 5 or 10% of FF in the group
353 culture system. However, 5% of FF in individual culture conditions boosted the ICM/TCN ratio
354 and substantially decreased the AC/TCN ratio. These results could be associated with the
355 greater availability of nutrients in FF supplemented oocytes, including essential amino acids
356 such as L-alanine and glycine, as previously speculated by Cruz et al. [15]. The availability of
357 nutrients may have also played a role in the cumulus expansion. An adequate cumulus
358 expansion during IVM is a vital indicator of oocyte viability and competence [59,60], and in
359 coincidence with embryo development and quality, the best results for cumulus expansion were
360 found by supplementing the maturation medium with 5% FF. Collectively, our data support
361 that 5% of FF may provide the ideal concentration of molecules (e.g., amino acids, extracellular
362 vesicles, miRNA, steroids, and growth factors), which resulted in greater cumulus expansion
363 and the subsequent production of embryos of high quality for individual culture conditions.

364 5. Conclusion

365 We demonstrated that 5% FF supplementation from dominant follicles (12 to 20 mm) in
366 a serum-free individual embryo production system improved the cumulus expansion and
367 increased the embryo development and quality. The group culture system did not benefit from
368 the addition of FF to the maturation media, except for some aspects of embryo quality

369 parameters, which slightly improved with the addition of 5 or 10% of FF to the maturation
370 medium. Results presented in this study are the highest in embryo development in a complete
371 serum-free setup under fully individual culture conditions. The ability to track and monitor
372 individual embryonic development is of great interest for identifying markers associated with
373 embryo viability and quality. Thus, the results presented here might be considered as a first step
374 to optimize the individual production of blastocysts that can be adopted in more fundamental
375 experiments.

376 **CRediT authorship contribution statement**

377 N.A.D. performed the laboratory work for all the experiments and wrote the first draft of
378 the manuscript. A.R., K.P., A.A., D.A.V., and P.V.D. assisted with the experiments. N.A.D.,
379 O.B.P., and A.V.S. designed the experiment. A.V.S. provided the funding. O.B.P. analyzed the
380 data. O.B.P., J.L.M.R.L., and A.V.S. helped with the drafting of the manuscript. All authors
381 read the final draft and agreed to its submission for publication.

382 **Declaration of competing interest**

383 The authors have no conflicts of interest.

384 **Acknowledgments**

385 This project was funded by by "BOF GOA 2018000504 (GOA030-18 BOF) Ex Ovo".
386 Krishna Chaitanya Pavani and Osvaldo Bogado Pascottini were funded by Fonds voor
387 Wetenschappelijk Onderzoek–Vlaanderen (FWO, Research Foundation, Flanders) under
388 project numbers 1228821N and 12Y5220N, respectively.

389 **References**

390 [1] Lonergan P, Rizos D, Gutierrez-Adan A, Fair T, Boland MP. **Oocyte and embryo**
391 **quality: effect of origin, culture conditions and gene expression patterns**. Reprod Domest
392 Anim 2003;38:259-67. doi:10.1046/j.1439-0531.2003.00437.x.

393 [2] Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. **Consequences of bovine oocyte**
394 **maturation, fertilization or early embryo development in vitro versus in vivo:**
395 **Implications for blastocyst yield and blastocyst quality**. Mol Reprod Dev 2002;61:234-48.
396 https://doi.org/10.1002/mrd.1153.

397 [3] Wydooghe E, Vaele L, Piepers S, Dewulf J, Van Den Abbeel E, De Sutter P, et al.
398 **Individual commitment to a group effect: Strengths and weaknesses of Bovine embryo**
399 **group culture**. Reproduction 2014;148:519-29. https://doi.org/10.1530/REP-14-0213.

400 [4] Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL, Schoolcraft WB. **Oocyte**
401 **environment: Follicular fluid and cumulus cells are critical for oocyte health**. Fertil Steril
402 2015;103:303-16. https://doi.org/10.1016/j.fertnstert.2014.11.015.

403 [5] Gilchrist RB, Lane M, Thompson JG. **Oocyte-secreted factors: Regulators of cumulus**
404 **cell function and oocyte quality**. Hum Reprod Update 2008;14:159-77.
405 https://doi.org/10.1093/humupd/dmm040.

406 [6] Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. **Manipulation of**
407 **Follicular Development to Produce Developmentally Competent Bovine Oocytes**. Biol
408 Reprod 2002;66:38-43. https://doi.org/10.1095/biolreprod66.1.38.

409 [7] Sirard MA, Blondin P. **Oocyte maturation and IVF in cattle**. Anim Reprod Sci
410 1996;42:417-26, https://doi.org/10.1016/0378-4320(96)01518-7.

411 [8] Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. **Follicular**
412 **fluid content and oocyte quality: from single biochemical markers to metabolomics**.
413 Reprod Biol Endocrinol 2009;7:40. doi:10.1186/1477-7827-7-40.

414 [9] Driancourt MA, Thuel B. **Control of oocyte growth and maturation by follicular cells**
415 **and molecules present in follicular fluid**. A review. Reprod Nutr Dev 1998;38;345-62.
416 https://doi.org/10.1051/rnd:19980401

417 [10] Harper KM, Brackett BG. **Bovine blastocyst development after in vitro maturation in**
418 **a defined medium with epidermal growth factor and low concentrations of**
419 **gonadotropins**. Biol Reprod 1993;48:409-16. doi: 10.1095/biolreprod48.2.409.

420 [11] Wang S, Liu Y, Holyoak GR, Bunch TD. **The effects of bovine serum albumin and**
421 **fetal bovine serum on the development of pre- and postcleavage-stage bovine embryos**
422 **cultured in modified CR2 and M199 media**. Anim Reprod Sci 1997;48:37-45.
423 https://doi.org/10.1016/S0378-4320(97)00041-9.

424 [12] Somfai T, Inaba Y, Watanabe S, Geshi M, Nagai T. **Follicular fluid supplementation**
425 **during in vitro maturation promotes sperm penetration in bovine oocytes by enhancing**
426 **cumulus expansion and increasing mitochondrial activity in oocytes**. Reprod Fertil Dev
427 2012;24:743-52. https://doi.org/10.1071/RD11251.

428 [13] Ali A, Coenen K, Bousquet D, Sirard MA. **Origin of bovine follicular fluid and its**
429 **effect during in vitro maturation on the developmental competence of bovine**
430 **oocytes**.Theriogenology 2004;62:1596-606.
431 https://doi.org/10.1016/j.theriogenology.2004.03.011.

432 [14] Lopes JS, Canha-Gouveia A, París-Oller E, Coy P. **Supplementation of bovine**
433 **follicular fluid during in vitro maturation increases oocyte cumulus expansion,**
434 **blastocyst developmental kinetics, and blastocyst cell number**. Theriogenology 2019
435 126:222-9. https://doi.org/10.1016/j.theriogenology.2018.12.010.

436 [15] Cruz MHC, Saraiva NZ, da Cruz JF, Oliveira CS, Del Collado M, Fernandes H,
437 et al. **Effect of follicular fluid supplementation during in vitro maturation on**
438 **total cell number in bovine blastocysts produced in vitro**. Rev Bras Zootec 2014;43:120-6.
439 https://doi.org/10.1590/S1516-35982014000300003.

440 [16] Avery B, Strobech L, Jacobsen T, Bogh I, Greve T. **In vitro maturation of bovine**
441 **cumulus-oocyte complexes in undiluted follicular fluid effect on nuclear maturation,**
442 **pronucleus formation and embryo development**. Theriogenology 2003;59:987-99.
443 https://doi.org/10.1016/s0093-691x(02)01139-1.

444 [17] Tesarik J, Mendoza C. **Nongenomic effects of 17 beta-estradiol on maturing human**
445 **oocytes: relationship to oocyte developmental potential**. J Clin Endocrinol Metab 1995
446 80:1438-43. doi: 10.1210/jcem.80.4.7714121. PMID: 7714121.

447 [18] Gupta S, Choi A, Yu HY, Czerniak SM, Holick EA, Paolella LJ, et al. **Fluctuations in**
448 **total antioxidant capacity, catalase activity and hydrogen peroxide levels of follicular**
449 **fluid during bovine folliculogenesis**. Reprod Fertil Dev 2011;23:673-80.
450 https://doi.org/10.1071/RD10270.

451 [19] Tesarik J , Mendoza C. **Direct Non-Genomic Effects of Follicular Steroids on**
452 **Maturing Human Oocytes: Oestrogen Versus Androgen Antagonism**. Hum Reprod
453 Update 1997;3:95-100. https://doi.org/10.1093/humupd/3.2.95.

454 [20] Botero-Ruiz W, Laufer N, DeCherney AH, Polan ML, Haseltine FP, Behrman HR. **The**
455 **relationship between follicular fluid steroid concentration and successful fertilization of**
456 **human oocytes in vitro**. Fertil Steril 1984;41:820-6. https://doi.org/10.1016/s0015-
457 0282(16)47892-1.

458 [21] Kreiner D, Liu HC, Itskovitz J, Veeck L, Rosenwaks Z. **Follicular fluid estradiol and**
459 **progesterone are markers of Preovulatory oocyte quality**. Fertil Steril 1987;48:991-4.
460 https://doi.org/10.1016/S0015-0282(16)59597-1.

461 [22] Goovaerts IGF, Leroy JLMR, Van Soom A, De Clercq JBP, Andries S, Bols PEJ. **Effect**
462 **of cumulus cell coculture and oxygen tension on the in vitro developmental competence**

463 **of bovine zygotes cultured singly**. Theriogenology 2009;71:729-38.
464 https://doi.org/10.1016/j.theriogenology.2008.09.038.

465 [23] Takeo S, Kimura K, Shirasuna K, Kuwayama T, Iwata H. **Age-Associated deterioration**
466 **in follicular fluid induces a decline in bovine oocyte quality**. Reprod Fertil Dev
467 2017;29:759-67. https://doi.org/10.1071/RD15228.

468 [24] Richards JS. **Maturation of ovarian follicles: actions and interactions of pituitary**
469 **and ovarian hormones on follicular cell differentiation**. Physiol Rev 1980;60:51-89. doi:
470 10.1152/physrev.1980.60.1.51.

471 [25] Pascottini OB, Catteeuw M, Van Soom A, Opsomer G. **Holding immature bovine**
472 **oocytes in a commercial embryo holding medium: high developmental competence for**
473 **up to 10 h at room temperature**. Theriogenology 2018;107:63-9.
474 https://doi.org/10.1016/j.theriogenology.2017.10.040.

475 [26] Pavani K.C, Hendrix A, Van Den Broeck W, Couck L, Szymanska K, Lin X, et al.
476 **Isolation and characterization of functionally active extracellular vesicles from culture**
477 **medium conditioned by bovine embryos in vitro**. Int J Mol Sci 2018;20:38.
478 https://doi.org/10.3390/ijms20010038.

479 [27] Bunel A, Jorssen EP, Merckx E, Leroy JL, Bols PE, Sirard MA. **Individual bovine in**
480 **vitro embryo production and cumulus cell transcriptomic analysis to distinguish**
481 **cumulus-oocyte complexes with high or low developmental potential.** Theriogenology
482 2015;83:228-37. https://doi.org/10.1016/j.theriogenology.2014.09.019.

483 [28] O'Doherty EM, Wade MG, Hill JL, Boland MP. **Effects of culturing bovine oocytes**
484 **either singly or in groups on development to Blastocysts**. Theriogenology 1997;48:161-69.
485 doi:10.1016/S0093-691X (97)00199-4.

486 [29] Nishio M, Hoshino Y, Tanemura K, Sato E. **Effect of single-oocyte culture system on**
487 **in vitro maturation and developmental competence in mice**. Reprod Med Biol
488 2014;13:153-59. doi:10.1007/s12522-014-0177-1.

489 [30] Wise T. **Biochemical analysis of bovine follicular fluid: albumin, total protein,**
490 **lysosomal enzymes, ions, steroids and ascorbic acid content in relation to follicular size,**
491 **rank, atresia classification and day of estrous cycle**. J Anim Sci 1987;64:1153-69.
492 https://doi.org/10.2527/jas1987.6441153x.

493 [31] Badinga L, Driancourt MA, Savio JD, Wolfenson D, Drost M, De La Sota RL, et al.
494 **Endocrine and ovarian responses associated with the first-wave dominant follicle**. Biol
495 Reprod 1992;47:871-83. doi: 10.1095/biolreprod47.5.871.

496 [32] Landau S, Braw-Tal R, Kaim M, Bor A, Bruckental I. **Preovulatory follicular status**
497 **and diet affect the insulin and glucose content of follicles in high-yielding dairy cows**.
498 Anim Reprod Sci 2000;64:181-97. doi: 10.1016/s0378-4320(00)00212-8.

499 [33] Wydooghe E, Heras S, Dewulf J, Piepers S, Van den Abbeel E, De Sutter P, et al.
500 **Replacing serum in culture medium with albumin and insulin, transferrin and selenium**
501 **is the key to successful bovine embryo development in individual culture.** Reprod Fertil
502 Dev 2014;26:717-24. https://doi.org/10.1071/RD13043.

503 [34] Abramoff MD, Magelhaes PJ, Ram SJ. **Image processing with ImageJ**. Biophoton Int
504 2004;11:36-42.

505 [35] Wydooghe E, Vandaele L, Beek J, Favoreel H, Heindryckx B, De Sutter P, et al.
506 **Differential apoptotic staining of mammalian blastocysts based on double**
507 **immunofluorescent CDX2 and active caspase-3 staining**. Anal Biochem 2011;416:228-30.
508 https://doi.org/10.1016/j.ab.2011.05.033.

509 [36] Park KW, Iga K, Niwa K. **Exposure of bovine oocytes to EGF during maturation**
510 **allows them to develop to blastocysts in a chemically-defined medium**. Theriogenology
511 1997;48:1127-35. https://doi.org/10.1016/S0093-691X(97)00345-2.

512 [37] Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. **Sex determines**
513 **the expression level of one third of the actively expressed genes in bovine blastocysts**.
514 Proc Natl Acad Sci Unit States Am 2010;107:3394-9.10.1073/pnas.0913843107.

515 [38] Chitwood JL, Rincon G, Kaiser GG, Medrano JF, Ross PJ. **RNA-seq analysis of single**
516 **bovine blastocysts**. BMC Genom 2013;14:350.

517 [39] Hagemann U, Weilert LL, Beaumont SE, Tervit HR. **Development of bovine embryos**
518 **in single in vitro production (sIVP) systems**. Mol Reprod Dev 1998;51:143-7.
519 https://doi.org/10.1002/(SICI)1098-2795(199810)51:2%3C143::AID-MRD3%3E3.0.CO;2-Q.

520 [40] Carolan C, Lonergan P, Khatir H, Mermillod P. **In vitro production of bovine embryos**
521 **using individual oocytes**. Mol Reprod Dev 1996;45:145-50.
522 https://doi.org/10.1002/(SICI)1098-2795(199610)45:2%3C145::AID-MRD6%3E3.0.CO;2-T.

523 [41] Heras S, De Coninck DI, Van Poucke M, Goossens K, Pascottini OB, Van Nieuwerburgh
524 F, et al. **Suboptimal culture conditions induce more deviations in gene expression in male**
525 **than female bovine blastocysts**. BMC Genom 2016;17:72. DOI 10.1186/s12864-016-2393-
526 z.

527 [42] Kolkman I, De Vliegher S, Hoflack G, Van Aert M, Laureyns J, Lips D, et al. **Protocol**
528 **of the Caesarean section as performed in daily bovine practice in Belgium.** Reprod
529 Domest Anim 2007;42:583-9. doi: 10.1111/j.1439-0531.2006.00825.x. PMID: 17976064.

530 [43] Boland MP, Lonergan P, O'Callaghan D. **Effect of nutrition on endocrine parameters,**
531 **ovarian physiology, and oocyte and embryo development**. Theriogenology 2001;55:1323-
532 40. doi: 10.1016/s0093-691x(01)00485-x.

533 [44] Walters AH, Pryor AW, Bailey TL, Pearson RE, Gwazdauskas FC. **Milk yield, energy**
534 **balance, hormone, follicular and oocyte measures in early and mid-lactation Holstein**
535 **cows**. Theriogenology 2002;57:949-61. https://doi.org/10.1016/S0093-691X(01)00688-4.

536 [45] Bazzoli I, De Marchi M, Cecchinato A, Berry DP, Bittante G. **Factors associated with**
537 **age at slaughter and carcass weight, price, and value of dairy cull cows**. J Dairy Sci
538 2014;97:1082-91. https://doi.org/10.3168/jds.2013-6578.

539 [46] Clemente M, de La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D,
540 Lonergan P. **Progesterone and conceptus elongation in cattle: a direct effect on the**
541 **embryo or an indirect effect via the endometrium?** Reproduction 2009;138:507–17.
542 https://doi.org/10.1530/REP-09-0152.

543 [47] Shimada M, Kawano N, Terada T. **Delay of nuclear maturation and reduction in**
544 **developmental competence of pig oocytes after mineral oil overlay of in vitro maturation**
545 **media.** Reproduction 2002;124:557-64. 10.1530/rep.0.1240557.

546 [48] Adams GP, Jaiswal R, Singh J, Malhi P. **Progress in understanding ovarian follicular**
547 **dynamics in cattle**. Theriogenology 2008;69:72-80.
548 https://doi.org/10.1016/j.theriogenology.2007.09.026.

549 [49] Opsomer G, Grohn YT, Hertl J, Coryn M, Deluyker H, de Kruif A. **Risk factors for post**
550 **partum ovarian dysfunction in high producing dairy cows in Belgium: a field study**.
551 Theriogenology 2000;53:841-57. https://doi.org/10.1016/S0093-691X(00)00234-X.

552 [50] Crowe MA, Diskin MG, Williams EJ. **Parturition to resumption of ovarian cyclicity:**
553 **comparative aspects of beef and dairy cows**. Animal 2014;8:40-53.
554 https://doi.org/10.1017/S1751731114000251.

555 [51] Hatler TB, Hayes SH, Laranja da Fonseca LF, Silvia WJ. **Relationship between**
556 **endogenous progesterone and follicular dynamics in lactating dairy cows with ovarian**
557 **follicular cysts**. Biol Reprod 2003;69:218-23. 10.1095/biolreprod.102.012179.

558 [52] Carolan C, Lonergan P, Monget P, Monniaux D, Mermillod P. **Effect of follicle size and**
559 **quality on the ability of follicular fluid to support cytoplasmic maturation of bovine**
560 **oocytes**. Mol Reprod Dev 1996;43:477-83. doi: 10.1002/(SICI)1098-
561 2795(199604)43:4<477:AID-MRD10>3.0.CO;2-X.

562 [53] Wydooghe E, Vandaele L, Heras S, De Sutter P, Deforce D, Peelman L,et al. **Autocrine**
563 **embryotropins revisited: how do embryos communicate with each other in vitro when**
564 **cultured in groups?** Biol Rev Camb Philos Soc 2017;92:505-20.
565 doi:10.1111/brv.12241.Epub 2015 Nov 26. PMID: 26608222.

566 [54] Stokes PJ, Abeydeera LR, Leese HJ. **Development of porcine embryos in vivo and in**
567 **vitro; evidence for embryo' cross talk' in vitro**. Dev Biol 2005;284:62-71. doi:
568 10.1016/j.ydbio.2005.05.001.

569 [55] Gopichandran N, Leese HJ. **The effect of paracrine/autocrine interactions on the *in***
570 ***vitro* culture of bovine preimplantation embryos**. Reproduction 2006;131:269-77. doi:
571 10.1530/rep.1.00677.

572 [56] O'Neill C. **The potential roles for embryotrophic ligands in preimplantation embryo**
573 **development**. Hum Reprod Update 2008b;14:275-88. doi: 10.1093/humupd/dmn002.

574 [57] Knijn HM, Gjørret JO, Vos PL, Hendriksen PJ, Van der Weijden BC, et al.
575 **Consequences of In Vivo Development and Subsequent Culture on Apoptosis, Cell**

576 **Number, and Blastocyst Formation in Bovine Embryos**. Biol Reprod 2003;69:1371-8.
577 https://doi.org/10.1095/biolreprod.103.017251.

578 [58] Van Soom A, Boerjan ML, Bols PE, Vanroose G, Lein A, Coryn M, et al. **Timing of**
579 **compaction and inner cell allocation in bovine embryos produced in vivo after**
580 **superovulation**. Biol Reprod 1997;57:1041-9. https://doi.org/10.1095/biolreprod57.5.1041.

581 [59] Chen L, Russell PT, Larsen WJ. **Functional significance of cumulus expansion in the**
582 **mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass**.
583 Mol Reprod Dev 1993;34:87-93. doi: 10.1002/mrd.1080340114. PMID: 8418823.

584 [60] Furnus CC, de Matos DG, Moses DF. **Cumulus expansion during in vitro maturation**
585 **of bovine oocytes: relationship with intracellular glutathione level and its role on**
586 **subsequent embryo development**. Mol Reprod Dev 1998;51:76-83. doi:
587 10.1002/(SICI)1098-2795(199809)51:1<76::AID-MRD9>3.0.CO;2-T. PMID: 9712320.

Highlights

- Five percent FF supplementation in an individual embryo production system improved the cumulus expansion and increased embryo development and quality.

- The blastocyst production in group culture system did not benefit from the addition of FF to the maturation media.

- Results presented in this study are the highest in embryo development under fully individual culture conditions.

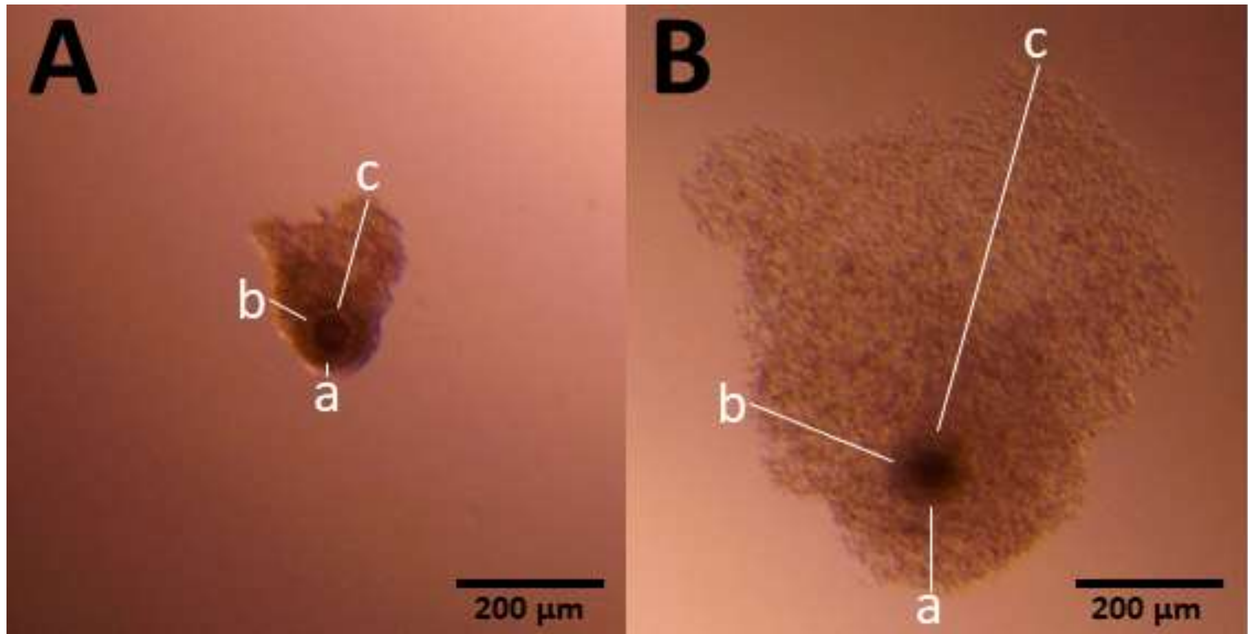

Figure 1. The cumulus-oocyte cells (COCs) expansion was measured at three places: shortest (a), medium (b), and the longest (c) distance between the zona pellucida and the extreme of the cumulus cells. Pictures of COCs were taken prior to (A) and after 22 h (B) maturation.

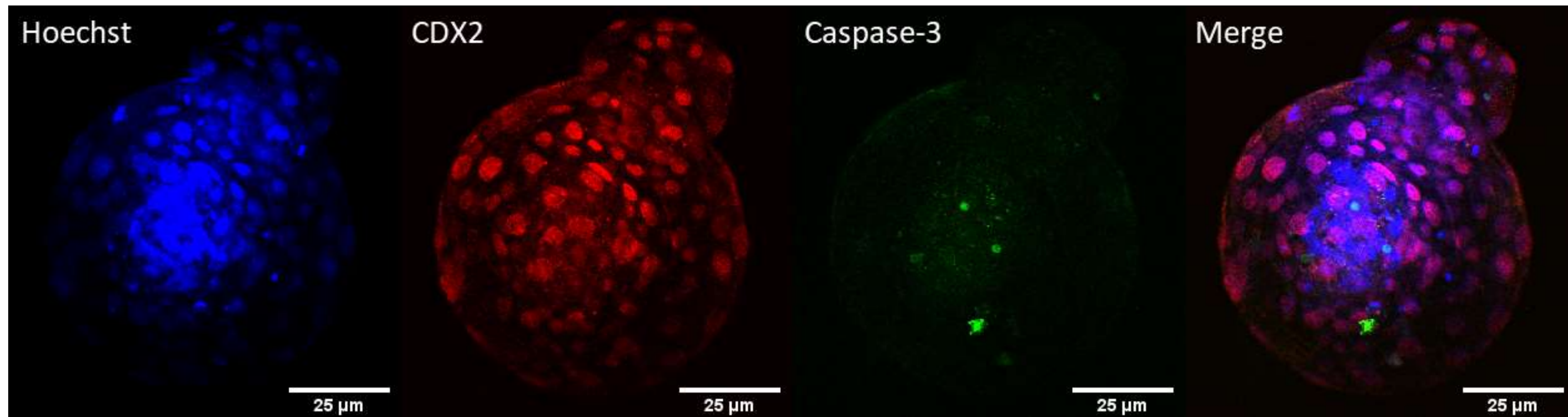

Figure 2. Differential-apoptotic stained was performed to assess the day 8 blastocysts quality. Hoechst 33342 stained all the blastomeres in blue. CDX2 uniquely stained the trophectoderm cells in red. Caspase-3 marked all apoptotic cells in green. Pictures were acquired from an individually cultured blastocyst (control group).

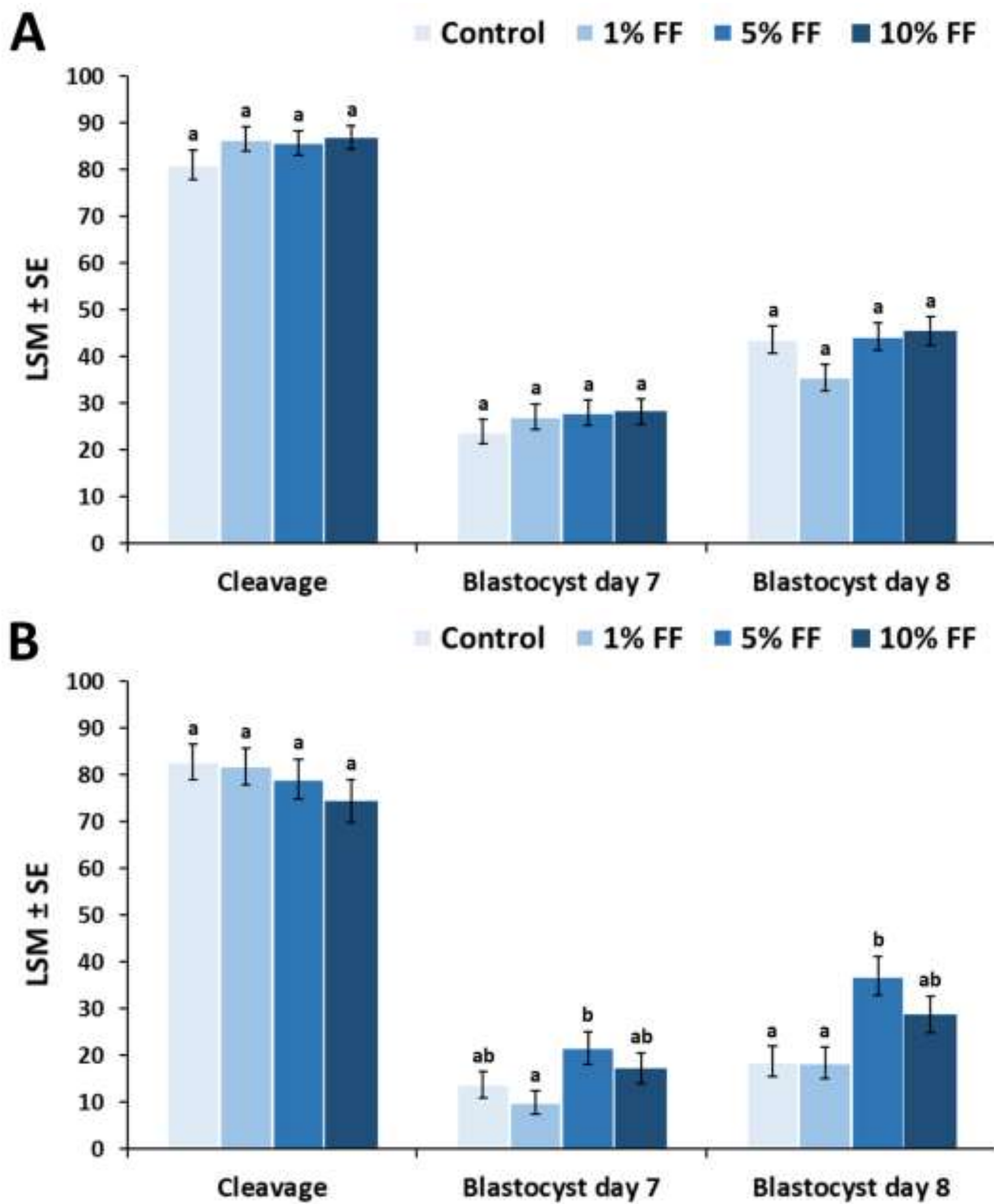

Figure 3. Cleavage, day 7, and day 8 blastocyst rates expressed as a percentage from presumed zygotes. Blastocysts were *in vitro* produced in groups (A; above, n = 1,056) and individually (B; below, n = 567). Maturation media was supplemented with 0 (control), 1, 5, and 10% follicular fluid (FF). Different superscripts (a and b) represent statistical differences ($P < 0.05$) among groups. Results are expressed as least square means ± standard error (LSM ± SE).

Table 1. Total cell number (TCN), trophectoderm cells (TE), inner cell mass (ICM), apoptotic cells (AC), ICM/TCN ratio, and AC/TCN ratio of day 8 blastocyst differentially stained. Blastocysts were *in vitro* produced in groups (n = 338) and individually (n = 248). Maturation media were supplemented with 0 (control), 1, 5, and 10% of follicular fluid (FF). Different superscripts per column (a, b, and c) represent statistical differences ($P < 0.05$) among groups. Results are expressed as least square means ± standard error.

Treatment	No. of blastocysts	Cell numbers				ICM/TCN ratio	AC/TCN ratio
		TCN	ICM	TE	AC		
Control group	91	152 ± 2.9[a]	73.9 ± 2.5[a]	77.1 ± 1.1[a]	4.5 ± 0.1[a]	47.5 ± 0.9[a]	3.0 ± 0.1[a]
1% FF group	70	135 ± 3.2[b]	56.4 ± 2.7[b]	78.3 ± 1.2[a]	4.4 ± 0.1[a]	40.7 ± 0.9[b]	3.3 ± 0.1[a]
5% FF group	94	164 ± 2.9[ac]	83.9 ± 2.4[c]	80.5 ± 1.1[a]	4.2 ± 0.1[a]	50.4 ± 0.8[a]	2.5 ± 0.1[b]
10% FF group	83	157 ± 3.1[c]	69.4 ± 2.5[a]	87.8 ± 1.1[b]	3.6 ± 0.1[b]	43.1 ± 0.9[b]	2.3 ± 0.1[b]
Control individual	61	98.0 ± 1.9[a]	36.9 ± 1.5[a]	61.1 ± 1.6[a]	2.7 ± 0.1[a]	37.4 ± 1.2[a]	0.5 ± 0.1[ab]
1% FF individual	50	87.6 ± 2.1[b]	36.3 ± 1.6[b]	50.7 ± 1.4[a]	2.7 ± 0.2[a]	41.7 ± 1.3[a]	0.5 ± 0.1[a]
5% FF individual	76	112 ± 1.7[c]	53.4 ± 1.3[a]	59.2 ± 1.4[b]	2.5 ± 0.1[a]	47.2 ± 1.1[b]	0.5 ± 0.1[b]
10% FF individual	61	100 ± 1.9[c]	40.3 ± 1.5[a]	60.6 ± 1.6[a]	3.1 ± 0.1[a]	39.7 ± 1.2[a]	0.6 ± 0.1[a]