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Title: Supplementation of bovine follicular fluid during *in vitro* maturation increases oocyte cumulus expansion, blastocyst developmental kinetics, and blastocyst cell number.

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Abstract

Bovine follicular fluid (bFF) is the natural milieu for oocyte growth and development. However, its value as supplementation to *in vitro* maturation medium is still questioned due to inconsistent results. In this study we hypothesized that adding 10% of follicular fluid as well as heat treating it to inhibit the complement system, would produce higher quality embryos. To do so, experiments were conducted to compare the effect of bFF and heat-treated bFF (bFFin) on oocyte competence assessed by different parameters such as nuclear and cytoplasmic maturation, IVF efficiency, in vitro embryo development and embryo survivability post-vitrification. No differences on nuclear maturation nor cortical granules migration were observed but differences were found on oocyte's cumulus cell expansion, with bFF group having the highest increase (79.0±3.7%). bFFin had a negative impact on IVF efficiency (58.6±3.2%), but no differences were found between bFF (62.9±3.2%) and control (72.8±3.0%). Although the cleavage and blastocyst rate were similar between groups, the day 6 embryo development rate was higher in bFFin group, suggesting an accelerated developmental kinetics. Hatched blastocysts from the bFF group showed a higher cell count than the control group (241.3±20.1 and 185.8± 10.0, respectively), and bFFin embryos showed values in between (214.9±14.0). No difference on survivability post-vitrification was found between groups, although the blastocyst stage had a significant impact on the survival rate across all groups. In conclusion, using bFF as supplementation to maturation medium showed a higher benefit when comparing to the standard supplementation by having oocytes with higher cumulus expansion rate,

faster development of embryos and higher number of cells per embryo. Inactivation of bFF lowered IVF efficiency but didn't compromise blastocyst development and quality.

Keywords

Bovine follicular fluid; maturation; heat-inactivation; embryo; vitrification

1. Introduction

Four decades ago, the birth of the first *in vitro* fertilization (IVF) derived calf [1] opened the doors to a commercially driven desire to produce fast and low-cost embryos while also overcoming infertility problems of high genetic merit animals. However, this calf was not entirely *in vitro* produced, since *in vivo* matured oocytes were used. Since then, an extensive and comprehensive research focused on *in vitro* production of embryos has been conducted [2]. It was not until 1987 that Fukuda *et al.*[3] were able to finally perform *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC), followed by embryo transfer of fresh and frozen-thawed embryos, pregnancy to term and live offspring. Although several attempts to improve the efficiency of *in vitro* maturation, fertilization and culture have been performed, the outcome (viable blastocyst formation) remains unsatisfactory, being around 30-40% [4–6].

The quality or competence of the female gamete plays a key role in determining the blastocyst yield [4,7] and that feature can be assessed by its ability to achieve nuclear maturation together with cytoplasmic maturation [5]. Nuclear maturation refers to the capacity of the oocyte to resume meiosis. However, it is not enough to name an oocyte as competent by its nuclear status (reviewed by [7]), otherwise the difference in embryo quality found between *in vivo* or *in vitro* matured oocytes used for IVF, would not be observed [4,5,8,9]. Even if what defines cytoplasmic maturity in the oocyte is not yet completely understood, some features have been associated with this type of maturation such as: changes in the organelles (like mitochondria and cortical granules migration), accumulation of specific molecules (carbohydrate, lipids, mRNA and other proteins), the grade of communication between cumulus cells and the oocyte, that has influence on the meiotic maturation and metabolism of the oocyte, as well as enhances further development and promotes sperm capacitation [10–12] and even the secretion of oocyte-derived growth factors [7,13–15].

Among the strategies to reach an optimal maturation of bovine oocytes *in vitro*, the addition of follicular fluid to the maturation media seems a plausible option since it is the natural milieu for the oocytes in the ovarian follicle. This fluid contains proteins, cytokines, growth factors, steroids, metabolites and other undefined factors [16].There have been several reports [17–27] showing the effect of the follicular fluid on IVM, IVF and blastocyst rate but the results aren't coherent. Kim *et al.* [25] used 10, 30 and 60% bovine follicular fluid (bFF) and the data showed that lower concentrations resulted in higher number of cells in blastocysts when compared to control and that the higher concentration (60%) decreased blastocyst rate, suggesting that high concentrations of bFF might have an inhibitory effect over meiosis. Cruz *et al.* [22], using 0, 25, 50, 75 and 100% bFF in IVM medium, demonstrated that 50% of bFF increases the inner cell mass number count, but in these experiments 10% of fetal bovine serum was present in all media and could have a confounding effect on the interpretation of these results. Somfai *et al.* [21] compared the effects of supplementation between polyvinylpyrrolidone, bovine serum albumin (BSA), calf serum or bFF at 5%, and the results showed that follicular fluid supplementation promotes sperm penetration by enhancing cumulus expansion and ATP levels in oocytes, though blastocyst rate remained the same.

One of the issues brought up by researchers on the use of bFF is the inhibitory effect on the resumption of meiosis [25], even when collecting with heparin. Heat-treatment of bFF was used in other experiments as a way to inhibit the complement system, arguing that this could be the factor causing the maturation problems, but either the concentration used was too high [27], or there was no comparison with untreated bFF [19].

Current standard IVM protocols from different laboratories [28–30] still include 10% Fetal bovine/calf serum in the maturation medium. Different studies recently published suggesting that the use of natural fluids during the *in vitro* procedures could help producing embryos with less epigenetic and gene expression aberrations than standard protocols [29,31]. Bearing this in mind, we hypothesize that adding the natural fluid where *in vivo* maturation occurs (bFF), in absence of bovine serum, would

produce better results. Additionally, the comparison with the heat-treated bFF would allow us to clarify if inhibition of the complement system is necessary and beneficial.

To test our hypothesis, we conducted three experiments to evaluate the effect of bFF (either heat inactivated or not) on oocyte competence assessed by different parameters related to nuclear and cytoplasmic maturation, followed by IVF efficiency, *in vitro* embryo development and survivability post-vitrification.

2. Material and methods

All chemicals were purchased from Sigma-Aldrich Chemical Company (Madrid, Spain), unless otherwise indicated.

2.1 Oocyte collection

Ovaries collected at a local slaughterhouse (Matadero Orihuela, S.A., Murcia - Spain), from postpubertal crossbred beef cattle, were transported in a thermo-flask with saline solution (0.9% wt/vol, supplemented with 100 mg/L of kanamycin sulfate) less than 2 hours after slaughter. Arrival temperature ranged between 32 and 35ºC and ovaries were washed with saline solution two times prior to use. Follicles between 2 and 8 mm were aspirated using a 10mL syringe coupled with an 18ga needle. Cumulus-oocyte-complexes (COC) were rinsed three times in collection medium, consisting of TCM-199 (with Hanks' salts) supplemented with 4.2mM sodium bicarbonate, 10mM HEPES, 2mM glutamine, 1% wt/v polyvinyl alcohol (PVA), 50IU/mL penicillin and 50µg/mL streptomycin. Only COCs with compact cumulus cells and uniform ooplasm were selected.

2.2 Bovine Follicular Fluid

The bFF samples (NaturARTs Cow BFF) were purchased from Embryocloud (Murcia, Spain). According to the manufacturer´s information, bFF was obtained by aspiration of superficial follicles with size between 4-7mm of abattoir ovaries, from animals at the early follicular stage of the estrus cycle. The fluid was centrifuged (3000 rpm, 30min at 4ºC), the supernatant was aspirated, filtered in sequence with 0.45µm and 0.22µm filters, aliquoted and stored at -20ºC until selling. The same batch was used for all

experiments. Quality control of the batch including endotoxin tests was performed by the manufacturer.

Heat-Inactivation of follicular fluid was performed using a thermoblock set at 56ºC for 30 min, immediately prior to use.

2.3 Oocyte maturation

COC's previously collected were washed twice in maturation medium, consisting of TCM-199 (with Earle's salts), supplemented with 26.2mM sodium bicarbonate, 0.2mM sodium pyruvate, 2mM glutamine,50µg/mL gentamycin, 10IU/mL equine chorionic gonadotropin (Foligon; Intervet International BV, Netherlands) and 10IU/mL human chorionic gonadotropin (Veterin Corion; Divasa Farmavic, Spain), divided in groups of 30 to 50 and transferred to four-well dishes (Nunc®, cell culture tested) in a total volume of 500µl, with further supplementation, in a concentration of 10% (v/v), of either fetal bovine serum (FBS, control), bovine follicular fluid (bFF) or bovine follicular fluid heat-inactivated (bFFin). Incubation took place at 38.5ºC, humidified atmosphere with 5% CO_2 for 22 to 24h. After maturation, COCs were either denuded with gentle pipetting in Dulbecco's phosphate-buffered saline (DPBS) and hyaluronidase (0.2% in DPBS) for experiment 1, or transferred to IVF medium for experiment 2 and 3. For nuclear maturation assessment, oocytes were considered matured when the first polar body was visible and the metaphase plate was present. A total of 846 oocytes in 8 replicates were used for experiment 1.

2.4 Cumulus Oocyte Complex expansion measurement

This evaluation was done according to the methods described by Romero-Aguirregomezcorta [32]. Photographs of the oocytes were taken using a Nikon SMZ-10A stereomicroscope under X10 magnification, before incubation and after incubation. Measurements were performed using ImageJ software (http://imagej.nih.gov/ij), and a mean of three measurements of each oocyte (longest, shortest and a medium distance between the *zona pellucida* and the extreme of the cumulus cells) was calculated. Results represent the percentage of increment of cumulus cells measurements from time 0 to 24h. Three replicates were measured with a total of 386 oocytes examined.

148 *2.5 In vitro fertilization*

149 Thirty minutes before IVF, COCs were rinsed twice and transferred to Fert-TALP
150 medium [33] - supplemented with 2µg/mL heparin and 50µg/mL gentamycin. Frozen
151 semen from two bulls with known fertility was used. Thawing was performed in a
152 water bath at 38ºC for 30sec, and the semen was transferred to a 15mL Falcon tube
153 with 10mL Sperm-TALP medium ([34] with modifications -114 mM sodium chloride,
154 3.2mM potassium chloride, 0.5mM magnesium chloride hexahydrate, 10.0 sodium
155 lactate) supplemented with 50µg/mL gentamycin, and incubated at 38ºC for 10 min.
156 Sperm was centrifuged at room temperature at 700 X g for 3 min and the supernatant
157 was discarded. Sperm motility and concentration were assessed and sperm was
158 diluted in Fert-TALP prior to insemination at a final concentration of $1x10^6$spz/mL. IVF
159 took place at 38.5ºC, humidified atmosphere, with 5% CO_2 for 18 to 20h. IVF
160 parameters assessed were the following: penetration rate (mean percentage of
161 oocytes penetrated by at least one spermatozoon), monospermy rate (mean
162 percentage of oocytes penetrated by only one spermatozoon), mean number of sperm
163 per oocyte (S/O), male pronucleus formation (MPN – assessed by the presence of at
164 least two pronuclei), mean number of sperm bound to the *zona pellucida* (S/ZP) and
165 IVF efficiency (percentage of oocytes that were penetrated and monospermic). Five
166 replicates were used for this experiment, totalling 691 putative zygotes.

167 *2.6 In vitro culture*

168 Eighteen to 20h post-insemination (hpi), potential zygotes were put in a 15mL Falcon
169 tube with 2mL of DPBS and vortexed for 3 min. Presumptive zygotes were washed
170 twice in DPBS and three times in culture medium (synthetic oviduct fluid Holms
171 (HSOF)[8] supplemented with 5% FBS) before transferring them to 25µl microdrops
172 covered with paraffin oil (NidOil™, Nidacon, Sweden) in a maximum load of 25
173 embryos/drop. Incubation took place at 38.5ºC, 5% CO_2, 5% O_2 for 8 days. Cleavage
174 rate was assessed 48hpi and development rates were recorded at days 6, 7 and 8 post-
175 insemination.

176 *2.7 Fixation and cell staining*

Fixation took place 22 to 24h post-IVM in the case of oocytes, 22 to 24 hpi for putative zygotes and 8 days post-insemination for blastocysts. Matured oocytes and zygotes were denuded with gentle pipetting and hyaluronidase (0.2% in DPBS) prior to fixation. All cells were incubated in glutaraldehyde (0.5% in DPBS) for 30 min at room temperature. After washing in DPBS, cells were stained with Hoechst (33342, 1µg/mL) for 30 min in the dark at room temperature. The mounting media used was DPBS with glycerol and Hoechst (1:1:0.001) and the slides were sealed with nail polish. Evaluation took place under an epifluorescence microscope (Leica® DM4000B LED, Germany) under X200 and X400 magnifications.

2.8 Cortical granules staining

All steps were performed at room temperature. Protocol is the same as described by Coy *et al.*[35]. DPBS without calcium nor magnesium supplemented with PVA (0.5% v/v) was used, and between each step of the protocol, oocytes were washed three times in DPBS for 5 min. *Zona pellucida* of denuded matured oocytes was removed by incubating the oocytes in pronase (0.5% v/v DPBS) until the detachment was visible. The oocytes were fixed with paraformaldehyde (3.7% v/v DPBS) during 30 min. Permeabilization of the membrane was made using Triton X-100 (0.1% v/v DPBS) for 10 min. Thirty minutes of incubation with LCA-FITC (1% v/v DPBS) in the dark followed by another 30 min of nucleus staining with Hoechst, also in the dark. Using the SlowFade-Antifadekit® (Invitrogen, UK), oocytes were washed three times in equilibration buffer solution, put in a slide containing 3.5µL of anti-fade mounting medium and covered with a coverslip. Slides were kept in the dark at 4ºC before evaluation by confocal microscope (Nikon® Eclipse 90i, Japan). Distribution of cortical granules through the oocyte was evaluated according to Hosoe and Shioya [36] classification: type I being a distribution of granules in clusters, type II being granules dispersed and partly clustered, type III being granules all dispersed and type IV being no visible granules. Oocytes that were degenerated, or in telophase/anaphase I, were discarded from evaluation. Six replicates were used with a total of 460 oocytes evaluated.

2.9 Vitrification, Warming and survivability of embryos

Vitrification of blastocysts was made using the Cryotop® open system with its vitrification media (Kitazato-Dibimed, Spain). On day 7 post-insemination, only blastocysts of stage code 6 and 7 (according to Bo and Mapletoft [37]) were selected for vitrification process. All steps performed according to manufacturer instructions. Briefly, embryos were put into the Equilibration Solution (ES) for 13 min, then Vitrification Solution (VS) for 30s and another 30s in VS finalized by putting the embryos on the cryotop sheet and submerging it into liquid nitrogen (-196ºC). Embryos were vitrified in groups of 2 to 4 embryos per cryotop, group within treatment and stage code (6 or 7) and stored until thawing. A total of 72 embryos were vitrified from five replicates. Warming was also performed using a warming media (Kitazato-Dibimed, Spain) as described by manufacturer: the cryotop was submerged in Thawing Solution (TS) for 60s at 37ºC, then progressively passed through Dilution Solution (DS) for 3 min, Washing Solution (WS) for 5 min, and WS again for 1 min. Warmed embryos were washed twice in culture medium and put in 25µL microdrops (one cryotop warmed per microdrop) covered with paraffin oil. Viability was assessed by the ability of the embryo to re-expand or hatch for 72h after warming, according to Lopera-Vásquez *et al.*[38].

2.10 Statistical analysis

Data in percentages were modelled according to the binomial mode of variables and arcsine transformation to achieve normal distribution. All the data were analysed by one-way analysis of variance (ANOVA). When ANOVA revealed significant differences, Tukey post-hoc tests were used. Differences were considered significant when $p<0.05$. The software used was IBM SPSS Statistics (v22.0).

2.11 Experimental design

All oocytes were matured using a supplement to maturation medium that consisted of either FBS (control), bFF or inactivated bFF (bFFin). Experiment 1 consisted of the evaluation of nuclear and cytoplasmic maturation by assessing statuses 24h post-IVM; Experiment 2 evaluated IVF parameters, 22 to 24 hpi, and Experiment 3 assessed of embryo development, quality and survivability post-vitrification.

3. Results

3.1 Experiment 1

Nuclear statuses assessed at 24h of maturation showed no significant differences between groups. The control group had a maturation rate of 72.9±2.7%, whereas the bFF and bFFin groups had rates of 72.1±2.7% and 77.1±2.5%, respectively (values indicate mean % ± S.E.M.).

Cortical granules distribution showed that rates of type III oocytes with a metaphase II plate were of 55.2±4.9% for control group, 55.2±4.6% for bFF group and 51.8±4.2% for bFFin group, without statistically significant differences. Rates of metaphase I oocytes or metaphase II oocytes with other type of cortical granules distribution (I and II) also did not have any significant difference between groups (data not shown).

Cumulus cell expansion (Table 1) showed a significantly higher expansion rate for the bFF (79.0±3.7%) than control (68.6±1.4%) and the bFFin group (62.1±3.1%).

3.2 Experiment 2

Sperm penetration rate was not significantly different between control and bFF but significant differences were found between control and bFFin groups, where the latter resulted in a lower penetration rate observed (Table 2). Monospermy, mean number of sperm per oocyte (S/O), and male pronucleus formation (MPN) showed no significant differences among groups. The mean number of sperm bound to *zona pellucida* (S/ZP) was different between groups, with the bFFin group showing the lowest value and control the highest. The efficiency of the IVF was also different with the highest value for the control group and the lowest for the bFFin group. However, there were no significant differences between the bFF group and the other two groups.

3.3 Experiment 3

Cleavage, blastocyst rate and kinetics of blastocyst development

Cleavage rate of presumptive zygotes as well as mean blastocyst formation weren't significantly different between groups (Table 3). At day 6 of embryo development (Figure 1), bFFin group showed the highest percentage of expanded blastocysts,

266 significantly different from the control group, while the bFF group showed a value in
267 between the other two groups. However, this difference disappeared by day 7 of IVC
268 (Figure 2).

269 *Embryo total cell number*

270 As for day 8 embryo total cell count, mean values were significantly different between
271 groups in hatched blastocysts (Table 4 and Figure 3). The bFF group had the highest
272 value while the control group had the lowest and bFFin had a value in between the
273 other two groups.

274 *Embryo survivability post-warming*

275 Survivability results (Figure 4) show that the stage of the embryo – blastocyst or
276 expanded blastocyst – was critical for survivability: all blastocysts (stage 6) were dead
277 by 48h after warming. No statistical differences were found between groups, but there
278 was a tendency in expanded embryos survivability from bFF and bFFin groups to have
279 higher values in t=4h and t=24h (p=0.051 and p=0.081 respectively) than control
280 group. Hatched embryos were stained and total cell number is shown in Table 5.

281 **4. Discussion**

282 Even though current bovine IVP systems provide consistent results, the quality of these
283 embryos is still far from optimal. The embryos obtained *in vitro* are of lesser quality
284 than those obtained *in vivo* [4,39], clearly expressing the lack of specific components in
285 chemically defined media. The IVM medium has not suffered major changes over the
286 years and it is still very similar to the one used for culture and growth of cells [40].
287 However, one cannot compare immature oocytes to other diploidic cells since the
288 latter won't undergo the process of meiosis, donate half of the genetic data, and
289 further host the future embryo. The maturation *in vivo* occurs within the follicle
290 surrounded by follicular fluid and even though the inorganic components can be more
291 easily traced and mimicked, the organic components are mostly missing under *in vitro*
292 conditions. Thus, by including natural components such as the bFF, we intended to
293 mimic the natural environment of oocyte development during follicular growth.

The follicular fluid supplementation as the main protein source has been used in several studies [16–27,41–43] but the origin of this fluid, whether it came from smaller or larger follicles, from pre-ovulatory or post-ovulatory phases, concentrations in IVM media, heat-treatment or not, or even its exclusive use as the main supplementation, was different between these studies. These differences were crucial for the outcomes and consequently for the conclusions of those experiments. Lonergan *et al.*[26] compared supplementation of maturation medium with fluid derived from follicles of different sizes and their results showed that 20% bFF from large follicles (>6mm) had a higher blastocyst yield than using 10% of either serum or bFF from small follicles (2-6mm). However, that difference was not observed when the amount of bFF from small follicles increased to 20% or when compared to 10% bFF from large follicles. Ali *et al.* [19] also compared different sizes of follicles but the bFF was heat-treated before use. They showed that 5% of bFFin from large follicles had the highest blastocyst outcome, when compared to the same concentration of bFFin from small follicles, BSA or no supplementation at all. But, in contrast to Lonergan *et al.* results, the IVP production decreased when the amount of bFF increased to 10%. This could be due to the heat treatment or different maturation protocols. Thus, in our study, we decided to compare supplementation of heat treated bFF and untreated bFF, at a fixed concentration to *in vitro* maturation media versus the most common supplementation (FBS) as control. Additionally, the same follicular phase was used to collect all follicular fluid to avoid different results attributed to the phase of the estrus cycle when the fluid was collected [44,45].

Heat inactivation of serum is still a common practice in cell-culture laboratories and its goal is, between others, inactivation of the complement system. Though nowadays the utility of the practice is questionable [46], we were not aware if the levels of complement factors present in bFF would be high enough to prevent embryo development, thus justifying our bFFin group. It had been tested before [17] that heating of bFF affected negatively the blastocyst yield. Nevertheless, those experiments included serum in the IVM medium and the origin of the bFF was from pre-ovulatory follicles so that their results are not comparable to ours.

As stated earlier, the developmental capacity of an oocyte doesn't rely only on nuclear maturation. Our maturation data shows no significant differences regarding nuclear status which is in discordance to a previous study [27], that reported lower maturation rates when using bFF or bFFin as a supplement. However, the concentration used was far superior (50%) which itself could be the reason for those results, since others reported [25] that high concentrations of bFF might inhibit normal resumption of meiosis.

Cortical granules distribution pattern had no significant differences between groups, but we did find differences on cumulus expansion rate. The bFF group had the highest expansion and this is supported by a previous study [21]. The authors used 5% bFF during IVM and suggested that its use promotes the sperm penetration by enhancing the cumulus expansion as well as mitochondrial re-distribution. Despite this fact, we did not find a significant difference regarding IVF efficiency between the bFF group versus the control group, although we did find a negative effect of inactivating the bFF, since it lowered the efficiency when compared to control group. If this difference is related to the lower level of cumulus expansion of the bFFin group (vs. bFF), remains to be further investigated, but the fact that the number of sperm bound to *zona pellucida* was also significantly lower, indicates that there might be a relationship between both.

Cleavage and blastocyst rates did not differ between groups. This was an unexpected outcome for the bFFin group since its IVF efficiency rate had previously dropped (experiment 2). We theorized that heat treatment might have affected some of the growth factors present in the follicular fluid and, as a consequence, cumulus cell expansion was not so extensive which resulted in lower penetration rates when compared to control. Since the growth factors alone are not the only molecules responsible for maturation [42], it could be plausible that the developmental capacity of oocytes matured with bFFin is high enough to compensate that drawback. This reinforces the idea that changes in the early stages of embryo production are critical for the IVP efficiency.

Although the yield of IVP remained the same, the speed at which the embryos developed as well as their quality differed. Embryos from bFFin group developed faster

than those from control but similar to bFF, which is interesting because it is known [8,47,48] that embryos produced with FBS have increased development rate, but until know, there was no information about changes in embryo kinetics after using exclusively bFF or bFFin in maturation medium. One of the issues brought up by the faster development of embryos produced with serum is that the more advanced embryo stage is not accompanied by cell proliferation [8], which was not the case with the bFF/bFFin groups. These groups developed faster (bFFin vs. control) and the growth was parallel with cell proliferation (bFF vs. control), indicating a possibility of higher quality of embryos from bFF and bFFin groups. Nevertheless, the increase in quality was not translated into a higher cryotolerance at a significant rate, despite a tendency being observed, probably due to the criteria of selecting only embryos on stage 6/7 to cryopreserve, which limited the amount of eligible embryos.

The composition of the follicular fluid is the core element for the differences found in these experiments. Proteomic studies refer that the proteins found in follicular fluid are mostly plasma-matched proteins [49], but in lesser concentrations [16]. Cytokines and growth factors, like EGF, TGF-β, IGF-I, activin and inhibin, have been highly linked to higher developmental competence of oocytes and embryos [41,50,51]and are also present in the follicular fluid. Similarly, steroids in the fluid like estradiol are crucial for cytoplasmic maturation [49]. Additionally, abundance of glucose and lactate as well as fatty acid free lipids are a valuable source of energy for COC's [30,49]. Recently, the presence of extracellular vesicles found in the follicular fluid have shown to give a protective effect over the oocyte during heat-shock [52]. Moreover, the exosomes present in the serum are not the same as the ones in the follicular fluid, producing different cumulus expansion as well as levels of gene expression [53]. Altogether, bFF differs in the type, quantities and quality of the components from serum, providing a richer environment to the COC maturation process.

It has been stated before [4] that the initial oocyte quality is a key factor for blastocyst yield while the embryo culture influences the blastocyst quality. However, our experiments show that changes in the *in vitro* maturation medium can also influence the blastocyst quality, at least in terms of cell numbers. Our goal was to increase the developmental potential of oocytes in order to produce higher quality embryos,

386 resembling more *in vivo* embryos. These minor changes in the IVP media can have a
387 great impact on the embryos [31] as well as later on the offspring, giving rise to
388 different genotypes and phenotypes[54].

389 **5. Conclusion**

390 In conclusion, the use of bFF improved IVP comparing to the standard IVM
391 supplementation (FBS), by having higher cumulus expansion rate, faster development
392 of embryos as well as a higher number of cells per embryo. Inactivation of the bFF
393 lowered the IVF efficiency but it did not compromise the blastocyst development and
394 quality. Additional studies should focus on analysing the gene expression of these
395 embryos in order to understand if inactivation of the bFF has any effect at the
396 molecular level. Moreover, it is still important to develop standardized protocols to
397 collect and store batches of follicular fluid as well as oviductal and uterine fluids for IVF
398 and EC giving consistent results in order to introduce its use in the current methods for
399 producing bovine embryos in vitro.

400

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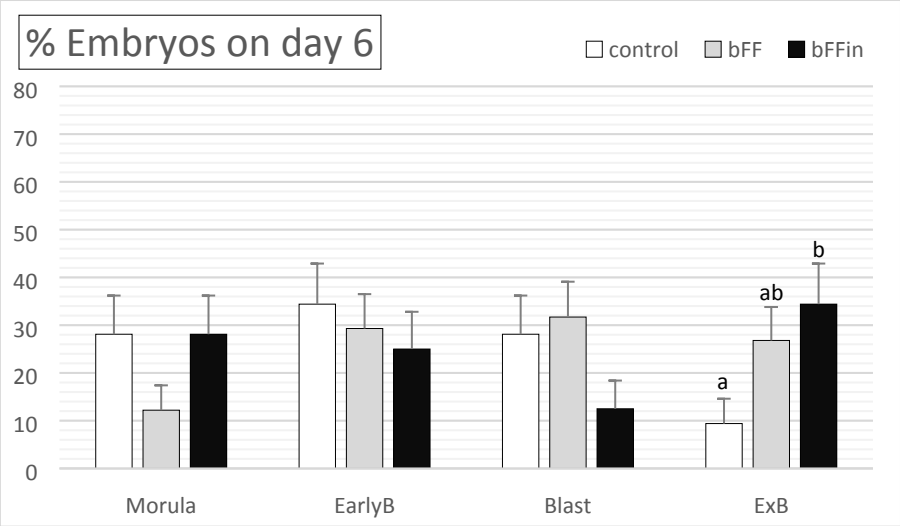

Figure 1. Kinetics of embryo development on day 6 of culture after 24h of maturation with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium. Embryo stage: Morula, Early blastocyst (EarlyB), Blastocyst (Blast), Expanded blastocyst (ExB). Thirty two, 41 and 32 embryos from control, bFF and bFFin (respectively), from 10 replicates, evaluated on day 6. Values represent mean %± S.E.M. Different letters represent statistical difference, $p<0.05$.

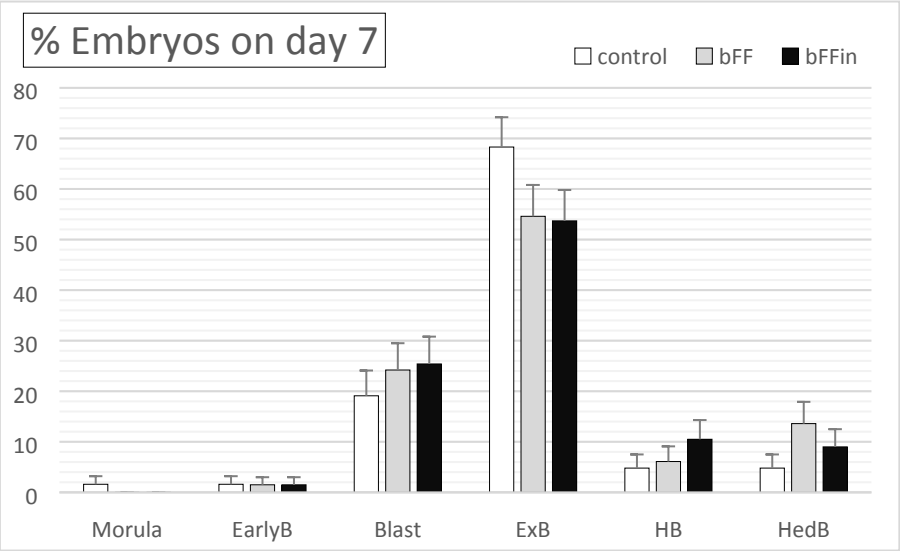

Figure 2. Kinetics of embryo development on day 7 of culture after 24h of maturation with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium. Embryo stage: Morula, Early blastocyst (EarlyB), Blastocyst (Blast), Expanded blastocyst (ExB), Hatching blastocyst (HB) and Hatched blastocyst (HedB). Sixty three, 66 and 67 embryos from control, bFF and bFFin (respectively), from 10 replicates, were evaluated on day 7. Values represent mean %± S.E.M.

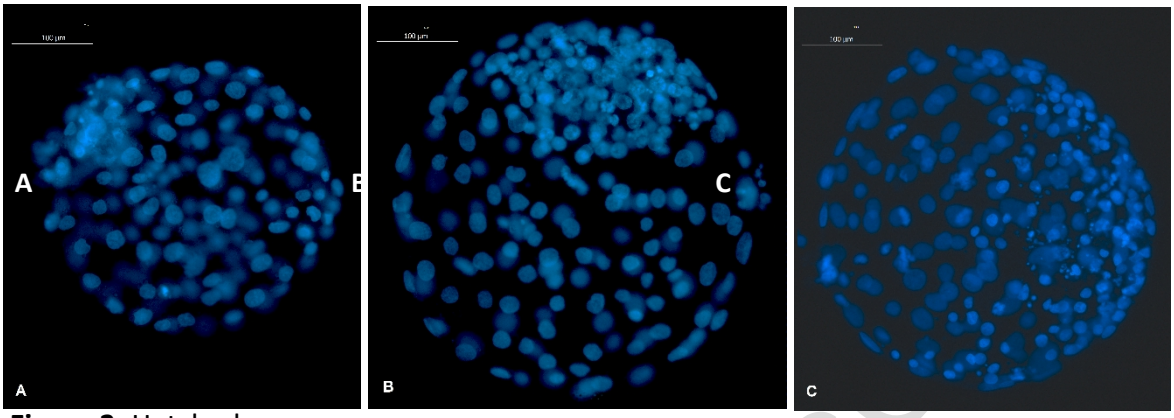

Figure 3. Hatched embryos stained with Hoechst. Control group (A), bFF group (B) and bFFin group (C).

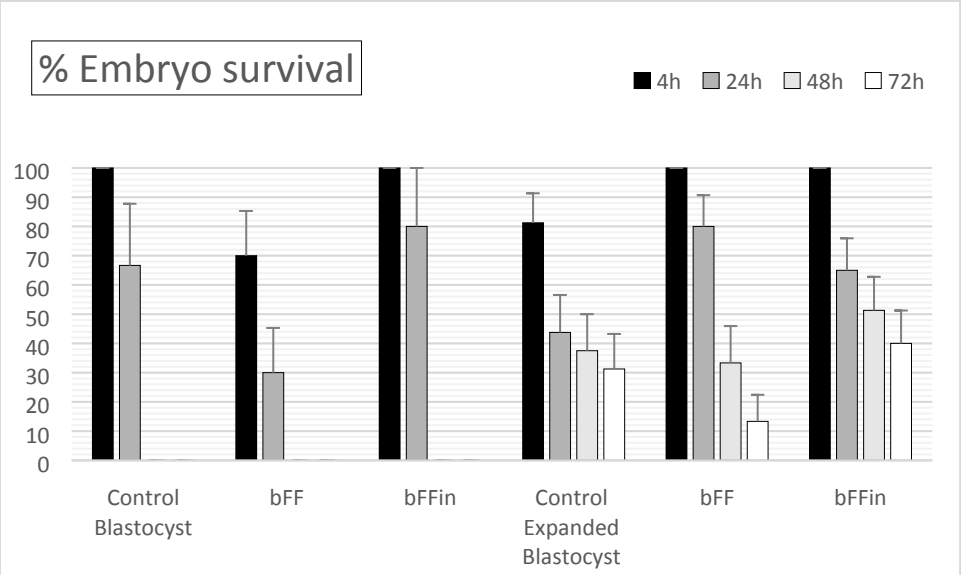

Figure 4. Survivability post-vitrification/warming of day 7 bovine embryos after 24h of maturation with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium. Data are separated by type of embryo - Blastocyst or Expanded blastocysts - and respective group. Values represent mean %± S.E.M.

Highlights

- The supplementation of maturation medium with bovine follicular fluid shows higher expansion of cumulus cells in oocytes as well as higher total cell number in *in vitro* grown embryos.

- Inactivating the follicular fluid decreases the efficiency of the IVF but not of blastocyst production. It also promotes earlier development of embryos on day 6 of culture.

- Bovine follicular fluid is shown to be a better alternative to fetal bovine serum supplementation during *in vitro* maturation of bovine oocytes.

Table 1 Increment of cumulus cell expansion during in vitro maturation of bovine oocytes from 0 to 24h with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium

	n	% Cumulus expansion after 24h
Control	139	68.6±1.4[a]
bFF	123	79.0±3.7[b]
bFFin	124	62.1±3.1[a]

Values for cumulus expansion represent % increment ± S.E.M. Different letters indicate statistical difference, $p<0.05$.

Table 2. *In vitro* fertilization parameters for bovine oocytes after 24h of maturation with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium.

	N	Penetration	Monospermy	S/O	MPN	S/ZP	Efficiency
Control	228	90.8±1.9[a]	80.2 ± 2.8	1.3	85.5±2.5	4.5[a]	72.8±3.0[a]
bFF	229	83.0±2.5[ab]	75.8±3.1	1.3	87.4±2.4	3.3[b]	62.9 ± 3.2[ab]
bFFin	234	78.2±2.7[b]	74.8±3.2	1.3	85.3±2.6	2.0[c]	58.6±3.2[b]

S/O = spermatozoa per oocyte; MPN = Male Pronucleus formation; S/ZP = Spermatozoa bound to Zona Pellucida; Efficiency = Oocytes that were penetrated and monospermic. Values represent mean % ± S.E.M, except S/O and S/ZP that are represented by mean numbers. Different letters represent statistical difference, $p<0.05$.

Table 3. *In vitro* culture results on day 2 (Cleavage rate) and day 8 (Blastocyst formation) for bovine oocytes after 24h of maturation with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium.

	n	% Cleavage	% Blastocyst
Control	269	85.5±2.2	24.2±2.6
bFF	272	83.1±2.3	25.7±2.7
bFFin	270	82.6±2.3	27.8±2.7

Values represent mean % ± S.E.M.

Table 4. Total cell number in bovine embryos after 24h of maturation with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium.

	n	Blast	n	ExB	n	HB	n	HedB
Control	2	117.0±24	13	130.9±6.8	0	-	17	185.8± 10.0[a]
bFF	2	98.0±20	6	120.0±10.8	8	171.9±11.9	17	241.3±20.1[b]
bFFin	3	108.0±18.2	7	150.9±10.8	5	150.2±16.1	21	214.9±14.0[ab]

Embryo stage: Blastocyst (Blast), Expanded blastocyst (ExB), Hatching blastocyst (HB) and Hatched blastocyst (HedB). Values represent mean of total cell number ± S.E.M. Different letters represent statistical difference, $p<0,05$.

Table 5. Total cell number for hatched bovine embryos after vitrification/warming maturated 24h of with either fetal bovine serum (FBS), bovine follicular fluid (bFF) or heat inactivated bFF (bFFin) added into the maturation medium.

	n	Hatched blastocysts
Control	3	188.7±25.8
bFF	2	205.0±24.0
bFFin	7	173.0±14.8

Values represent mean of total cell number ± S.E.M. No statistical analysis was performed with these data.