

Effect of Fetal Serum and Follicular Liquor Supplementation on the *In Vitro* Production of Bovine Embryos

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ABSTRACT

To optimize *In Vitro* Fertilization (IVF) results in bovines by lowering costs, comparing the efficiency of Fetal Calf Serum (FCS) (high cost) with respect to bovine Follicular Fluid (bFF) (low cost) and by quantifying embryo production. Cumulus-Oocyte-Complexes (COC) obtained from a slaughter house, transported at 37°C in saline solution, were classified according to their quality in A, B, C and D. A and B oocytes were washed with modified Phosphate-Buffered Saline (m-PBS) and three groups were randomly formed (GC; G1; G2) cultured in drops of the respective media (10 COC/drop of 100 µL), covered with mineral oil and placed in an incubator (38,5°C, 5% CO₂ y 95% humidity). Maturation was done in Tissue Culture Medium (TCM-199) and antibiotics, for 22h and developed in CR1aa medium and antibiotics. The Control Group (CG) was supplemented during both maturation and development stages with 5% FCS and 10% bFF; G1 with 5% FCS during maturation and development and G2 with 10% bFF during maturation and development. We obtained bFF from follicles larger than 15 mm, it was centrifuged (800G) inactivating it. At fertilization Bracket and Oliphant (BO) medium was used. Zygotes were evaluated every 48 h for 8 days since insemination, counting the Division Rate (DR) and the Embryo Development (ED) of compacted morulae. X² Test was applied. For RD, G2 differs from G1 and CG (p<0.05), CG and G1 do not differ significantly (p>0.05). During development no significant differences were found between groups (p>0.05). Results show that when using FCS, bFF or a combination of both, development results are similar. It is assumed that it is possible to substitute FCS with bFF.

Keywords: *In Vitro* Fertilization, Oocytes, Culture Media, Protein Supplementation, *In Vitro* Maturation, *In Vitro* Development

1. INTRODUCTION

In vitro production of bovine embryos is a biotechnology that makes it possible to obtain a higher number of calves per year from a single female for variable production objectives, in particular genetic advances, however, the rate of viable embryos obtained for embryo transfer by this method is still low (López *et al.*, 2005). The first calves obtained by IVF were reported in 1987 (First and Parrish, 1987). In 1992, the

first two calves from Latin America were born at the Laboratorio de Biotecnología de la Facultad de Veterinaria, Uruguay (reported by the press). The increment in the use of the Ovum Pick Up (OPU) technique, mainly in cows with genetic value, calls for a new need to increase the efficiency in the production of embryos by IVF, lowering at the same time its costs. One of the problems is the supplementation of the culture media with FCS because of its high cost (Triana *et al.*, 1997). bFF is easily available and can be obtained at a

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minimal cost, by preovulatory follicular aspiration. Research that contributes to a higher efficiency and a lower cost of IVF has a direct influence in production.

Some of the benefits of using serum are: it contributes with proteins; protection of the embryos in culture medium from toxic substances (e.g., heavy metals); provides growth factors; lowers the surface tension of the medium, preventing the embryos from attaching to instruments (culture plaques, pipettes, tubes) (Mucci *et al.*, 2006). Research has shown that the addition of bFF to maturation and *in vitro* development media for bovine embryos has a favorable effect (Larocca *et al.*, 1993; 1997; Romero-Arredondo and Seidel, 1994), since it also has hormones needed for maturation. There are also studies done that compare the effect of 0.6% bovine Serum Albumin (bSA), 10% Estrous Cow Serum (ECS) and 10% FCS as protein sources for the production of embryos (López *et al.*, 2005). High concentrations of FSH (Suchanek *et al.*, 1988), hCG (Ellsworth *et al.*, 1984) and LH (Cha *et al.*, 1986), associated with estrogens present in bFF have been reported to promote ovocyte maturation and are related to ovocytes that have a high probability of being fertilized (Revelli *et al.*, 2009). From a physiologic standpoint, follicular liquor provides an important microclimate for ovocyte development, being produced from plasma theca and the secretion from granulosa and follicular theca cells (Revelli *et al.*, 2009).

The aim of this study is to compare the performance of FCS with that of bFF on the embryos produced, with the objective of evaluating its efficiency in the production of embryos and if it is possible to substitute FCS with bFF in IVF. With this project we try to determine if bFF behaves equally or better than FCS under the same conditions within the IVF culture of bovine ovocytes, so as to determine if it is possible to substitute FCS.

2. MATERIALS AND METHODS

Ovocytes were obtained from bovine ovaries from an abattoir less than three hours after slaughter and transported in isotonic saline solution at 37°C. Follicles measuring between 2 and 8 mm in diameter were aspirated using a 5ml syringe and an 18G needle. Aspiration medium was m-PBS, with 5% FCS and antibiotics. COC were classified under stereoscopic microscope 40× as class A when having more than 3 layers of cumulus cells and a homogeneous cytoplasm; class B, with less than three layers of cells or with cumulus cells that do not completely cover the ovocyte and an irregular cytoplasm; C, naked and D, degenerated (IETS standards). Those of A and B quality were washed

2 times in m-PBS. Three groups were formed, randomly chosen with the same number of COC. CG: maturation culture was done in TCM-199 supplemented with FCS 5%, bFF 10% and antibiotics, in 100 µL drops, 10 ovocytes per drop covered with mineral oil, in an incubator (38°C, 5% CO₂ in air and 95% relative humidity) for 22 h. For development CR1aa medium were used + 5% FCS + 10% bFF + antibiotics, in incubator, for 8 days. Group 1 (G1): cultured for maturation in TCM-199 supplemented with FCS 5% and antibiotics. In the development medium CR1aa + 5% FCS and antibiotics, with the same method as described for CG. Group 2 (G2): Cultured for maturation in TCM-199 supplemented with bFF 10% and antibiotics. For development CR1aa + bFF 10% and antibiotics was used.

bFF was obtained from follicles measuring more than 15mm, centrifuged twice at 800g, supernatant inactivated at 56°C (Calvo *et al.*, 2004), aliquoted and conserved at -20°C.

After maturation all COC were inseminated *in vitro*. A tested semen straw, was added with BO semen wash with caffeine and heparin. It was centrifuged at 450g for 5 min and the supernatant discarded. The semen pellet was adjusted at 1-2 million sperm/mL with diluting BO solution, after the total concentration was calculated using a Neubauer chamber. Drops of 100 µL were formed with the semen, covered in mineral oil, COC from each group were placed in the drops and then incubated at 38°C, with 5% CO₂ in the air and 95% relative humidity for 5-6 h. After insemination gametes were washed in TCM-199 with Hepes and then were denuded with a sharpened Pasteur pipette. Fertilized COC were cultured for their development as described for each group. Cleavage was studied at 48h with an inverted microscope at 200×. Divided embryos were counted and compared to the number of COC fertilized for each group. Embryo development was assessed every 48h until day 8 from insemination, counting compacted morulas and/or blastocysts (transfer stages). All the experiments were done using the same serum lot and semen from the same bull.

12 replics were done and results compared using χ^2 test (Siegel and Castellan, 1988).

3. RESULTS

Forty eight hours after IVF in the CG, from a total of 181 COC, 79 divided (DR: 43,6 p.100) and 29 developed into compacted morulae (ED/DR: 36,7 p.100); in G1, form 183 COC, 70 divided (DR: 38,3 p. 100) and 37 developed into compacted morulae (CM) (ED/DR: 52,9 p.

100); in G2 from 173 COC, 97 divided (DR: 56 p. 100) and 40 developed a CM (ED/DR: 41,2 p. 100).

For DR, G2 differs significantly from G1 and CG ($p < 0.05$), while CG and G1 do not show significant changes ($p > 0.05$). For the development phase statistics showed no differences between the groups ($p > 0.05$).

During cleavage, G2, supplemented only with bFF, performed better, while during development all three groups behaved in a similar way. The results show that either using FCS, bFF or a combination of both; similar results are obtained for development, suggesting that FCS could be replaced with bFF in the bovine IVF culture.

4. DISCUSSION

Serum is considered a variable and undefined component (Gardner and Lane, 1998), which creates variations in media used and interferes with the repeatability of the results obtained. Most researchers use hormones and FCS in the media (Pinyopummintr and Bavister, 1994). In this sense there are differences in the rate of production between the different trials published where serum is used as a proteic supplement (Hammond *et al.*, 2000).

The effect produced by the additions of bFF to the maturation medium has been studied and they show positive results (Romero-Arredondo and Seidel, 1994; Sirard *et al.*, 1995), coinciding with the results of this study. Calvo *et al.* (2004) by adding 10% bFF to a medium containing FCS obtained better results with respect to the group without bFF. The present study shows a significant difference in the cleavage rate when bFF is used alone during maturation and development, not being significant in the embryos obtained, these results are similar to the ones aforementioned, taking into account a better performance when only bFF was used during maturation and development.

Choi *et al.* (1998), used bFF as an additive to the culture medium without obtaining any benefit on the development rate. The author concludes that there is a need for further research, using bFF derived from preovulatory follicles.

5. CONCLUSION

The present work, which deepens in the study of the effects of bFF on IVF found that given the results obtained it is possible to substitute FCS with bFF in the IVF cultures.

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