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Effect of the Addition of Follicular Fluid on *in vitro* Maturation Media for Bovine Oocytes

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Abstract: The objective of the present research was to evaluate the effect of the addition of bovine Follicular Fluid (FF) to the *In vitro* Maturation media (IVM) of bovine oocytes. The Cumulus Oocyte Complexes (COC) were obtained by aspiration with PBS+bovine fetal serum (CS)+Antibiotics (ATB) from follicles measuring 2-5 mm in diameter from ovaries collected from a slaughter house. The FF was extracted from 15 mm follicles and was inactivated. In total, 718 COC (A and B) were selected and were randomly divided into two groups: G1 (Control, 329 COC), TCM+5% CS+ATB; G2 (FF, 389 COC), TCM-199/10% FF+ATB. Each group was then subdivided into 4 subgroups: [G1T1> 18h (69 COC); 20h (82 COC); G1T3: 22h (99 COC) and G1T4: 24 h (100 COC)]. COC were matured at 38.5°C, 5% CO^2 and 99% humidity. The IVM of oocytes was evaluated by observing the first polar body using a stereoscopic comb (40X) and by visualizing the metaphasic plate of oocytes stained with 1% acetic orceine under the microscope (400X). The total number of mature COC for each group was: [G1T1: 18h (17), G1T2: 20h (28), G1T3: 22h (31) and G1T4: 24h (48); G2T1: 18h (38), G2T2: 20h (46), G2T3: 22h (47) and G2T4: 24h (49)]. Chi-square test was used with a significance level of 5%. These results show that the addition of FF enhaces the maturation percentages at 18, 20 and 22 h, with the best results obtained at 22 h. At 24 h percentages are similar between the 2 groups.

Key words: Follicular fluid, maturation media, bovine oocytes, addition

INTRODUCTION

In vitro Maturation (IVM), which includes nuclear and cytoplasmic maturation, was reported in various species in 1965 (Hinrichs, 1993). As it is known, meiosis is halted until the oocytes are exposed to gonadotrophins (endogenous or exogenous), until when meiosis continues (Romero and Seidel, 1996). When the Cumulus Oocyte Complexes (COC) are removed from the follicles meiosis begins spontaneously.

Various authors have indicated that for the maturation process to take place there must be a hormonal balance within the follicle (most importantly regarding steroid hormones) (Del Campo *et al*., 1992; Benau and Storey, 1988; Callesen *et al*., 1986; First and Parrish, 1987).

In an *in vitro* system this natural balance is sought by adding Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and estradiol 17 B to the maturation media (TCM-199). In some laboratories instead of adding hormones they add bovine Follicular Fluid (FF) (Romero and Seidel, 1994; Atef *et al*., 2004; First and Parrish, 1987; Gordon, 1994; Irritani *et al*., 1992; Larocca *et al*., 1993, 1997).

These authors have stated that the addition of FF to the IVM is enough to produce the maturation, cumulus cell expansion and future development of the oocyte.

The objective of the present research was to evaluate the effect of the addition of Bovine Follicular Fluid (FF) to the *In vitro* Maturation media (IVM) on bovine oocytes.

MATERIALS AND METHODS

The Cumulus Oocyte Complexes (COC) were obtained by aspiration of follicles measuring 2-5 mm in diameter from ovaries collected from a slaughter house and transported to the laboratory within 4 h, in physiologic solution at 37°C. FF was extracted from follicles measuring 15 mm in diameter, was inactivated at 56°C for 30 min and after centrifugation the supernatant was aspirated.

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Aspiration media was Phosphated saline solution (PBS, Gibco, Grand Island, NY) + Fetal Serum (FS, Gibco, Grand Island, NY) + Antibiotics [(ATB, Sigma Chemical Company): Penicilin (100 UI mL^{-1}) + Streptomycin (100 μg mL^{-1})] filtered (0.22 μm). 718 COC were selected based on cytoplasmic quality and the number of cell layers of the granulosa (= 3). COC's were randomly divided into 2 groups: G1 TCM-Control, 329 COC (Gibco, Grand Island, NY)+5% FS+ATB; G2 (FF, 389 COC) + 5% FS+ATB. Each group was further divided into 4 subgroups [G1T1: 18h (69 COC); G1T2 20 h (82 COC); G1T3: 22h (76 COC); G1T4: 24h (102 COC); G2T1: 18h (90 COC); G2T2: 20h (100 COC); G2T3: 22h (99 COC); G2T4: 24h (100 COC)]. COC were matured at 38.5°C, 5% CO^2 and 99% humidity. Oocyte IVM was evaluated by observing the first polar body using a stereoscopic comb (40X) and by visualizing the metaphasic plate of oocytes stained with 1% acetic orceine under the microscope (400X). Results were analized by Chi-squared test, evaluating the number of mature oocytes for each range of time and for each media.

RESULTS AND DISCUSSION

Differences were considered significant at $p<0.05\%$. The total number of mature COC for each group were: [G1T1: 18h (17), G1T2 20 h (28), G1T3: 22h (31), G1T4: 24h (48), G2T1: 18 h (38), G2T2: 20h (46), G2T3: 22 h (47), G2T4: 24 h (49)].

Chi-squared test was used with a significance level of 5%. For G2T1 vs G1T1 significance level was >0.01; for G2T2 vs G1T2 > 0.01; for G2T3 vs G1T3 > 0.01, while for G2T4 vs G1T4 the difference was not significant, $p>0.05$. Percentages for G2T4 vs G1T4 96.08% vs 88.89 (Table 1 and Fig. 1).

This study evaluated FF as a component of bovine oocyte maturation media.

Nowadays, it is important to have an economic source of embryos, either to be able improve livestock genetics and also to carry out research involving new genetic tecnology. Today using IVF in the laboratory it is possible to obtain from immature oocytes, embryos in each stage of maturation, utilizing different culture media.

Results by Sirard *et al.* (1989) were used as background. He investigated how oocytes mature by adding TCM-199, FS and gonadotropic hormones to the media, from hour 0 upto 24 h, while fixating and staining every 3 h. 95% of oocytes reached Metaphase II.

Choi (1998), who employed bovine FF as an additive for the maturation media, did not obtain any beneficial effect.

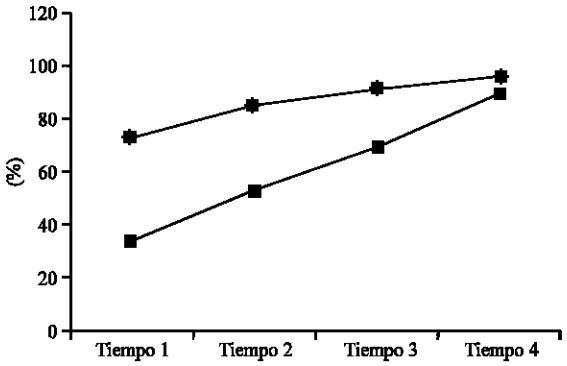

Fig. 1: Maturation percentage in relation to time for each media

Table 1: Percentage of mature oocytes in relation to time for each culture media

Group	(T1) time 1	(T2) time 2	(T3) time 3	(T4) time 4
Control (%)	32.69 (17/52)	51.85 (28/54)	68.88 (31/45)	88.89 (48/54)
FF (%)	72.55 (38/52)	85.19 (46/54)	90.38 (47/52)	96.08 (49/51)

The results of our study show that the addition of FF improve maturation percentages at 18, 20 and 22 h. At 24 h percentages for each group are similar. The number of oocytes should be increased so as to better evaluate the group G1T4.

Results also indicate that the addition of FF to the maturation media allow for shorter maturation times of 18-20 h, obtaining maturation percentages above 70%.

Xiang *et al.* (2007) compared *in vitro* maturation using a media containing FS with one containing FF, but found no significant difference.

It is necessary to continue research in the field of maturation media in order to obtain lower cost alternatives, as this is a fundamental aspect for the viability of IVF technique.

FF allows for a more flexible protocol and is also more viable from an economic point of view, when compared to the use of hormones.

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