INTEREST OF BOVINE FOLLICULAR FLUID IN THE ENRICHMENT OF MEDIA FOR THE IN VITRO MATURATION OF BOVINE OOCYTES

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Abstract. *Our aim is to verify the interest of using bovine follicular fluid as an enrichment of basal media for the in vitro maturation of cow oocytes. The oocytes are collected from slaughterhouse ovaries as well as the follicular fluid used as an additive. The ovaries were transported at room temperature and the follicles aspirated within two hours of slaughter. The maturation was carried out in an oven at 38°C, 5% CO_2 and in an atmosphere saturated with humidity. In this work we tested three media with different proportions of bovine follicular fluid (0%, 10% and 50%) then we compared the expansion rates of the cumulus clouds with that obtained with a commercial medium that does not require CO_2.The best expansion rates were obtained with the basic medium added with 10% follicular fluid (57.64%), this mixture seems to be optimal because this result has been confirmed by data from the bibliography. The comparison with the commercial culture medium gave almost similar results (57.64% vs 56.16%) which is a positive result in itself despite the absence of statistical significance.*

Keywords: *Oocyte maturation, follicular fluid, culture medium, slaughter ovaries, cattle.*

INTRODUCTION

Oocyte maturation is the final stage of a long evolution that will allow it to successfully ensure fertilization and early embryonic development. This maturation follows a preparation of the oocyte cytoplasm which takes place gradually during folliculogenesis. From birth until the day of ovulation, the oocyte remains blocked in meiotic prophase (germinal vesicle stage) thanks to an intrafollicular inhibition system involving factors such as OMI (oocyte meiotic inhibitor) (Drion et al, 1996; Sirard et al 1989). It only resumes its meiosis if, having successfully completed its growth within a follicle destined for ovulation, it finally receives the release signal from the gonadotropic hormones. The knowledge acquired on the various physiological phenomena and the factors involved in maturation allow us to draw some lessons for the implementation of in vitro maturation techniques.

The oocyte maturation technique was first identified in 1968 in Ireland by Joe Sreenan (1970). The oocyte spontaneously resumes meiosis in vitro, but the cytoplasmic aspects of maturation strongly depend on the composition of the maturation medium. Gonadotropic hormones have an action on the expansion of the cumulus, other factors such as EGF (epidermal growth factor) have an action on nuclear and cytoplasmic maturation in several species including cattle (Lonergan et al, 1996). Growth factors belonging to the insulin family are widely involved in the control of folliculogenesis (Monget et Monniaux, 1995) and IGF-I (insulin-like growth factor-1) could act in synergy with EGF in the control of oocyte maturation (Lorenzo et al, 1995). The activin

present in the follicular fluid is also involved in the cytoplasmic maturation of the bovine oocyte (Silva and Knight, 1998). When an oocyte is cultured, the spontaneous resumption of meiosis deprives the oocyte of a preparation necessary for optimal development. In cattle, it is possible to obtain meiotic blockade, in order to increase developmental competence, using cocultures of cumulus-oocyte complex and granulosa and theca cells.

The follicular fluid contains several elements that are essential for maturation. It includes steroid hormones such as estrogens with varying concentrations during the different phases of the cycle (Klumpp 2004), energy substrates such as glucose and fructose as well as proteins that stimulate oocyte maturation (Saranya et al, 2015). The beneficial effect of follicular fluid on maturation has been demonstrated in several species including pigs (Gallardo et al, 2001), sheep (Sun et al, 1999), horses (Bogh et al, 2004; Hinrichs et al 2004) and cattle (Avery et al, 2003). Follicular fluid plays a role in the expansion of cumulus cells (Aguilar et al, 2001), enhances cytoplasmic maturation (Gallardo et al, 2001) and improves embryonic development (Algryani et al, 2004).

Our aim is to verify the contribution of an enrichment based on bovine follicular fluid (BFF) on the rate of maturation of oocytes obtained from slaughterhouse ovaries, in comparison with a basic medium alone and a medium commercial maturation without CO2.

MATERIAL AND METHODS

Collection of ovaries. In our work, 162 cow ovaries were collected in the slaughterhouses of El Harrach (Algiers) and Boufarik (Blida). The ovaries are transported within 2 hours after slaughter to the laboratory in an isotonic saline solution at a temperature of 20 to 25°C. Among the ovaries collected, 14 were discarded because they presented various anomalies: atrophy, smooth ovaries, fibrosis and cysts.

Oocyte harvest and classification. A total of 1202 superficial follicles with a diameter less than or equal to 4mm are punctured using an 18G needle mounted on a 5cc syringe. The collected follicular fluid is placed in petri dishes previously filled with PBS at room temperature and squared to facilitate the search for COCs (cumulus oocyte complexes).

The search for oocytes was carried out under an inverted microscope at the lowest magnification. COCs are classified based on morphological criteria into four classes (Kouamo et al, 2014), thus 495 COCs of different classes were obtained. Only class 1 and 2 COCs are kept for maturation, they are rinsed three times in PBS (Barceló-Fimbres et al., 2015) or MOFA rinsing place (depending on the destination medium) before being used.

Collection and conditioning of follicular fluid. The follicular fluid is aspirated from follicles with a diameter greater than 8mm, using a syringe, and is then put into dry tubes to be centrifuged at 2000 rpm for 10 minutes. The supernatant is removed and centrifuged a second time in the same way. The supernatant is removed once again and placed in identified Eppendorf tubes to be either used immediately or frozen at -20C° for later use. Before using the stored follicular fluid, the tube is thawed and kept in a water bath at 56°C for 30 minutes (Karami Shabankareh et al., 2011).

Maturation media. Four media were used in our experiment, three media based on TCM199 (tissue culture medium 199) and different proportions of follicular fluid and one commercial medium (without CO_2). The composition of the media is as follows:

- Medium I: TCM199 base medium with 0% follicular fluid
- Medium II: TCM199 base medium enriched with 10% follicular fluid
- Medium III: TCM199 base medium enriched with 50% follicular fluid
- Medium IV: oocyte maturation medium not requiring CO_2 MOFA (MOFA Global LLC, Verona, WI, USA)

Oocyte maturation

The COCs selected for in vitro maturation are placed in multiwell cell culture dishes. Each well is filled beforehand with 0.5 ml of culture medium; a number of 4 to 8 COCs are placed in each well. The culture dishes are then introduced into an oven at a temperature of 38°C, in an atmosphere at 5% CO_2 and saturated with humidity for 24 hours. The COCs intended for the MOFA medium are placed in the tubes conditioned by the manufacturer at the rate of 7 to 9 COCs per tube and cultured in an oven at 38° C without CO_2.

Statistical analysis. During this work, three questions were asked; whether there is a difference in the rate of expansion of the cumulus between the four media, depending on the proportion of BFF and finally between the first three media (1, 2, 3) and MOFA. To answer these three questions, a chi-square test of homogeneity was used. The difference was considered significant for $p<0.05$.

RESULTS

To assess the maturation rate of COCs, we considered the expansion of the cumulus as an indicator (Figure 1).

Medium effect. The question here is whether these four media differ overall from each other, in other words if there is an effect of the nature of the culture medium on the rate of oocyte maturation. Table 1 shows the expansion rates of COCs from different media, no significant difference was reported (p= 0.7053).

Table 1.

Expansion rate of COCs in the four maturation media

Culture media	Oocytes in culture	Expanded oocytes n (%)	X^2	p-value
Medium I	86	42 (48.83)	1.4011	0.7053
Medium II	85	49 (57.64)		
Medium III	106	47 (44.33)		
Medium IV	73	41(56.16)		
Total	350	179 (51.14)		

Effect of the composition of medium I, II and III

Statistical analysis shows no significant difference between the expansion rate of medium I, II and III (Table 1) (p= 0.5721). This means that the proportion of BFF has

no effect on the expansion of the cumulus even if a numerical advantage was noted for the medium II added with 10% of BFF.

Effectiveness of medium IV compared to the rest

The question is whether the first three media taken together differ from the Medium IV (MOFA medium)

Table 2.

Difference in expansion rate of media containing BFF and MOFA

Culture media	**Oocytes in culture**	**Expanded oocytes n (%)**	**X^2**	**p-value**
Media I, II and III together	277	138 (49.81)	0.29379	0.5878
Medium IV	73	41(56.16)		

Statistical analysis revealed no difference (p=0.5878), which is considered a positive result with regard to the cost price of the two types of media.

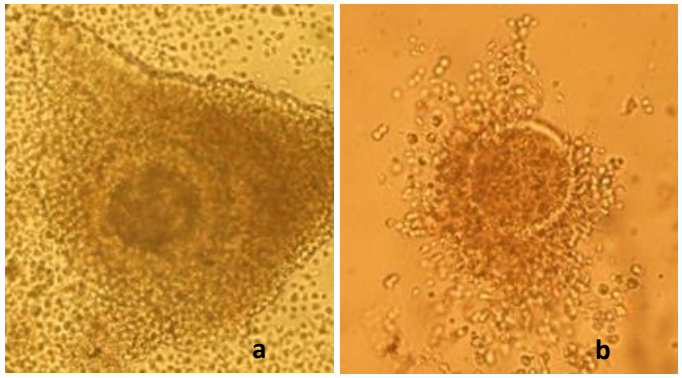

Figure 1. COCs de classe 1 avant (a) et après (b) maturation

DISCUSSION

In vitro oocyte maturation is largely influenced by the conditions of collection and transport of the ovaries from the slaughterhouse, the quality of the oocytes and the composition of the media used. In our work, the ovaries were collected and transported to the laboratory within 4 hours following slaughter at ambient temperature, Klumpp (2004) consider that the optimal temperature for storing the ovaries until aspiration is the ambient temperature, Rosenkranz (1993) found greater expansion of the cumulus when the ovaries were transported at 20C° compared to 30 and 37C°.

We used the aspiration technique and obtained a COC recovery rate of 41.18% with an average of 3.05 COCs/ovary, Kumar et al (1997) reported an average of 3.10, an improvement which can go to 6.25 COCs/ovary can be obtained by the slicing technique. An increase of up to 200 COCs/ovary was obtained by Fouladi et al (1998) using the technique of enzymatic digestion with trypsin.

In our work we obtained an overall maturation rate of 51.14%, the best result is recorded by medium II (10% BFF) with 57.64%. Several studies (Romero-Arredondo and Seidel, 1994; Elmileik et al, 1995; Kim et al, 1996; Choi et al, 1998; Avery et al, 2003) have shown that follicular fluid can be a beneficial supplement in maturation of the cow. On the other hand, Ayoub and Hunter (1993) recorded a drop in the rate of maturation; in fact the follicular fluid leads to a delay in nuclear maturation and inhibits the latter in cattle (Gallardo et al., 2001) and in pigs (Driancourt et al., 1998). This explains the rate we obtained in our study (44.33%) by adding the basal medium with 50% BFF.

According to Dostal et al (1996) and Choi et al (1998), follicular fluid from large follicles has a reduced inhibitory effect on oocyte maturation compared to follicular fluid harvested from small and medium follicles. Other studies have identified several substances such as hormones in the follicular fluid which would prevent the resumption of oocyte meiosis (Takahashi et al, 1986), this problem can be avoided by adding epidermal growth factor (EGF) which will induce the resumption of meiosis in bovine oocytes (Nandi et Al, 2002).

Gallardo et al (2001) showed that bovine follicular fluid inhibits nuclear maturation but enhances cytoplasmic maturation and pronuclei formation. According to Aguilar et al (2001), the slowing down of nuclear maturation gives the oocyte more time for the synthesis and storage of proteins and ribonucleoproteins, and therefore improves its developmental competence. A study found that follicular fluid promotes cytoplasmic oocyte maturation during IVM and suggests that its major role is to provide protection against oxidative stress (Grupen and Armstrong, 2010), which gives follicular fluid a beneficial effect on the rate of maturation and developmental competence.

Concerning the MOFA medium, we obtained a maturation rate of 56.16%, it is almost similar to that obtained by the medium supplemented with 10% of BFF. No statistically significant difference was reported between the MOFA medium and the three other media used. The same observation was made by Barceló-Fimbres et al (2015) who found no difference between the cleavage rates of matured oocytes in the MOFA medium compared to the control (83.1% and 84.1% respectively).

CONCLUSION

Our work allowed us to confirm the effectiveness of BFF in the expansion of the cumulus which is the preliminary step to fertilization. Our results, especially for the medium supplemented with 10% BFF, are very promising and similar to those obtained by the MOFA commercial medium, which is in itself an advantage because the BFF is available free of charge and in large quantities. It would be interesting to test the same medium with other enrichments such as gonadotropins, serums and growth factors. The ultimate test for this medium would be to go to fertilization and quantify the rate of oocyte cleavage.

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