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In this study, the co-authors: H.H.V. Correia, L.F. Lima, F.G.C. Sousa, A.C.A. Ferreira, J. Cadenas and V.M. Paes performed the experimental protocols, acquisition of data, and participated in drafting the full manuscript. Bênner Geraldo Alves; contributed to the analysis of the data. A. Shikanov and J.R. Figueiredo participated in substantial contribution to conception and revising it critically for important intellectual content. All the authors in this manuscript have read and approved the final version.

Activation of goat primordial follicles *in vitro*: Influence of alginate and ovarian tissue

Running Head: Effect of alginate and ovarian tissue on follicle activation

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Abstract

The present study aimed to evaluate the effect of three culture systems on caprine primordial follicle activation in vitro: follicles cultured either in the isolated form within alginate (Isolated follicles + Alginate treatment), or enclosed in ovarian tissue (in situ), with or without alginate (Fragment + Alginate, and Fragment alone treatments, respectively). After culture, the Isolated follicles + Alginate treatment presented a percentage of morphologically normal follicles (MNF) similar to both the non-cultured control and the Fragment Alone treatments. Nevertheless, Fragment + Alginate treatment showed a significant reduction in the number of MNF when compared to the other treatments. Regarding follicle development, our results showed that regardless of the alginate, the presence of ovarian tissue limited primordial follicle activation during in vitro culture. Remarkably, the Isolated primordial follicle + Alginate treatment was the only one that significantly promoted follicle activation and increased both follicle and oocyte diameters during IVFC, pointing out a higher cell proliferation. In conclusion, the presence of ovarian tissue with or without alginate limited follicle development (activation) after culture. Nevertheless, when primordial follicles were isolated and encapsulated in alginate they presented suitable survival rates, higher rates of follicle activation and continued to grow throughout the culture period.

Keywords: 3D culture system; Alginate; Ovarian tissue; Primordial follicles; Goat.

Introduction

Preantral follicles (primordial and primary) are the largest ovarian follicle population and represent an important source of potentially competent oocytes for further use in assisted reproductive technologies. Nevertheless, primordial follicles (PFs) are quiescent within the ovary, and usually activate and enter growth phase after exposure to activating or removal of inhibitory stimuli (John et al., 2008). However, the mechanisms underlying PF activation and growth are still not well defined.

The in vitro follicle culture (IVFC) is an outstanding tool to study the control of early folliculogenesis. Evidence has suggested that PFs need a stiff, similar than of the ovarian cortex, to initiate their development in vitro (Woodruff and Shea, 2011). Telfer et al, (2008) reported that the in vitro culture (IVC) of the ovarian cortical fragments has shown limited results regarding follicle growth. Moreover, differences among fragments in terms of follicular population, number and density of the tissue may occur (Hornick et al., 2012). To minimize the effects of heterogeneity of the ovarian fragments several groups proposed to isolate, encapsulate and culture PFs within an inert three-dimensional (3D) biomimetic matrix, such as alginate (Shikanov et al., 2011; Laronda et al., 2014). Despite promising results with secondary and multiple primary follicle culture, this culture system has not demonstrated a proper development of PFs *in vitro*. Furthermore, there is no experimental data that compares under the same conditions isolated PFs encapsulated in alginate with PFs enclosed in ovarian tissue embedded or not in alginate. Therefore, the present study aims to compare the survival and development rates among isolated PFs embedded in alginate with those enclosed in ovarian fragments in the presence or absence of alginate.

Materials and methods

Research ethics

Alternatives to animal testing are the development and implementation of test methods that avoid the use of live animals. One of the major alternatives to in vivo animal testing is in vitro cell culture. In line with this ethical issue, the present study aimed to evaluate the effects of the tested substance (alginate) on in vitro folliculogenesis using caprine follicles recovered from slaughterhouse ovaries. This source of ovarian material represents a by-product of the food industry and is more readily acceptable than euthanasia of animals specifically for scientific purposes.

Culture medium and chemicals

Unless mentioned otherwise, the culture media and other chemicals used in the study were purchased from Sigma Chemical Co. (St. Louis, USA). The basic culture medium consisted of α-MEM (pH 7.2 - 7.4) supplemented with 1.25 mg/mL bovine serum albumin, 1% ITS (10 µg/mL insulin, 5.5 µg/mL transferrin and 5 ng/mL selenium), 2 mM glutamine and 2 mM hypoxanthine, which was referred to as α-MEM$^+$.

Experimental protocol

Ovaries (n=10) were collected at a local slaughterhouse from 5 adult mixed-breed goats, washed and transported within 1 h to the laboratory in MEM-HEPES at 4°C. Ovarian cortex tissue samples from each ovarian pair were cut into 18 slices (approximate size 3x3x1 mm) (Figure 1). Twelve of the eighteen fragments were submitted to the mechanical isolation of PFs (Lucci et al, 1999). A total of thirty PFs were isolated per repetition. Groups of 10 follicles were embedded in 10-µl drop of alginate (**Isolated primordial follicle + Alginate treatment**). The remaining six fragments were distributed randomly among the following treatments: **Fragment + Alginate treatment** (one fragment/20-µl drop of alginate) and **Fragment alone treatment** (fragment without any matrix)**.** The ovarian fragments and isolated follicles were either immediately fixed (**Non cultured control**) or in vitro cultured for 1 or 7 days and thereafter fixed for histological analysis as described previously (Lima et al., 2016). Both isolated follicles and the fragments were cultured in 48-well culture dishes containing 500 µL of α-MEM$^+$ at 39°C in 5% CO_2 in air.

The experiment was replicated 5 times and the culture media were replaced every other day.

Follicle and tissue encapsulation in alginate

For encapsulation, each group of 10 isolated follicles or fragments of ovarian cortex were washed with 100 µL drops of alginate to remove the holding medium. Sodium alginate (55–65% guluronic acid) hydrogel was prepared as described in previous reports (Xu et al., 2006).

Morphological analysis and evaluation of follicular growth in vitro

Follicle stage and survival were assessed on serial sections. The unilaminar follicles were classified according to their developmental stage as primordial (one layer of flattened granulosa cells or one layer of a mixture of flattened and cuboidal granulosa cells) or primary (from one to less than two complete layers of cuboidal granulosa cells). Also, these follicles were still classified as histologically normal or atretic. To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before and after culture as previously described (Lundy et al 1999). Overall, 150 follicles were evaluated for each group (30 follicles/group/repetition).

Statistical analysis

All statistical analyses were carried out using Sigma Plot 11.0 (Systat Software Inc.). Comparison of means was analyzed by ANOVA followed by the Student-Newman-Keuls as a post hoc test, whereas follicular integrity and development were analyzed by chi-square test. Data was presented as percentage and mean±standard error of mean (SEM) and P-value <0.05 was considered statistically significant.

Results

Morphological features of normal follicles (MNF) before and after IVFC are shown (Figure 2A). From D1 onwards, the Fragment + Alginate treatment showed a significantly lower percentage of MNF when compared to the other treatments (Figure 2B).

After culture, there was a reduction in the percentage of PFs with a concomitant increase ($P<0.05$) in the percentage of developing follicles (primary follicles) in all treated groups compared to their correspondent controls (Figure 2C). On D7 of culture the highest ($P<0.05$) percentage of primary follicles was achieved in the isolated primordial culture treatment.

A significant increase in follicle and oocyte diameter was observed in Isolated primordial follicles + Alginate treatment from D0 to D7. In contrast, oocyte diameter decreased from D0 to D7 in the Fragment + Alginate and Fragment alone treatments ($P<0.05$). Only in the Isolated primordial follicles + Alginate treatment the volume filled by granulosa cells in preantral follicles increased from D0 to D7 and was higher than Fragment + Alginate treatment on D7 ($P<0.05$) (Table 1).

Discussion

The present study compared for the first time the effect of three culture systems (isolated PFs embedded in alginate matrix with those enclosed in ovarian fragments in the presence or absence of alginate) on caprine PF development in vitro. Our results showed that the use of alginate as a 3D matrix for isolated follicles promoted survival and follicle activation compared to the other two culture systems.

The IVC of follicles in the isolated form within alginate or those cultured only enclosed in ovarian tissue ensured appropriate preservation of follicular morphology. Nevertheless, the IVC of fragments encapsulated in alginate had detrimental effect on the percentage of MNF suggesting that the extra pressure exerted by the alginate on the tissue negatively affected follicle survival, possibly because alginate hydrogels are non-degradable and have a relatively stable elasticity module (Laronda et al., 2014). An additional layer of hydrogel around already dense tissue could also limit the diffusion of nutrients and oxygen towards the cell during IVC further affecting follicle growth and survival.

Isolated follicles demonstrated activation rates 1.3 times higher compared to other treatments. The method of encapsulation of isolated PFs in alginate promoted follicle activation and increased both follicle and oocyte diameters during IVFC. It has been suggested that alginate mimics the role of the extracellular matrix in vivo (Telfer and Zelinski, 2013), maintaining follicular 3D structure, as well the contact between the oocyte and the surrounding granulosa cells, which facilitated the exchange of substances that are essential for follicular activation and survival (Sadeghnia et al., 2016). In contrast, presence of ovarian tissue around PFs restrained their activation during IVFC, suggesting increasing density and reduced diffusion during culture.

In conclusion, the presence of ovarian tissue limited follicle development during IVC. Nevertheless, when PFs were isolated and encapsulated in alginate they presented suitable survival rates, higher rates of follicle activation and continued to grow throughout the culture period.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

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Conflict of interest

None of the authors have any conflict of interest to declare.

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Figure legends

Figure 1. Experimental design to assess the effect alginate encapsulation on the development of isolated primordial follicle or enclosed in ovarian tissue after 0, 1 or 7 days of IVC.

Figure 2. Representative images (a), percentage of morphologically normal follicles (b) primordial and primary follicles (c) after 0, 1 or 7 days of IVC. ^{AB}Differences among groups within the same day; ^{ab}Differences between days within the same group; *Differences from D0 within the same group ($P<0.05$).

Table legends

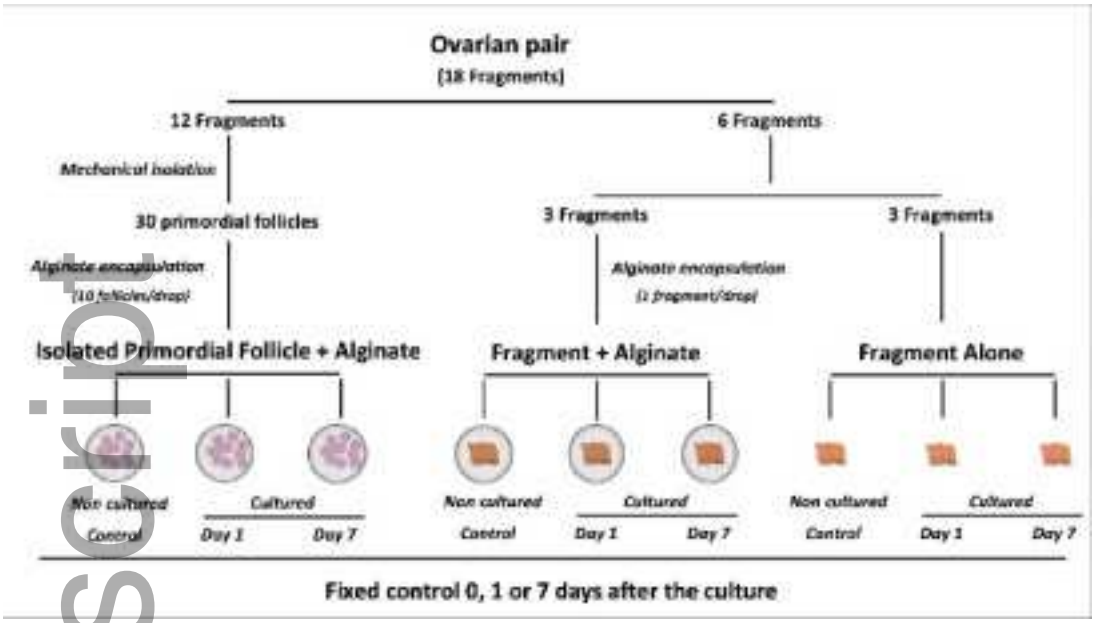
Table 1. Follicular, oocyte diameters (µm) and volume (mean±SEM) filled by granulosa cells in preantral follicles subjected to IVC. ^{A,B,C}within a column and ^{a,b}within a row and the same and point ($P<0.05$).

Treatment	Follicles			Oocyte			Volume (μm^3)		
	D0	D1	D7	D0	D1	D7	D0	D1	D7
Fragment Alone	28.1 ± 0.7^{Aa}	27.6 ± 0.7^{ABa}	26.3 ± 0.8^{Aa}	21.1 ± 0.5^{Aa}	19.4 ± 0.6^{Ab}	18.5 ± 0.7^{Ab}	7055.9 ± 676.6^{Aa}	6933.2 ± 559.7^{Aa}	6336.3 ± 655.4^{ABa}
Fragment + Alginate	27.9 ± 0.6^{Aa}	28.7 ± 1.1^{Aa}	26.2 ± 0.9^{Aa}	21.3 ± 0.4^{Aa}	20.5 ± 0.9^{Aab}	18.6 ± 0.7^{Ab}	6447.4 ± 533.4^{Aa}	7590.7 ± 871.1^{Aa}	6063.3 ± 643.2^{Aa}
Isolated primordial follicles + Alginate	21.6 ± 0.03^{Ba}	25.8 ± 0.7^{Bb}	27.1 ± 0.8^{Ab}	13.9 ± 0.6^{Ba}	16.7 ± 0.5^{Bb}	17.4 ± 0.7^{Ab}	3751.18 ± 232.9^{Ba}	6778.5 ± 562.2^{Ab}	7931.3 ± 670.7^{Bb}

Table 1: Follicular, oocyte diameters (µm) and volume (mean ± SEM) filled by granulosa cells in preantral follicles subjected to in vitro culture

^{A,B,C} within a column ($P<0.05$)

^{a,b} within a row and the same and point ($P<0.05$)



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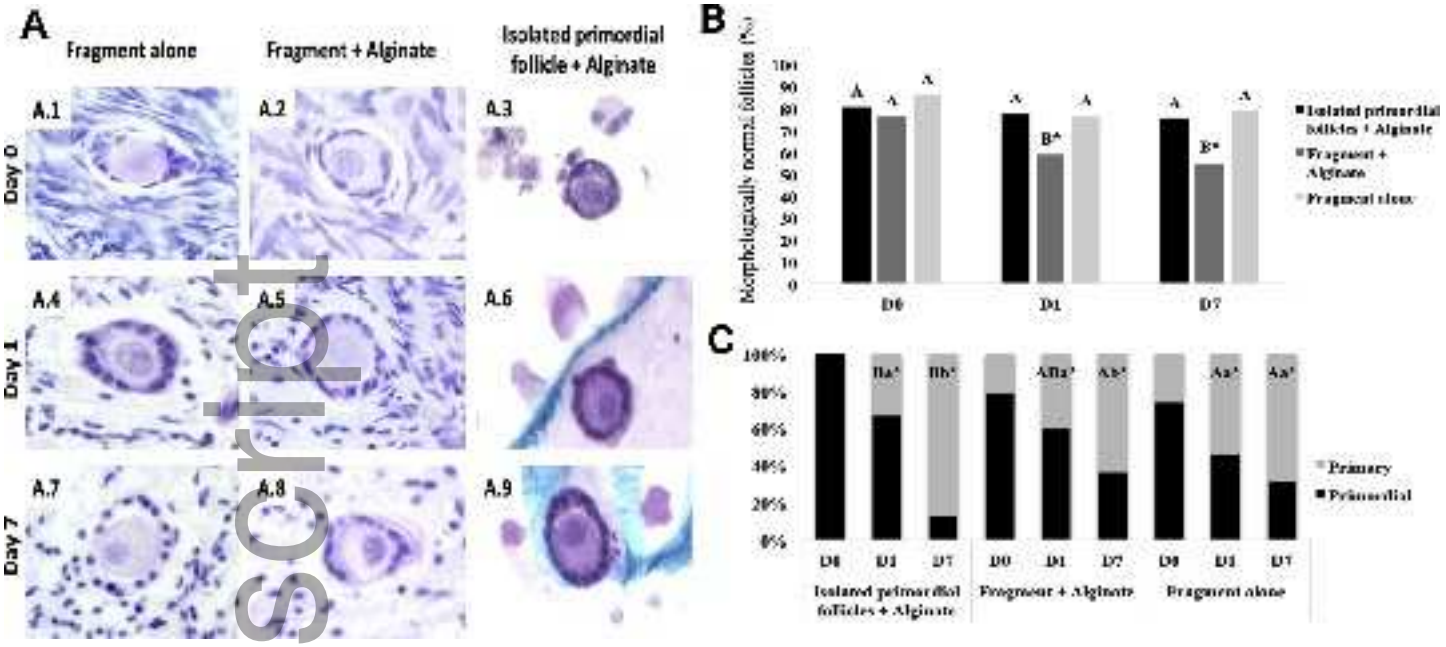

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