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Short Communication

Kit ligand promotes the transition from primordial to primary follicles after *in vitro* culture of ovine ovarian tissue

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Summary

This study evaluated the effects of kit ligand (KL) on the morphology and development of ovine preantral follicles (fresh control) and after 7 days of *in vitro* culture in α-Minimal Essential Medium (α-MEM; control medium) or the presence of KL (1, 10, 50, 100 or 200 ng/ml). There was an increase in the percentage of primary follicles at the concentration of 100 ng/ml KL, compared with the fresh control, control medium (α-MEM) and the other KL concentrations. Follicle diameter was significantly higher than the control medium only at concentrations of 50 and 100 ng/ml KL. In conclusion, 100 ng/ml KL promoted the transition from primordial to primary follicles (follicular activation) after *in vitro* culture of ovine ovarian tissue.

Keywords: Activation, Kit ligand, Oocyte, Ovary, Sheep

Introduction

The *in vitro* culture of ovarian cortex to initiate primordial follicle growth and produce developing follicles for later oocyte maturation and embryo production is a promising fertility preservation strategy. As early stages of follicle growth are not completely understood, any finding on primordial follicle development is of great importance. Relatively few substances have been effective to promote the transition from primordial to primary follicles *in vitro* [growth and differentiation factor-9: Martins *et al.*, 2008; epidermal growth factor (EGF): Celestino *et al.*, 2009; kit ligand (KL): Celestino *et al.*, 2010; insulin-like growth factor I: Martins *et al.*, 2010; leukemia inhibitory factor: Nobrega Jr *et al.*, 2011], highlighting the importance of KL.

In vitro studies have shown that KL, besides promoting the initiation of primordial follicle growth, called follicular activation (Nilsson & Skinner, 2004; Lima *et al.*, 2012), also maintained survival, and promoted oocyte maturation (Reynaud *et al.*, 2000; Jin *et al.*, 2005) and granulosa cell proliferation (Otsuka & Shimasaki, 2002) in different species. Muruvi *et al.* (2005) cultured isolated ovine primordial follicles with different concentrations of KL (0, 10 or 100 ng/ml) and observed that the addition of 100 ng/ml KL increased oocyte diameter compared with non-treated controls. A recent study has demonstrated that the association of KL (100 ng/ml), follicle stimulating hormone (FSH), EGF, basic fibroblast growth factor (bFGF) and glial-derived neurotropic factor (GDNF) promoted ovine primordial follicle activation during 18 days of *in vitro* culture (Esmaielzadeh *et al.*, 2013). However, it was not possible to conclude that KL had an effect on the *in*

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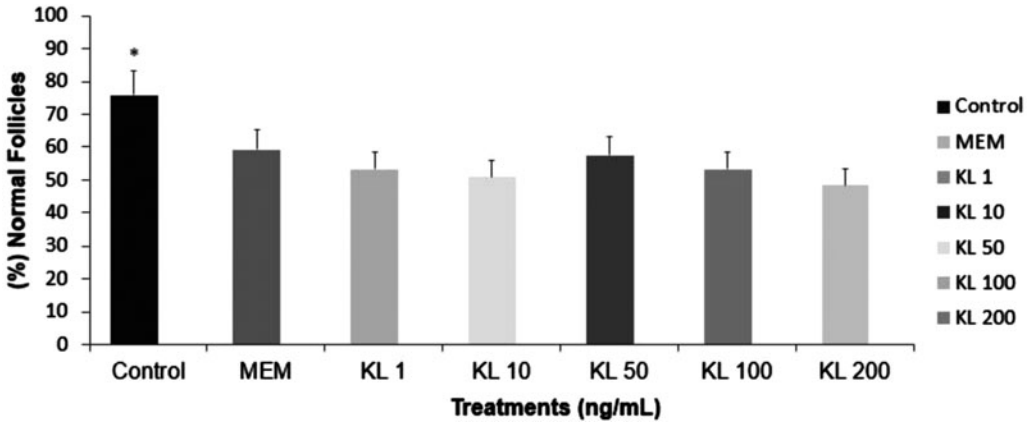

Figure 1 Percentages [(mean ± standard error of the mean (SEM)] of morphologically normal follicles in the fresh control and after 7 days of *in vitro* culture in different concentrations of KL. ∗Differs significantly from fresh control ($P < 0.05$).

vitro activation of ovine primordial follicles because it was not tested alone in the medium, which remains to be determined. Therefore, this study aimed to evaluate the effect of KL on the morphology and growth of ovine primordial follicles *in situ*.

Materials and methods

Unless noted otherwise, the chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ovaries from four adult mixed-breed sheep were collected at a local slaughterhouse, washed once in 70% alcohol and Minimal Essential Medium (MEM) supplemented with HEPES and antibiotics (100 mg/ml penicillin and 100 mg/ml streptomycin) and transported to the laboratory in MEM–HEPES at 4°C (Chaves *et al.*, 2008).

Ovarian cortex samples from each ovarian pair were cut into seven slices (approximately 3 × 3 × 1 mm). One slice was randomly selected and immediately fixed for histology (fresh control) and the remaining slices were cultured individually in 1 ml of medium in 24-well culture dishes for 7 days, at 39°C and 5% CO_2 in air. The basic culture medium (control) consisted of α-MEM (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with ITS (10 μg/ml insulin, 5.5 μg/ml transferrin and 5.0 ng/ml sodium selenite), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/ml bovine serum albumin (BSA), and 50 μg/ml ascorbic acid and then referred as α-MEM$^+$. For the experimental conditions, the control medium was supplemented with KL (human recombinant stem cell factor, H8416) at 1, 10, 50, 100 or 200 ng/ml. The culture medium was replenished every other day.

Tissues from all treatments were fixed in 4% buffered paraformaldehyde (Dinâmica, São Paulo, Brazil) for 18 h and destined to histology. The tissues were cut into 5-μm sections, stained with hematoxylin–eosin and then examined by microscopy (Nikon, Tokyo, Japan; ×400 magnification). Follicles were classified as primordial (one layer of flattened or flattened and cuboidal granulosa cells around the oocyte), primary (one layer of cuboidal granulosa cells) or secondary (two or more layers of cuboidal granulosa cells around the oocyte) (Ribeiro *et al.*, 2014), and as morphologically normal or atretic, according to the presence or absence of pyknotic nucleus, retracted oocyte, and/or disorganized granulosa cells detached from the basement membrane. To evaluate follicular activation, only morphologically normal follicles with a visible oocyte nucleus were recorded, and the proportion of primordial and growing follicles was calculated at day 0 (fresh control) and after 7 days of culture. In addition, oocyte and follicle diameters were measured using Image Pro-Plus® software (Media Cybernetics Inc., Silver Spring, MD, USA).

The percentages of normal, primordial and primary follicles were submitted to analysis of variance (ANOVA) and Tukey's test. Differences were considered to be significant when $P < 0.05$.

Results and Discussion

After culture, all treatments reduced ($P < 0.05$) the percentage of morphologically normal follicles compared with the fresh control (Fig. 1), similarly to the results found by Lima *et al.* (2012). This result may be explained by removal of the ovaries from the animal, which reduces the supply of oxygen and energy to these organs, leading to tissue necrosis (Wongsrikeao *et al.*, 2005). However, no difference ($P > 0.05$) was observed between control medium and KL treatments. Other authors have also shown no effect of KL on follicle survival (Muruvi *et al.*, 2005; Hutt

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Table 1 Mean oocyte and follicular diameter (mean ± standard deviation) in the fresh control and after *in vitro* culture of ovine preantral follicle in different concentrations of KL

Treatment group	Oocyte diameter (μm)	Follicle diameter (μm)
Fresh control	34.38 ± 3.66	50.98 ± 2.80
MEM^{+}	33.85 ± 4.46	51.45 ± 4.35^{b}
KL 1 ng/ml	34.69 ± 3.99	53.61 ± 3.4^{b,c}
KL 10 ng/ml	34.14 ± 4.40	51.29 ± 5.30^{b}
KL 50 ng/ml	37.02 ± 4.72	57.12 ± 4.99^{*,a}
KL 100 ng/ml	37.11 ± 6.74	56.49 ± 8.68^{*a,c}
KL 200 ng/ml	32.69 ± 5.27	50.8 ± 7.06^{b}

*Differs significantly from fresh control ($P < 0.05$).
$^{a, b}$Different letters denote significant differences within the column ($P < 0.05$).

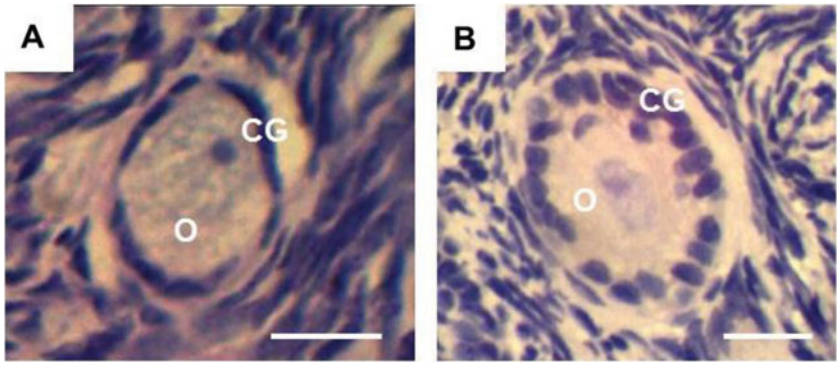

Figure 2 Normal (*A*) primordial follicle in the fresh control and (*B*) primary follicle after culture in medium containing 100 ng/ml KL. Hematoxylin–eosin staining. (O, oocyte; CG, granulosa cells. Scale bar: 20 μm; ×400 magnification).

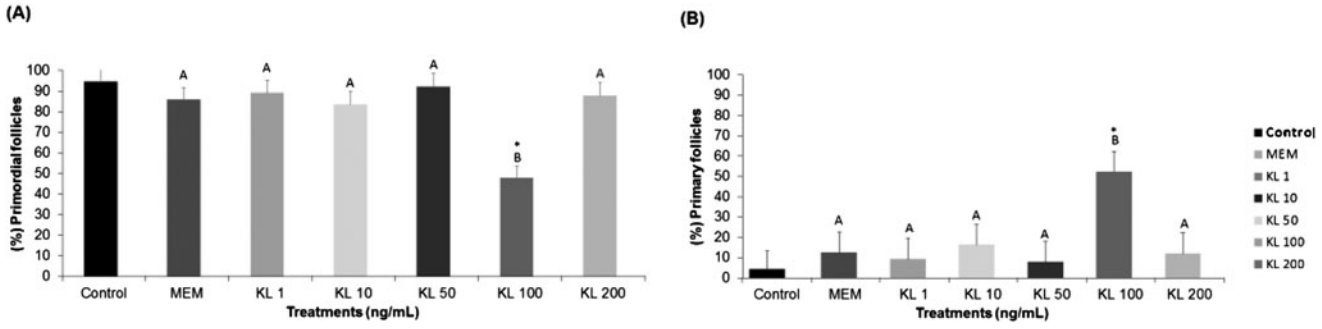

Figure 3 Percentages of normal primordial (*A*) and primary (*B*) follicles in the fresh control and in medium containing KL after 7 days of *in vitro* culture. ∗Differs significantly from fresh control ($P < 0.05$). ^{A,B}Different letters denote significant differences among treatments (different media) ($P < 0.05$).

et al., 2006). This result could be explained due to the basic culture medium (α-MEM^{+}) composition, which is rich in nutrients (amino acids, carbohydrate and vitamins) that may contribute to follicular survival (Faustino *et al.*, 2013). Moreover, our control medium was supplemented with insulin and ascorbic acid, important substances for the maintenance of viability *in vitro* (Adhikari & Liu, 2009; Rosseto *et al.*, 2009). Nevertheless, after using a basic medium less rich in nutrients than ours, Celestino *et al.* (2010) observed a positive effect on caprine follicular survival at 50 ng/ml KL.

There was a reduction ($P < 0.05$) in the percentage of primordial follicles (Figs 2*A* and 3*A*) and an increase in the primary follicles (52.2%) (Figs 2*B* and 3*B*) at 100 ng/ml KL, compared with the fresh control (4.4%), α-MEM (12.7%) and other KL concentrations (9.4; 16.4; 7.8 and 12.1% primary follicles for 1, 10, 50 and 200 ng/ml KL, respectively). This is an important finding as few substances have demonstrated this capacity,

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especially in FSH-free medium and after a short period of 7 days of culture. Other studies have shown that KL promotes the transition from primordial to primary follicles in rodents (Nilsson & Skinner, 2004; Wang & Roy, 2004) and goats (Celestino *et al.*, 2010, Lima *et al.*, 2012). In our study, follicle diameter was significantly higher than the control medium only at concentrations of 50 and 100 ng/ml KL (Table 1). This substance may have a role in the early follicular development through Akt activation (a signalling molecule known for increasing cell proliferation and survival, as well as protein synthesis) and inhibition of FKHRL1 (Akt substrate and transition factor that leads to apoptosis and cell cycle arrest) in the oocyte (Reddy *et al.*, 2005).

In conclusion, 100 ng/ml KL promotes the transition from primordial to primary follicles (follicular activation) after *in vitro* culture of ovine ovarian tissue, being an important step for further follicular growth, maturation and *in vitro* production of embryos.

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Declaration of interest

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

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