Short communication

Kit ligand and insulin-like growth factor I affect the in vitro development of ovine preantral follicles

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A B S T R A C T

We investigated the effect of KL and IGF-I, alone or in combination, on the in vitro survival, growth and antrum formation of sheep preantral follicles, and subsequent oocyte developmental competence. Preantral follicles ∼200 μm were isolated and cultured for 18 days in basic medium (control) added or not by KL, IGF-I or both. Every six days follicular survival, growth and antrum formation was evaluated. At the end of the culture period, the oocytes underwent in vitro maturation (IVM) and their viability and chromatin configuration were assessed. To evaluate oocyte quality and its developmental competence, parthenogenetic activation and in vitro fertilization (IVF) of the oocytes recovered from in vitro grown preantral follicles were performed. Medium supplementation with KL improved follicular integrity and antrum formation. IGF-I promoted the increase in follicular diameter at the end of the in vitro culture when compared to control, but not differently from KL. Follicles cultured in the presence of KL presented the highest percentages of meiosis resumption (92.3%). Therefore, these oocytes were submitted to parthenogenetic activation and IVF resulting in a percentage of 35.6% of parthenotes and 23.1% of 2- or 4-cells embryos. Medium supplementation with KL results in increased rates of antrum formation, as well as in meiosis resumption, resulting in the production of embryos using parthenogenetic activation or IVF.

1. Introduction

In vitro embryo production from in vitro grown caprine and ovine preantral follicles (PFs) has been reported (Arunakumari et al., 2010; Saraiva et al., 2010; Magalhães et al., 2011). Barbini et al. (2011) developed sheep preantral follicles in vitro, resulting in meiotic and developmental competent oocytes. The in vitro production of embryos from immature oocytes enclosed in PFs requires reliable methods of evaluation. One method consists of in vitro fertilization (IVF) of in vitro matured (IVM) oocytes. Another valuable tool to analyze oocyte quality is the parthenogenetic activation, which has been applied to evaluate the competence of oocytes recovered from in vitro developed sheep PFs (Barboni et al., 2011; Luz et al., 2012). PFs can be in vitro cultured (IVC) in a variety of
systems using complex culture media supplemented with hormones (Cecconi et al., 1999, 2008) and several growth factors such as leukemia inhibiting factor (LIF) (Reynaud and Driancourt, 2000), kit ligand (KL) (Tisdall et al., 1997) and insulin-like growth factor 1 (IGF-I) (Webb et al., 2004), which are known to play a role in sheep folliculogenesis. In sheep in vitro studies, the presence of LIF in the culture medium improved the development of sheep PFs, meiosis resumption, and promoted the production of parthenogenesises (Luz et al., 2012). Furthermore, follicular survival, antrum formation, oocyte growth and meiosis resumption were obtained when goat secondary follicles were in vitro cultured in the presence of KL (Lima et al., 2011). Also, IGF-I was able to maintain follicular survival, as well as promote follicular activation, growth and meiosis resumption in sheep (Arunakumari et al., 2010). As KL and IGF-I play a role in folliculogenesis, such factors, alone or in combination, may be important for the in vitro development of sheep PFs. Therefore, our aim was to evaluate if KL and IGF may enhance the in vitro development of isolated sheep PFs.

2. Methods

Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, USA). Ovaries were collected at a local slaughterhouse from twenty adult (1–3 years old), cross-breed ewes, making a total of 5 repetitions (4 ewes/repetition). The ovaries were washed in 70% alcohol followed by two rinses in Minimum Essential Medium (MEM) containing HEPES and antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin), and transported within 1 h to the laboratory in MEM at 4 °C. In the laboratory, ovarian cortical slices (1–2 mm thick) were placed in MEM plus HEPES. PFs ~200 µm in diameter were visualized under a stereomicroscope (SMZ 645, Nikon, Tokyo, Japan) and manually dissected from the strips of ovarian cortex using 26 gauge needles. After isolation, PFs (n = 200) were transferred to 100 µL drops containing fresh medium under mineral oil to further evaluate follicular quality. PFs with a visible oocyte, surrounded by granulosa cells, an intact basement membrane and no antral cavity were selected for IVF. After selection, 117 PFs were cultured in 100 µL drops of IVC medium in Petri dishes (60 mm × 15 mm, Corning, USA) under mineral oil for 18 days at 39 °C and 5% CO2 in air in basic IVC medium (control; n = 29), or in basic medium supplemented with 50ng/ml KL (n = 30), 50ng/ml IGF-I (n = 29), or 50ng/ml KL + 50ng/ml IGF-I (n = 29). The basic IVC medium (control) was selected based on a previous study (Luz et al., 2012), i.e. α-MEM (pH 7.2–7.4) supplemented with 3 ng/ml bovine serum albumin (BSA), ITS (insulin 10 µg/ml; transferrin 5.5 µg/ml; and sodium 5 ng/ml), 2 mM glutamine, 2 mM hypoxantin, 50 µg/ml of ascorbic acid and 50 ng/ml of LIF. IVC medium was replenished every six days until Day 18. Only PFs showing intact basement membrane, bright and homogeneous granulosa cells and absence of morphological signs of degeneration, such as darkness of the oocytes and surrounding cumulus cells, or deformed oocytes, were classified as intact. Every six days (days 0, 6, 12 and 18) intact follicles were analyzed regarding: (i) antrum formation, and (ii) the diameter, measured as the mean of two perpendicular measures of each preantral follicle using an ocular micrometer (100x magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). Intact follicles without antrum formation after IVC were classified as degenerated.

2.1. In vitro maturation (IVM) of ovine oocytes from in vitro cultured (IVC) follicles

At the end of the culture period, the oocytes were carefully harvested from intact follicles using 26-G needles under a stereomicroscope. Only oocytes greater than 110 µm in diameter with a homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for IVM. The recovery rate was calculated by dividing the number of oocytes greater than 110 µm by the number of viable follicles at day 18 of culture multiplied by 100. The selected cumulus-oocyte complexes were washed three times in maturation medium composed of TCM199 + sodium bicarbonate (TCM199B) supplemented with 0.5 µg/ml FSH, 5 µg/ml LH, 1 µg/ml 17β-estradiol, 10 ng/ml recombinant epidermal growth factor (EGF), 0.911 nM/L pyruvate, 100 µM/L cysteamine, 50 ng/ml recombinant insulin-like growth factor 1 (IGF-I) and 1% BSA. After washing, the oocytes were transferred to 50 µL drops of maturation medium under mineral oil and then incubated for 40 h at 39 °C with 5% CO2.

2.2. Assessment of oocyte viability and chromatin configuration

After the maturation period, the oocytes were subjected to viability analysis. To this end, the oocytes were incubated in 100 µL drops of 2 µM ethidium homodimer-1 supplemented with 4 µM calcein-AM and 10 µM Hoechst 33342 at 37 °C for 15 min. After this, the oocytes were washed three times in TCM199 and were visualized under fluorescence microscopy (Nikon Eclipse 80i, Tokyo, Japan). Oocytes were considered viable if the cytoplasm was positively stained with calcein-AM (green) and not stained with ethidium homodimer-1 (red). The Hoechst staining was used to evaluate the nuclear chromatin configuration.

2.3. Assessment of oocyte developmental competence

Oocytes obtained from in vitro cultured PFs were submitted to parthenogenetic activation (n = 14) or to IVF (n = 60). For this, IVC was performed in the presence of Control + KL (50 ng/mL), based on the highest rates of antrum formation in this medium. Subsequently, IVM was performed as described above. In vivo matured oocytes (n = 65) obtained by puncture from four ovaries were used as control.

2.4. Parthenogenetic activation using oocytes from ovine in vitro developed PFs

The oocytes grown and in vitro matured were activated by exposure to 5 µm ionomycin in TCM-HEPES supplemented with 0.4% BSA for 5 min followed by a 3.5 h incubation in 2 mM 6-DMAP in SOFaa + 0.4% BSA at 39 °C. The activated oocytes were cultured in SOFaa + 10% FCS at 39 °C under 5% CO2 for 5 days. Cleavage and embryo formation rates were determined on Days 2 and 6, respectively.

2.5. In vitro fertilization (IVF) of oocytes from in vitro developed PFs

Following IVM, IVF-TALP was used for sperm-oocytes co-incubation. To this end, droplets (50 µL) of IVF-TALP were prepared in a 60 µm Petri dish, covered with mineral oil and allowed to equilibrate in the incubator for 1 h before sperm-oocytes co-incubation. Frozen semen from a Dopper ram (Alta Genetics, Uberaba, Minas Gerais, Brazil – 0.25 mL straw, 50 × 105 spermstraw) from each batch was thawed at 37 °C for 30 s. The thawed semen was layered onto a discontinuous Percoll gradient column in a 15 mL centrifuge tube (bottom layer 1 mL 90% and top layer 1 mL 45%) (Parrish et al., 1995). The tube was then centrifuged at 1,800 rpm for 10 min, and the supernatant was discarded after leaving 100 µL of the sperm pellet. The sperm concentration in the pellet was determined using a hemocytometer and then adjusted to 2 × 106 mL-1 with capacitation-Tyrode’s albumin lactate pyruvate (CAP-TALP) supplemented with fatty acid free (BSA-6 mg/mL). The diluted semen was then mixed with CAP-TALP (1:1, v/v) containing 20 µg/mL heparin (final concentrations in the IVF droplet = 10 µg/mL heparin) and incubated for 15 min at 39 °C under 5% CO2 in a humidified air atmosphere. After 27 h of maturation, 14 COCs were expanded, removed from the IVM droplets, washed three times in washing TALP and then once in IVF-TALP.

Next, the COCs were transferred to each droplet of IVF-TALP (10 COCs/drop), after which 2 µL PHE (20 µM penicilline, 10 µM hypotaurine and 1 µM epinephrine) and 5 µL capacitated sperm suspension were added to each fertilization droplet. Spermatozoa and COCs were co-incubated for 24 h at 39 °C under 5% CO2 in a humidified air atmosphere. Following 24 h of insemination, presumptive zygotes were washed three times in SOF medium and cultured in 100 µL droplets of embryo culture medium consisting of SOFaa supplemented with 5% fetal bovine serum and 1% BSA, then covered with mineral oil. The dishes were placed in a 5% CO2 incubator maintained at 39 °C for 6 d. After in vitro culture (IVC), the presumptive zygotes were assessed after protocol staining with ethidium, hoechst and calcein using the protocol described above.
Table 1
Recovery rate of oocytes >110 μm, viability and chromatin configuration of oocytes from in vitro grown ovine preantral follicles after 18 days of culture. A,B: Different uppercase letters indicate significant differences among treatments (P < 0.05).

<table>
<thead>
<tr>
<th>Recovery rate &gt;110 μm oocytes</th>
<th>% viable oocytes (n)</th>
<th>% of meiosis resumption (n)</th>
<th>% MII (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>62.0 (18/29)</td>
<td>88.8 (16/18)</td>
<td>44.4 (8/18)B</td>
</tr>
<tr>
<td>KL</td>
<td>43.3 (13/30)</td>
<td>84.6 (11/13)</td>
<td>92.3 (12/13)A</td>
</tr>
<tr>
<td>IGF-I</td>
<td>51.7 (15/29)</td>
<td>86.0 (13/15)</td>
<td>60.0 (9/15)A</td>
</tr>
<tr>
<td>KL + IGF-I</td>
<td>65.5 (19/29)</td>
<td>89.4 (17/19)</td>
<td>68.4 (13/19)AB</td>
</tr>
</tbody>
</table>

A,B: Different uppercase letters indicate differences among treatments (P < 0.05).

2.6. Statistical analysis

The follicles were considered to be the experimental unit. Data from discrete variables (intact follicles, antrum formation, recovery rate, viable oocytes, meiotic resumption, metaphase II) were analyzed as dispersion of frequency and compared using Chi-square test, with the results expressed in percentages. Follicular diameter, as a continuous variable, was initially submitted to Kruskal–Wallis and Bartlett tests to verify normality of distribution and homogeneity of variance among treatments, respectively. Data showed normal distribution, but did not show homoscedasticity, even after log transformation. Therefore, data were compared using Kruskal–Wallis tests. Results were expressed as mean ± Standard Error of Means (SEM) and differences were considered to be significant when P < 0.05.

3. Results

During IVC there was a progressive reduction in the percentage of follicles presenting intact basement membrane from 100% at Day 0, to 97.47 ± 1.60 at Day 6, 68.44 ± 4.94 at Day 12 and 10.00 ± 2.06 at Day 18. However, no significant effect of treatment group on these percentages was found. After 6 days IVC, antrum formation was observed. When compared to controls, the highest rate of increase was observed for follicles treated with KL (72.2 ± 0.95%) (P < 0.05), and IGF-I alone (48.2 ± 0.80%) or IGF-1 plus KL (40.8 ± 1.27%) were not different from controls (26.4 ± 0.98%).

Follicular diameter increased significantly from day 0 to day 18 in all treatments containing KL or IGF-I. When the treatments were compared with each other, at days 6 and 12 of culture, the follicular diameter was lower in the control than the other treatments. Follicles treated with IGF-I presented higher follicle diameter (720.66 μm) than controls (395.40 μm) by Day 18 and intermediate values were observed for KL (545.92 μm) and KN plus IGF-I (497.44 μm) treated follicles.

At the end of IVC, only those oocytes greater than 110 μm underwent in vitro maturation; no significant differences were observed among the groups regarding recovery rate and follicular viability. The only treatment with a significantly higher rate of meiosis resumption compared to control (P < 0.05) was KL, which resulted in 23% of MI oocytes (Table 1). Therefore, this treatment was selected for in vitro embryo production after parthenogenetic activation and IVF. The percentages of embryos produced after IVF from oocytes grown in vivo (29.0%) or in vitro (21.3%), as well as after parthenogenetic activation (35.6%) are shown in Table 2.

4. Discussion

In this study, we have shown the effects of KL with or without IGF-I, on the in vitro development of sheep PFs. Although follicular integrity was similar among treatments within each evaluated day of IVC, after days 6 of IVC, medium supplementation with KL resulted in an increased rate of antrum formation. According to Reynaud et al. (2000), blockage of KL results in an impairment of antrum formation.

The rate of antrum formation was constant from days 6 to 18 in all treatments, and it was accompanied by a low rate of intact follicles after 18 days IVC. This shows that our medium composition was inefficient to preserve follicular viability for 18 days. Cecconi et al. (2004) recovered healthy oocyte cumulus complexes after 10 days IVC of sheep preantral follicles. In a recent study Barboni et al. (2011), successfully in vitro cultured sheep PFs for 14 days, obtaining ~10% of 16 cells embryos after parthenogenetic activation of in vitro grown oocytes. Apart from our long-term IVC, our cultured medium was FSH-free, which is different from the culture medium used by the above cited authors (Cecconi et al., 2004; Barboni et al., 2011). After 18 days of IVC, medium supplementation with IGF-I resulted in an increased follicular diameter when compared to control. Also studying the in vitro development of sheep preantral follicles, Arunakumari et al. (2010) showed that IGF-I was able to support follicular development, but in combination with GH and FSH, which was not applied in the present study.

Table 2
Percentage and embryo production after IVF and parthenogenetic activation.

<table>
<thead>
<tr>
<th>Embryo stages</th>
<th>IVF</th>
<th>Parthenogenetic activation from in vitro grown oocytes n = 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo grown oocytes (n = 65)</td>
<td>In vitro grown oocytes (n = 60)</td>
</tr>
<tr>
<td>2 cells</td>
<td>21.5 (14)</td>
<td>6.6 (4)</td>
</tr>
<tr>
<td>3–4 cells</td>
<td>3.0 (2)</td>
<td>11.6 (7)</td>
</tr>
<tr>
<td>5–8 cells</td>
<td>3.0 (2)</td>
<td>3.3 (2)</td>
</tr>
<tr>
<td>≥8 cells</td>
<td>1.5 (1)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>Mórlula</td>
<td>0 (0)</td>
<td>1.6 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>29.0 (19)</td>
<td>23.1 (14)</td>
</tr>
</tbody>
</table>
Here, we demonstrated the efficiency of medium containing KL on the IVM of sheep oocytes harvested from preantral follicles, in which we obtained 92.3% of meiosis resumption, reaching 23% of oocytes in MII. It has been reported that 50 ng/ml KL is able to promote cytoplasmic maturation in mice (Reynaud et al., 2000), as well as induces granulosa cells differentiation in sheep (Muruvui et al., 2005).

Although we enriched IVM and IVF media as reported in the literature (Mara et al., 2013), the embryo developmental rates obtained from in vivo and in vitro matured oocytes were very low after IVF. According to Mara et al. (2013) sheep oocyte quality is season dependent in this may affect blastocyst production in vitro. Indeed, our experiment was performed in the dry season of the year in the Northeast from Brazil. Furthermore, the low embryonic development from IVM/IVF oocytes harvested from in vitro grown PFs was expected, since at the moment, successful protocols are available only for rodents. Oocytes grown in medium supplemented with KL and parthenogenetically activated were able to produce five parthenotes, one at morulae stage. The number of parthenotes was lower than that observed in our previous study (Luz et al., 2012), where we have obtained 58% of 8-cells stage parthenotes. We suggest that dry season in the present study did affect the quality not only of the mature oocytes but also from PFs (Cahill, 1981).

Ptak et al. (2006) reported that LIF improves cleavage rates in oocytes parthenogenetically activated, which was confirmed in a recent study performed by our group (Luz et al., 2012). Kim et al. (2009) using rat preantral follicles, reported a cleavage rate of 25.3%. However, only one blastocyst was produced. Also in mice, Lee et al. (2008) reached cleavage and blastocyst rates of 22.5% and 2.8%, respectively. Recently, Arunakumari et al. (2010) have cultured sheep preantral follicles for six days in a medium rich in thyroxine, IGF-I, GH and FSH, followed by IVM and IVF, resulting in the production of 10 (12.31%) embryos at morulae stage.

In conclusion, we found that KL improves antrum formation and meiosis resumption. IGF-I was related to the increase in the follicular diameter, but did not improve antrum formation when compared to KL treated group. Most importantly, oocytes harvested from sheep preantral follicle cultured in the presence of KL resulted in embryos produced by parthenogenetic activation, as well as by IVF. However, our present long-term IVC system of 18 days remains to be improved.

Competing interests

There is no conflict of interest.

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