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ONLY VEGF IS NECESSARY TO STIMULATE THE MEIOTIC COMPETENCE OF CAPRINE OOCYTES DURING IN VITRO MATURATION

(Apenas VEGF é necessário para estimular a competência meiótica de oócitos caprinos durante a maturação in vitro)

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RESUMO

Para o sucesso da maturação in vitro (MIV), faz-se necessário uma sincronização entre as maturação nuclear e citoplasmática. Desta forma, o presente estudo teve por objetivo comparar o efeito do fator de crescimento do endotélio vascular (VEGF) como fator de crescimento na maturação in vitro (MIV) de oócitos caprinos com os fatores de crescimento epidermal (EGF) e semelhante à insulina I (IGF-I) adicionados juntos. Complexos cúmulos oócito (CCOs) foram isolados por slicing de ovários caprinos (n=40) utilizando bisturi cirúrgico. Apenas oócitos ≥110 µm com citoplasma homogêneo circundado por pelo menos uma camada compacta e coesiva de células do cúmulus foram selecionados para MIV. O meio de cultivo de base foi o TCM199 suplementado com 1% de BSA, 5 µg/mL de hormônio luteinizante (LH), 0,5 µg/mL de hormônio folículo estimulante recombinante (rFSH®), 0,911 mMol/L de piruvato, 1 µg/mL de estradiol, e 100 µM de cisteamina, o qual foi denominado TCM199^{+}. Os CCOs selecionados foram distribuídos aleatoriamente nos seguintes tratamentos: 1) EGF e IGF-I, TCM199^{+} suplementado com 10 ng/mL de EGF e 50 ng/mL de IGF-I; 2) VEGF, TCM199^{+} suplementado com 100 ng/mL de VEGF. Após a MIV, foram utilizados marcadores fluorescentes para avaliar a configuração da cromatina (Hoechst) e a viabilidade oocitária (calceína-AM e etídio homodímero). De uma maneira geral, ambos os tratamentos VEGF (80.2 e 35.7%) e EGF e IGF-I (70.5 e 29.9%) apresentaram resultados similares de quebra da vesícula germinativa (GVBD) e metáfase II rates (MII), respectivamente, sendo o tratamento VEGF numericamente superior ao EGF e IGF-I. Desta forma, conclui-se que o VEGF atua de forma similar ao EGF e IGF-I na competência oocitária para retomar a meiose.

Palavras-chaves: Competência oocitária, metáfase II, cabra, fatores de crescimento.

ABSTRACT

For successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation. Thus, the aim of the present study was to compare vascular endothelial

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growth factor (VEGF) as a growth factor to the in vitro maturarion (IVM) to caprine oocytes instead of epidermal growth factor (EGF) plus insulin like growth factor I (IGF-I).Then, cumulus oocyte complexes (COCs) were pooled from slicing of caprine ovaries (n=40) using a surgical blade. Only oocytes ≥110 μm with homogeneous cytoplasm and surrounded by at least one compact layer of shiny and cohesive cumulus cells were selected to the IVM. The basic culture medium consisted of TCM199 supplemented with 1% of BSA, 5 μg/mL of luteinizing hormone (LH), 0.5 μg/mL of recombinant follicle-stimulanting hormone (rFSH®), 0.911 mMol/L pyruvate, 1 μg/mL estradiol, and 100 μM cysteamine named TCM199+. The selected COCs were randomly distributed in the following treatments: 1) EGF plus IGF-I, TCM199+ supplemented with 10 ng/ml of EGF and 50 ng/mL of IGF-I; 2) VEGF, TCM199+ supplemented only with 100 ng/mL of recombinant VEGF-A. After the IVM, oocytes were stained with fluorescent markers for the assessment of chromatin configuration (Hoechst) and to evaluate oocyte viability (calcein-AM and ethidium homodimer). Overall, both VEGF (80.2 and 35.7%) and EGF plus IGF-I (70.5 and 29.9%) treatments presented similar results of germinal vesicle breakdown (GVBD) and metaphase II rates (MII), respectively, being VEGF numerically greater than EGF plus IGF-I. Thus, we can infer that VEGF may act similar to EGF plus IGF-I in in vitro oocyte competence to resumes meiosis.

Key words: oocyte competence, metaphase II, goat, growth factors

INTRODUCTION

In vitro maturation (IVM) is the most critical part of the whole process of in vitro embryo production. For successful IVM, oocytes must undergo synchronically nuclear and cytoplasmic maturation. Growth factors also have been added to IVM to improve in vitro oocyte maturation. Epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) are routinely used (PARAMIO e IZQUIERDO, 2014) as a growth factor to enhance the media to in vitro oocyte maturation. However, this supplementation could makes the procedure more expensive.

It is well established that VEGF has a variety of physiological functions including some in the reproductive system and folliculogenesis (ARAÚJO *et al.*, 2011a). In caprine, VEGFR-2/KDR (a VEGF type II receptor), is expressed in oocytes of follicles from all developmental stages and in the cumulus cells of antral follicles (BRUNO *et al.*, 2009). The presence of this receptor on both oocytes (BRUNO *et al.*, 2009; YAN *et al.*, 2012; KERE *et al.*, 2014) and cumulus cells (BRUNO *et al.*, 2009), and in granulosa cells (ABIR *et al.*, 2010) is a strong evidence that VEGF may be involved on the acquisition of oocyte competence. In addition, the complex

VEGF/type II receptor activates the MAPK signaling pathway, and contributes to create an environment optimal for oocyte, which may promote oocyte maturation (YAN *et al*., 2012).

During follicular development, the production of follicular fluid is known to be enhanced by the increased follicular vascularization and permeability of blood vessels (van den HURK e ZHAO, 2005). Along with that, VEGF concentrations at follicular fluid increase as the antrum cavity became larger (KERE *et al*., 2014) and with increasing follicle size (BARBONI *et al*., 2000; GREENAWAY *et al*., 2004). Moreover, an in vitro study has shown similar results since, in preantral follicles, the production of VEGF during the culture period became larger throughout follicular diameter increases (FISHER *et al*., 2009), leading enhances in the in vitro growth rate these type of follicles (ARAÚJO *et al*., 2011b). Although no blood supplementation is available in the in vitro environment, the ability of exogenous VEGF to increase the permeability of the cells might have promoted the increased availability of nutrients and substances important for the growth of oocytes and the acquisition of meiotic competence (ARAÚJO *et al*., 2011b).

Despite strong evidence demonstrating VEGF may be involved on the acquisition of oocyte competence, we have hypothesize that VEGF could replace EGF plus IGF-I, which were routinely used to in vitro maturation of caprine oocytes. Therefore, the aim of this study was to compare VEGF as a growth factor to the meiosis resumption during in vitro maturation of caprine oocytes instead of EGF plus IGF-I.

MATERIAL AND METHODS

Chemicals and media

Unless mentioned otherwise, the culture media, VEGF and other chemicals used in the present study were purchased from Sigma Chemical Co (St Louis, MO).

Source of ovaries

Ovaries (n = 40) from 20 adult mixed-breed goats (one to three years old) were collected at a local slaughterhouse. The surrounding fat tissue and ligaments were removed, and the ovaries were washed in 70% alcohol, followed by two washes in minimum essential medium (MEM) plus HEPES (MEM-HEPES), supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin. The ovaries were placed into tubes containing 15 mL of MEM-HEPES, and then transported to the laboratory at 4 °C (CHAVES *et al*., 2008) within one hour. The animal ethical committee (n. 10610761-5/59) from State

Univeristy of Ceará (CEUA-UECE) approved the present study.

Collection and morphological evaluation of cumulus oocyte complexes (COCs)

Cumulus oocyte complexes (COCs) were pooled from slicing of caprine ovaries using a surgical blade. Only oocytes (≥110 μm) with homogeneous cytoplasm and surrounded by at least one compact layer of shiny and cohesive cumulus cells were selected using a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; 100x magnification) in TCM199 supplemented with HEPES and bovine serum albumin (BSA, 1%).

In vitro maturation (IVM) of caprine oocytes

The basic culture medium consisted of TCM199 supplemented with 1% of BSA, 5 μg/mL of luteinizing hormone (LH), 0.5 μg/mL of recombinant follicle-stimulanting hormone (rFSH®), 0.911 mMol/L of pyruvate, 1 μg/mL of estradiol, and 100 μM of cysteamine, named as TCM199^{+}. The selected COCs were randomly distributed in the following treatments: 1) EGF plus IGF-I, TCM199^{+} supplemented with 10 ng/ml of epidermal growth factor (EGF) and 50 ng/mL of insulin-like growth factor-I (IGF-I); 2) VEGF, TCM199^{+} supplemented with 100 ng/mL of human recombinant VEGF. After washing, the oocytes were transferred to 100 μL drops of maturation medium under mineral oil, and then incubated for 26 h at 39 °C with 5% CO_2 in air. At the end of the maturation period, oocytes were stained with 10 μM Hoechst 33342 (483 nm) for the assessment of chromatin configuration. The oocyte meiotic stage was analyzed for intact germinal vesicle (GV), germinal vesicle breakdown (GVBD), anaphase I (AI), metaphase II (MII) and meiotic resumption (including GVBD, MI, AI and MII). The concentrations of VEGF and the basic medium used were based on the previous experiments (ARAÚJO *et al.*, 2011b).

Viability assessment of oocytes matured in vitro

For a better evaluation of oocyte viability, after the IVM live/dead fluorescent staining was performed on caprine oocytes in 100 μL droplets of MEM mounted in glass slides with 4 μM calcein-AM and 2 μM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), followed by an incubation at 39 °C for 15 min. Finally, the oocytes were examined using a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan). The emitted

fluorescent signals of calcein-AM and ethidium homodimer were collected at 488 and 568 nm, respectively. While the first probe detected the intracellular esterase activity of viable cells, the later labeled the nucleic acids of non-viable cells by plasma membrane disruption. The oocytes were considered live if the cytoplasm was stained positively with calcein-AM (green) and if chromatin was not labeled with ethidium homodimer (red).

Statistical analysis

Data from meiotic resumption after in vitro maturation in each treatment were compared using the Chi-square test, with the results expressed as percentages. Differences were considered to be significant when $p<0.05$.

RESULTS AND DISCUSSION

The present study reports the effect of VEGF on the in vitro maturation of caprine oocytes grown in vivo. In the table 1 demonstrates all the stages of meiosis resumption from caprine oocytes after IVM. The VEGF treatment presented a lower percentage of germinal vesicle (GV, 19.0%) compared to EGF plus IGF-I treatment (27.7%) and a concomitant increase of metaphase II rate (MII, 35.7% to VEGF vs 29.9% to EGF plus IGF-I treatment), although there was no difference between treatments ($p>0.05$).

Table 1: Stage of meiosis from caprine oocytes after in vitro maturation using TCM 199 supplemented with EGF (10 ng/mL) and IGF-1 (50 ng/mL) or only TCM199 supplemented with VEGF (100 ng/mL).

	EGF plus IGF % (n)	VEGF % (n)
GV	27.7 (38)	19.0 (24)
GVBD	8.0 (11)	9.5 (12)
MI	27.7 (38)	31.0 (39)
AI	0.7 (1)	0.8 (1)
MII	29.9 (41)	35.7 (45)
Dead	5.8 (8)	4.0 (5)
Total	137	126

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI, Anaphase I; and MII, metaphase II. There was no difference among treatments ($p>0.05$).

For the first time, the present study compare the influence VEGF to in vitro maturation of caprine oocytes versus EGF plus IGF-I. The results of this study indicate that the addition of VEGF to the maturation medium show similar rate of the metaphase II, or even the germinal vesicle breakdown rates when compared to EGF plus IGF-I. Our basic medium containing BSA, pyruvate, and cysteamine which acts as protein, energy and antioxidant source, respectively (de MATOS *et al*., 2002; PARAMIO, 2010). The hormones used in basic composition (estradiol, LH, and FSH) are generally used in in vitro maturation protocols to improve nuclear and cytoplasmic oocyte maturation, as well as expansion of the surrounding cumulus cells in ovine (GULER *et al*., 2000) and caprine (PAWSHE *et al*., 1996) oocytes. The EGF and IGF has been used to stimulate oocyte maturation and promoting blastocyst development in sheep (GULER *et al*., 2000) and goat (ARAÚJO *et al*., 2011b). In our study, the rates of oocyte maturation were similar between EGF plus IGF-I, and VEGF treatments. Thus, the addition of only a growth factor to the basic maturation medium, as VEGF, make the maturation medium cheaper than the it supplemented with EGF and IGF-1 together.

Table 2. Percentage of caprine oocyte with meiotic resumption after in vitro maturation using TCM 199 supplemented with EGF (10 ng/mL) and IGF-1 (50 ng/mL) or only TCM199 supplemented with VEGF (100 ng/mL).

	EGF plus IGF	VEGF
GV	29.5 (38)	19.8 (24)
Meiotic resumption*	70.5 (91)	80.2 (97)
Total[#]	129	121

GV, germinal vesicle; *Meiotic resumption, including germinal vesicle breakdown, metaphase I, anaphase I, and metaphase II. [#]Total of the live oocytes, excluding the dead oocytes. There was no difference among treatments ($p>0.05$).

Moreover, **Table 2** demonstrates that overall VEGF treatment presented the higher germinal vesicle breakdown rate (GVBD, 80.2%) when compared to EGF plus IGF-I medium (70.5%; $p>0.05$). It should be noted that the rate of dead

oocytes was low in both treatments (4.0% to VEGF vs 5.8% to EGF plus IGF-I treatment).

Previously, we have found that VEGF added to the growth of caprine preantral follicles could significantly increase the rate of suitable oocytes more than 110 μm diameter for IVM and was able to stimulate meiotic resumption, enhances in vitro maturation in medium added of EGF plus IGF-I (ARAÚJO *et al*., 2011b). In oocytes grown in vivo, the addition of VEGF to the maturation medium significantly increases the percentage of sheep oocytes at MII, promotes a normal distribution of α-tubulin, and chromosomes in the spindle (CAO *et al*., 2009) and increase cytoplasmic VEGF content, and subsequently expression of its receptors (YAN *et al*., 2012). Moreover, in a similar way, Einspanier *et al*. (2002) and Luo *et al*. (2002) verified that VEGF also increased extrusion of the first polar body and improved development potential of bovine oocytes. Recently, similar to these results, VEGF was able to improve intracellular glutathione (GSH) levels after in vitro maturation in bovine species when was added plus cysteamine (100 μM/mL) (ANCHORDOQUY *et al*., 2015). Thus, all these studies together, suggests that the exposure of oocytes to VEGF is beneficial to the meiosis progression by improving the cytoskeleton organization (ARAÚJO *et al*., 2011b).

Taking all results together, we can infer that VEGF may act similarly in vitro oocyte competence to resumes meiosis when compared to EGF plus IGF-I, probably by their effects improving the cytoskeleton organization, creating an environment optimal for oocyte development. However, more studies must be done to found the optimal concentration of VEGF to the in vitro maturation.

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CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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