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Influence of caprine arthritis encephalitis on expression of ovulation related genes and activation of primordial follicles cultured in presence of phytohemagglutinin, epidermal growth factor or both

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ABSTRACT

This study investigated the levels of mRNA for *BMP15*, *GDF9* and *BMPR1B* in preantral follicles, as well as in cumulus oocyte complex (COC) and follicular walls from antral follicles of healthy and CAEV infected goats by real-time PCR. In addition, the influence of phytohemagglutinin (PHA) and EGF on activation of primordial follicles and on expression of mRNA for *TNFa* and its receptors in cultured ovarian tissue from healthy and infected goats was evaluated. The expression of *BMP15* and *GDF9* in primordial/primary follicles from infected goats was significantly higher than in healthy animals, whereas the expression of *BMP15* and *GDF9* in secondary follicles from infected goats was significantly lower. The expression of mRNA for *BMP15* in follicular wall of antral follicles from infected goats was significantly higher than in healthy goats. CAEV infection did not influence the ability of primordial follicles to develop *in vitro*, but the presence of EGF in culture medium significantly increased follicular diameter in healthy and infected animals. Ovarian tissue from infected goats cultured in medium with PHA had primordial follicles with larger diameters than those from healthy animals. An increase of mRNAs for *TNFα* was observed after culturing tissue in presence of both EGF and PHA in healthy animals, but this same treatment promoted a reduction of mRNAs for *TNFa* and increase of *TNFR2* transcripts in infected animals. In conclusion, CAEV influences the expression of mRNA for *BMP15* and *GDF9* in goat ovarian follicles and PHA and EGF differentially regulate the expression of *TNFa* and *TNFR2* in cultured ovarian tissue.

1. Introduction

Caprine arthritis encephalitis lentivirus (CAEV) infects goats of all ages and causes chronic arthritis, interstitial pneumonia and mastitis, compromising economically their productivity (Martínez-Navalón et al., 2013). The high prevalence of CAEV infection is a major concern in many

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parts of the world, particularly in industrialized countries where 80–95% of the breeding stock may be infected (Peretz et al., 1994; Rowe and East, 1997). The use of embryo transfer for virus disease control has been discussed in several species. In goats, Ali Al Ahmad et al. (2008) reported that embryo transfer can be safely used to produce CAEV-free neonates from infected CAEV donors, being a useful technique for a prophylactic health program against CAEV. Thus, ovulation rate can be a major determinant of embryo production efficiency in infected goats. Some studies have demonstrated the role bone morphogenetic protein 15 (BMP15), growth differentiation factor 9 (GDF9) and bone morphogenetic protein receptor 1 B (BMPR1B) in regulating ovulation in ovine species. Increased ovulation rates have been observed in ewes with a reduction in BMP15 and GDF9 synthesis, caused by natural mutation in these genes (McNatty et al., 2005; Galloway et al., 2000; Hanrahan et al., 2004). However, an increase in *BMP15* expression has been reported in prolific goat breeds (Cui et al., 2009; Yang et al., 2012; Pramod et al., 2013). Mutation in *BMPR1B* gene is also associated with increased ovulation rates in ovine species (McNatty et al., 2005; Fabre et al., 2006). Considering that Ali Al Ahmad et al. (2005) have shown the presence of CAEV in cumulus cells, but not in oocytes, it is hypothesized that the presence of CAEV in granulosa cells influences the expression of *BMP15*, *GDF9* and *BMPR1B* in the follicular compartments and can be associated with high ovulation rate in infected goats.

Considering that infected goats are generally discarded, the use of reproductive biotechnologies, such as embryo transfer and *in vitro* growth of primordial follicles, can be an alternative to use the oocyte reservoir of genetically superior and/or endangered goats that are infected by CAEV. Thus, it is very important to evaluate if CAEV influences early folliculogenesis *in vitro*. Several studies have shown that culture of primordial follicle is an alternative to study follicular development and atresia *in vitro* (Markström et al., 2002; Silva et al., 2004a; Hutt et al., 2006; Celestino et al., 2009; Araújo et al., 2010; Kim, 2012; Kalich-Philosoph et al., 2013; Portela et al., 2014; Ribeiro et al., 2014). The effects of several growth factors, such as epidermal growth factor (EGF) (Silva et al., 2004a) on activation and atresia of goat primordial follicles *in vitro* have been tested, but substances that are not produced by the ovaries, such as phytohemagglutinin (PHA) were still not evaluated. PHA is a lectin extracted from *Phaseolus vulgaris*, which bind to complex oligosaccharide containing N-acetylgalactosamine/galactose residues and is involved in cell to cell communication (Sell and Costa, 2003). Since paracrine communication between the oocyte and its enveloping granulosa cells is important for primordial follicle activation, it is hypothesized that PHA improves this activation and reduces the levels of atresia in cultured primordial follicles from healthy and infected goats. Follicular atresia occurs *via* apoptosis after binding of death ligands, including tumor necrosis factor alfa (TNFα) to specific receptors (Hsu et al., 1996; Van Wezel et al., 1999).

The aim of the present study is to evaluate (1) the influence of CAEV on *in vivo* expression of mRNAs for *BMP15*, *GDF9* and *BMPR1B* in goat ovarian follicles and (2) to investigate the effect of EGF, PHA or both on *in vitro* activation of primordial follicles and expression of mRNA for *TNFa* and its receptors in cultured ovarian cortical tissue of healthy and infected goats.

2. Materials and methods

2.1. Experiment 1: influence of CAE on the expression of mRNAs for BMP15, GDF9 and BMPR1B in goat ovarian follicles

Ovaries ($n = 20$) from healthy ($n = 5$) and infected ($n = 5$) goats were collected at Goat and Sheep National Research Center (Brazilian Agricultural Research Corporation – EMBRAPA). Infected animals were seropositive for CAEV by using caprine arthritis-encephalitis progressive pneumonia antibody test kit (Veterinary Diagnostic Technology, Inc., USA) and the results were confirmed as positive by PCR on leucocytes. This study was approved by Institutional Animal Ethics Committee (CEUA-UVA no. 10.1). Immediately postmortem, the ovaries were washed in 70% ethanol for 10 s following two times in saline solution (0.9% NaCl) containing antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). The ovaries were transported within 1 h to the laboratory in saline solution containing antibiotics at 4 °C (Chaves et al., 2008). Unless mentioned otherwise, the culture media, EGF, PHA and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

In the laboratory, the ovaries from each animal were stripped of surrounding fat tissue and ligaments, and then follicular isolation was performed. Primordial/primary follicles were isolated from ovarian tissue by using a mechanical procedure described by Lucci et al. (1999). To isolate secondary follicles, fine slices of the ovarian cortex (1–2 mm) were cut from the ovarian surface, using a sterile scalpel blade. Secondary follicles of approximately 0.2 mm diameter were identified under a stereomicroscope (SMZ 645; Nikon, Tokyo, Japan) and microdissected manually from strips of the ovarian cortex, using 26-gauge needles. Follicles were selected, which exhibited a visible oocyte, surrounded by two or more layers of granulosa cells and an intact basal membrane, and which lacked an antral cavity within their granulosa. Three groups of 10 primordial/primary follicles, as well as of 10 secondary follicles were collected from healthy or infected goats and stored at −80 °C until extraction of total RNA. COCs from healthy or infected goats were aspirated from antral follicle with an 18 gauge needle. After sedimentation, COCs from healthy or infected goats were recovered and selected using a stereomicroscope (SMZ 645; Nikon, Tokyo, Japan). Only COCs with homogenous cytoplasm and at least five compact layers of cumulus cells (Caixeta et al., 2013) were used and stored at −80 °C until extraction of total RNA. To collect the follicular walls from healthy or infected goats, antral follicles from 3 to 6 mm were isolated from the ovaries. The follicles were then cut in half with the aid of a scalpel blade according to the protocol used by (Richard and Sirard, 1996) and stored at −80 °C until extraction of total RNA.

Quantitative real-time PCR experiments were performed according to Bustin et al. (2009). To evaluate gene expression for *BMP15*, *BMPRIB* and *GDF9*, total RNA extraction was performed using Trizol® purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 800 μL of Trizol solution was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 × *g* for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz units/mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 μL RNAse-free water. The RNA concentration was estimated and 1 μg of total RNA was used for reverse transcription. Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70 °C and then cooled in ice. Reverse transcription was performed in a total volume of 20 μL, which was comprised of 10 μL of sample RNA, 4 μL 5X reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNAse out, 150 units Superscript III reverse transcriptase, 0.036 U random primers (Invitrogen, São Paulo, Brazil), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42 °C, for 5 min at 80 °C, and then stored at −20 °C. Negative controls were prepared under the same conditions, but without the inclusion of the reverse transcriptase. Quantification of mRNA was performed using GoTaq® qPCR Master Mix. PCR reactions were composed of 1 μL cDNA as a template in 7.5 μL of GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA), 5.5 μL of ultra-pure water, and 0.5 μM of each primer. The primers were designed by using the PrimerQuestSM