Animal Reproduction Science 170 (2016) 15–24

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Overexpression of hyaluronan synthase 2 and gonadotropin receptors in cumulus cells of goats subjected to one-shot eCG/FSH hormonal treatment for ovarian stimulation

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ARTICLE INFO

Article history:
Received 1 December 2015
Received in revised form 6 March 2016
Accepted 15 March 2016
Available online 16 March 2016

Keywords:
HAS2
FSHR
LHR
Connexin 43
Cumulus cell
Oocyte maturation

ABSTRACT

Hormonal ovarian stimulation may affect transcripts in somatic cells of cumulus-oocyte complexes (COCs) and affect the resulting oocyte quality. Here, in parallel with morphological classification and *in vitro* maturation (IVM) rate analysis, we investigated the expression of hyaluronan synthase 2 (*HAS2*), gonadotropic receptors (*FSHR* and *LHR*) and connexin 43 (*GJA1*) in cumulus cells (CCs) from goat COCs after multi-dose FSH (MD) or one-shot FSH/eCG (OS) treatments, using bovine COCs as control groups. The MD treatment produced more large follicles, and the resulting COCs had a better morphology and IVM rate than were obtained with OS. The OS treatment produced COCs with increased *HAS2*, *FSHR*, *LHR* and *GJA1* expression. This gene expression pattern was also observed in the CCs of COCs that showed poor morphological characteristics. On the other hand, the mRNA levels were more similar between groups after IVM; *FSHR* and *LHR* were the main genes that showed decreased expression. Some events that occurred in bovine CCs during IVM, such as cell expansion, increased *HAS2* expression and decreased *GJA1* expression, were less evident or did not occur in goat COCs. In conclusion, increasing *HAS2*, *FSHR*, *LHR* and *GJA1* expression in goat COCs does not confer greater meiotic competence to oocytes. Instead, it may result from poor regulation of gene expression in CCs by lower quality oocytes. Finally, cumulus expansion, together with *HAS2* upregulation and *GJA1* downregulation, seems to be more important for bovine COCs than for goat COCs. Additional studies are needed to investigate the importance of other HAS isoforms and connexins in goat COCs.

1. Introduction

In several ruminant species, *in vitro* embryo production (IVP) technology has become more widely used (van Wagtendonk-de Leeuw, 2006; Cox and Alfaro, 2007), especially in cattle, a species for which it has already reached commercial application (Pontes et al., 2011). However, there seems to be a consensus that *in vitro* maturation (IVM) is an important step for the success of IVP, i.e., it is necessary to produce competent mature oocytes that reach the blastocyst stage (for a review: Kane, 2003). The IVM of oocytes precedes and is required for successful *in vitro* fer-

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http://dx.doi.org/10.1016/j.anireprosci.2016.03.008
0378-4320/

tilization (IVF). The cumulus cells (CCs) surrounding the immature oocyte have an important function in oocyte development. One of their functions is the channeling of metabolites and nutrients to the oocyte to help stimulate germinal vesicle breakdown and direct development to metaphase II. In fact, it has been widely reported for several species that one of the first morphological indicators of successful oocyte maturation is expansion of the cumulus mass away from the oocyte (Lonergan and Fair, 2016).

One of the principal components of the expanded cumulus-oocyte complexes (COCs) is the glycosaminoglycan (GAG) hyaluronan (HA), which is synthesized at the cell membrane by hyaluronan synthases (HAS; Weigel et al., 1997). In mammals, Watanabe and Yamaguchi (1996) found that the HAS family consists of three known isoenzymes (HAS-1 to 3). In bovine CCs, the action of the HAS2 isoform is the most important source of HA (Schoenfelder and Einspanier, 2003).

The process of cumulus expansion is accompanied by modifications of gap junctions (Pandey et al., 2009), which contain transmembrane channels formed by hexamers of proteins belonging to the connexin family (for review: Nielsen et al., 2012). Connexin 43 (alpha 1 gap junction protein of 43 kDa, GJA1) is the main protein that builds these junctions between CCs of several animal species (Santiquet et al., 2013; Li et al., 2015). Gap junction communication between an oocyte and adjacent CCs and communication between somatic cells is critical for both nuclear and cytoplasmic maturation (Vozzi et al., 2001). In equine and porcine CCs, the beginning of meiotic resumption has been associated with the reduction of the connexin 43 protein level (Marchal et al., 2003). It has long been known that during IVM of bovine COCs, the connexin 43-positive gap junctions disappear (Sutovský et al., 1993).

Gonadotropins are often added to IVM media to induce cytoplasmic maturation and CC expansion. Follicle stimulating hormone (FSH) induces the expansion of mouse COCs *in vitro* (Salustri et al., 1990) and improves bovine IVF (Izadyar et al., 1998). Luteinizing hormone (LH) has beneficial effects on bovine oocyte maturation (Zuelke and Brackett, 1990). Nonetheless, for gonadotropins to act *in vitro*, the COCs must express mRNAs and proteins encoding the FSH and LH receptors (FSHR and LHR).

In goats, information is lacking regarding the IVM process. Thus, the aim of this study was to investigate, before and after IVM, gene expression (*HAS2*, gonadotrophin receptors and connexin 43) in the CCs of goat COCs obtained by laparoscopic ovum collection after ovarian stimulation with multi-dose FSH (MD) or one-shot FSH/eCG (OS) treatments. We compared the results with those of bovine COCs.

2. Materials and methods

2.1. Animal ethics and management

All procedures in this study were performed in compliance with the Ethics Committee on Animal Use at the State University of Ceará (Number protocol 5846717/2014). A total of 14 Canindé goats aged one to three years (mean body weight ± SEM, 32.6 ± 1.92 kg) were selected as oocyte donors. All animals were maintained indoors in groups of five per pen under controlled nutrition. They had access to a Tifton (*Cynodon dactylon*) pasture in the morning and received Tifton during the afternoon. Additionally, goats were supplemented with good-quality concentrate (20% crude protein) and had free access to water and minerals. Bovine ovaries, goat ovaries and goat brain tissues were obtained from a local slaughterhouse.

2.2. Hormone treatments

The animals were subjected to two ovarian stimulation protocols. Each hormonal treatment was performed in four sessions with seven animals per session per protocol. The estrous cycles of all oocyte donors were synchronized using intravaginal sponges impregnated with medroxyprogesterone acetate (60 mg; Progespon; Syntex, Buenos Aires, Argentina), which were inserted for 10 days, combined with an intramuscular (im) luteolytic injection of cloprostenol (50 μg; Ciosin; Coopers, São Paulo, Brazil) on the 7th day of progestagen treatment. The ovarian stimulation consisted of two experimental groups: the standard ovarian stimulation was carried out using multiple doses (MD) of pFSH (120 mg; Folltropin-V; Bioniche, Belleville, Canadá) distributed across five injections (30/30; 20/20; 20 mg) at 12 h intervals starting on the 7th day of progestagen treatment. The one-shot treatment (OS) used pFSH (70 mg) and a single dose of eCG (200 IU; Novormon; Syntex, Buenos Aires, Argentina) administered 36 h before sponge removal.

2.3. Cumulus-oocyte complex recovery

Follicle aspiration for oocyte harvest was performed through the laparoscopic method, as previously described by Baldassarre et al. (2003). In brief, goats were fasted for 36 h from food and water prior to oocyte recovery by laparoscopy (LOR). The surgery was performed under general anesthesia, starting with an intravenous anesthetic induction using thiopental at a dose of 20 mg/kg (Tiopentax 2.5%; Cristália, São Paulo, Brazil). Deep anesthesia was maintained with 3% isoflurane (Isoforine, Cristália, São Paulo, Brazil) with the help of inhalation anesthesia equipment (HB Hospital, São Paulo, Brazil). The COCs were aspirated from follicles >2 mm in diameter that were visible on the ovary's surface using a 22-gauge needle (Watanabe, São Paulo, Brazil) and a vacuum pump (WTA, Cravinhos, São Paulo, Brazil). The vacuum pressure was regulated at 35 mmHg. The collection medium used was TCM199 (Nutricell, Campinas, Brazil) buffered with 10 mM HEPES and supplemented with 20 UI/mL heparin, 0.2 mM pyruvate, 100 U penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin (Sigma-Aldrich, St. Louis, USA), and 10% fetal bovine serum (Life Technologies, New York, USA).

Bovine ovaries were transported from the slaughterhouse to the laboratory in a warmed (30–35 °C) 0.9% NaCl solution supplemented with 40 μg/mL gentamicin (Sigma-Aldrich, St. Louis, USA). The COCs were aspirated gently from follicles using an 18-gauge needle attached to a 10-mL syringe and recovered in the same collection medium used for goats.