Animal Reproduction Science 165 (2016) 1–10

Contents lists available at ScienceDirect

Animal Reproduction Science

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

Balance of insulin and FSH concentrations improves the *in vitro* development of isolated goat preantral follicles in medium containing GH

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ARTICLE INFO

Article history:
Received 30 June 2015
Received in revised form 5 October 2015
Accepted 30 October 2015
Available online 1 November 2015

Keywords:
Goat
Follicle development
Oocyte maturation
Insulin
FSH

ABSTRACT

The aim of this study was to evaluate the effect of different combinations of insulin and FSH concentrations in culture media containing GH on the *in vitro* follicle morphology, antrum formation, growth rates, estradiol (E2) production, oocyte viability and maturation as well as gene expression for *FSHR*, *GHR*, *INSR*, *CYP19A1*, *CYP17*, *3ßHSD*. Secondary follicles were individually cultured for 18 days in a basic medium containing 50 ng/mL GH supplemented with low insulin concentration (INS-LW: 10 ng/mL) or high insulin concentration (INS-HG: 10 μg/mL) alone or with a fixed FSH concentration (FSH100: 100 ng/mL) or with increasing FSH concentrations (FSH-SEQ: 100 ng/mL, days 0–6; 500 ng/mL, days 6–12; 1000 ng/mL days 12–18). In the INS-LW treatment was observed a higher ($P < 0.05$) incidence of normal follicles at day 18 of culture. However, overall higher ($P < 0.05$) follicular growth, oocyte diameter and meiotic resumption rates were obtained using INS-HG + FSH 100. The INS-HG and INS-HG + FSH100 treatments showed higher E2 production and mRNA levels for *CYP19A1*, *CYP17*, *3βHSD* when compared to INS-LW and INS-LW + FSH100. However, the addition of increasing FSH concentration, regardless of insulin concentration, did not improve the follicular growth, meotic resumption, E2 production or gene expression of steroidogenic enzymes when compared with INS-HG + FSH100. In conclusion, in presence of GH, a basic medium supplemented with 10 μg/mL insulin and 100 μg/mL FSH throughout the culture period, improves follicular and oocyte growth, oocyte meiotic resumption and E2 production from isolated preantral caprine follicles cultured *in vitro*.

1. Introduction

The follicular development depends upon a complex sequence of events within the cells during folliculogenesis. However, most follicles cultured *in vitro* gradually undergo atresia (Nayudu and Osborn, 1992). Therefore, the ultimate objective of *in vitro* culturing of ovarian tissue aims to recue

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http://dx.doi.org/10.1016/j.anireprosci.2015.10.010
0378-4320/

the preantral follicles before they become atretic in order to culture them up to maturation for the purpose of improving the relative yield of mature oocytes.

The composition of the culture medium is a key factor to promote the *in vitro* development of preantral follicles to more advanced stages. It is well known, follicles can be potentially influenced by growth factors and hormones produced by themselves, by stroma cells, or by other follicles (Fortune, 2003). These substances play a key role in controlling early folliculogenesis, acting by increasing steroidogenesis (Greisen et al., 2001), follicle activation (Ojeda et al., 2006; Martins et al., 2008; Celestino et al., 2010), growth (Skinner, 2005; Magalhães et al., 2009), inhibition of follicular atresia (Fortune, 2003), and stimulating oocyte competence (Araújo et al., 2011). Among the various hormones studied so far, it is important to highlight the influence of insulin, follicle-stimulating hormone (FSH) and growth hormone (GH) (Magalhães et al., 2011; Saraiva et al., 2011; Chaves et al. 2012).

The *in vitro* survival, antrum formation, growth and oocyte meiotic resumption rates of isolated goat preantral follicles were improved in a medium supplemented with increasing FSH concentration (from 100 to 1000 ng/mL) or in the presence of high insulin concentration (10 μg/mL) (Saraiva et al., 2011). The addition of 50 ng/mL of GH, to the latter mentioned medium resulted in the production of the first caprine embryo from preantral follicles grown *in vitro* and marks the greatest result reported in the literature for caprine species so far (Magalhães et al., 2011). However, recently it has been reported that the addition of lower concentration of insulin (10 ng/mL) proved to be more efficient in maintaining survival, promoting follicular development and meiosis resumption when the culture medium was supplemented with increasing FSH concentrations (FSH-SEQ: 100 ng/mL, days 0–6; 500 ng/mL, days 6–12; 1000 ng/mL days 12–18) (Chaves et al., 2012). Although insulin, FSH and GH have been used commonly for the *in vitro* culture of caprine preantral follicle yet, the suitable balance of insulin and FSH concentration in medium containing GH is not known. Therefore, the aim of this study was to evaluate the effect of different combinations of insulin and FSH concentrations in *in vitro* culture media containing GH on the *in vitro* follicle morphology, antrum formation, growth, estradiol (E2) production, oocyte viability and maturation as well as gene expression for *FSHR*, *GHR*, *INSR*, *CYP19A1*, *CYP17*, *3βHSD*.

2. Materials and methods

2.1. Chemicals and media

The reagents and chemical used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.2. Collection of ovaries, isolation, selection and culture of preantral follicles

Ovaries (n = 50) were collected at a local slaughterhouse from 25 adult (ages 1–3 years old) cross-breed goats, for a total of five repetitions. Immediately after slaughter, the ovaries were washed in alcohol (70%) followed by two washes in minimum essential medium (MEM) supplemented with penicillin (100 μg/mL), streptomycin (100 μg/mL) and HEPES (25 mM). The ovaries were transported to the laboratory in MEM within 1 h at 4 °C (Chaves et al., 2008).

In the laboratory, fat and connective tissue surrounding the ovaries were removed. Cortical slices (1–2 mm thick) were obtained with a surgical blade (under sterile conditions) and placed in a holding medium consisting of HEPES-buffered MEM. Preantral follicles that were approximately 200 μm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of ovarian cortex using 26-gauge (26 G) needles. After isolation, follicles were individually cultured in 100 μL drops containing fresh culture medium under mineral oil to further evaluate follicular quality. Follicles with a visible oocyte that were surrounded by granulosa cells and had an intact basement membrane and no antrum formation were selected for culture.

After selection, follicles were individually cultured in 100 μL drops of culture medium in Petri dishes (60 mm × 15 mm, Corning Incorporated, Corning, NY, USA). The basic culture medium consisted of α-MEM (pH 7.2–7.4) (Gibco; Invitrogen, Karlsruhe, Germany) supplemented with 3 mg/mL bovine serum albumin (BSA), 5.5 μg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 50 μg/mL ascorbic acid and 50 ng/mL bovine Growth Hormone from Bovine Pituitary Gland (GH). The GH concentration (50 ng/mL) was established in previous experiment (Magalhães et al., 2011). Fresh media was prepared immediately before use and pre-equilibrated for at least 1 h prior to use. Basic culture medium was supplemented according to the experimental design. The culture was carried out at 39 °C, in 5% CO_2 in air for 18 days. Every other day, 60 μL of medium was replaced in each drop, and at days 6 and 12 of culture all medium (100 μL) was replaced. The experiment was repeated five times and in total approximately 40 follicles were used in each treatment.

2.3. Experimental design

For the experimental conditions, preantral follicles were randomly distributed in the following treatments: (1) INS-LW, basic culture medium supplemented with low concentration (10 ng/mL) of human recombinant insulin; (2) INS-HG, basic culture medium supplemented with high concentration (10 μg/mL) of human recombinant insulin; (3) INS-LW + FSH100, basic culture medium supplemented with low concentration (10 ng/mL) of insulin associated with a fixed concentration (100 ng/mL) of bovine recombinant FSH (rFSH, Nanocore, Campinas, São Paulo, Brazil) throughout the entire culture period; (4) INS-HG + FSH100, basic culture medium supplemented with high concentration (10 μg/mL) of human recombinant insulin associated with a fixed concentration (100 ng/mL) of bovine recombinant FSH throughout the entire culture period; (5) INS-LW + FSH-SEQ, basic culture medium supplemented with low concentration (10 ng/mL) of human recombinant insulin associated with in increasing FSH