Small Ruminant Research 115 (2013) 71–76

Contents lists available at ScienceDirect

Small Ruminant Research

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

In vitro culture of goat preantral follicles from fetal ovaries

Rooh Ul Amin[a,c,1], K. Chandrashekar Reddy[a,c,2], K. Sadasiva Rao[a,c], K.B.P. Raghavender[b,c], A. Teja[a,c], T. Ramesh[a,c], G. Arunakumari[a,c,*]

[a] Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Rajendranagar, Hyderabad, India
[b] Department of Surgery and Radiology, College of Veterinary Science, Rajendranagar, Hyderabad, India
[c] Sri Venkateswara Veterinary University, Tirupati 517 502, India

ARTICLE INFO

Article history:
Received 16 May 2013
Received in revised form 12 August 2013
Accepted 15 August 2013
Available online 28 August 2013

Keywords:
Goat fetus
Preantral follicles
Thyroxin
Growth hormone
FSH
IGF-I
EGF

ABSTRACT

The objective of the present study was to investigate the influence of thyroxin (T_4), follicle stimulating hormone (FSH), growth hormone (GH), epidermal growth factor (EGF) and insulin like growth factor-I (IGF-I) individually or in combination on *in vitro* development of goat fetal preantral follicles (PFs). PFs (100–300 μm) were isolated from the fetal ovaries of more than 125 days of gestation and cultured for a period of 6 days. The development of the cultured PFs was assessed by the proportions of follicles exhibiting growth, increase in diameter, antrum formation, and extrusion of oocytes *in vitro*, if any. In the first experiment, PFs were cultured in tissue culture medium 199 supplemented with different individual concentrations of T_4 (0.5, 1 and 2 μg/mL), FSH (1, 2 and 4 μg/mL), GH (1, 2 and 4 mIU/mL), EGF (10, 25 and 50 ng/mL) and IGF-I (10, 15 and 20 ng/mL). The proportion of PFs exhibiting growth, increase in diameter and antrum formation were highest when the PFs were cultured at T_4:1 μg/mL, FSH:2 μg/mL, GH:1 mIU/mL, EGF:25 ng/mL or IGF-I:10 ng/mL. Among the different individual concentrations of growth factors and hormones, the extrusions of oocytes from cultured PFs were observed only in GH and EGF supplemented media. In the second experiment, the effect of different combinations of the above and hormones and growth factors at their best concentrations as determined in the first experiment was investigated. The average increase in diameter (50.56 ± 3.8 μm), the proportion of PFs exhibiting antrum formation (96.54 ± 2.4) and extrusion of oocytes (25.79 ± 1.8) were highest when cultured in T_4 + FSH + GH + EGF supplemented medium. However, the proportion of extrusion of oocytes was similar in T_4 + FSH + IGF-I, T_4 + FSH + GH + IGF-I and T_4 + FSH + EGF + IGF-I combinations. In conclusion, the *in vitro* development of goat fetal PFs could be significantly improved when the medium was supplemented with T_4 + FSH + GH + EGF.

1. Introduction

Mammalian ovary contains a large number of oocytes present in follicles of various stages (primordial, preantral and antral). The majority of these follicles undergo atresia during the follicle maturation from primordial to ovulatory stage. A major constraint for optimal utilization of the genetic potential of female animals is the limited number of ova produced at each estrous cycle. Multiple ovulation and embryo transfer (MOET) and *in vitro* production (IVP) of embryos using oocytes from antral follicles of slaughter house ovaries has enhanced the utilization of

* Corresponding author at: Department of Veterinary Gynaecology and Obstetrics, Teaching Veterinary Clinical Complex, College of Veterinary Science, Rajendranagar, Hyderabad, India. Tel.: +91 998 5024898; fax: +91 04024015143.
E-mail addresses: aruna.gangineni@gmail.com, siva.pemmasani@gmail.com (G. Arunakumari).
[1] Present address: Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, India.
[2] Present address: Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Korutla, Karimnagar, India.

0921-4488/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.smallrumres.2013.08.004

female genetic resources only to a limited extent. In addition to MOET and IVP of embryos from antral follicles, IVP of oocytes/embryos obtained from cultured primordial or preantral follicles (PFs) have the potential to maximize the utilization of female gametes (Eppig and O'Brien, 1996; Wu et al., 2001a, b; O'Brien et al., 2003; Gupta et al., 2008; Arunakumari et al., 2010; Saraiva et al., 2010; Magalhaes et al., 2011). Lack of a repeatable culture system for efficient production of competent oocytes from cultured PFs hampers the maximum utilization of oocytes. Several studies have been aimed at producing competent oocytes from cultured PFs of various species: laboratory animals (Wang and Roy, 2004; Hasegawa et al., 2004), farm animals (Cecconi et al., 1999; Wu et al., 2001a, b; Itoh et al., 2002; Tamilmani et al., 2005; Gupta et al., 2007; Arunakumari et al., 2007, 2010), non-human primates (Wandji et al., 1997), as well as humans (Carlsson et al., 2006). However, offspring have only been produced in mice using oocytes from cultured PFs (Eppig and O'Brien, 1996; O'Brien et al., 2003). In farm animals, success has been achieved up to embryo development using oocytes from cultured PFs in pig (Wu et al., 2001a, b), buffalo (Gupta et al., 2008), sheep (Arunakumari et al., 2010) and goat (Saraiva et al., 2010; Magalhaes et al., 2011). However, none of these studies progressed up to blastocyst stage, except in the pig (Wu et al., 2001b).

Chelikani et al. (1998) isolated and characterized the PFs of goat for the first time. Dulbecco's minimum essential medium (DMEM) supplemented with fetal bovine serum (FBS), follicle stimulating hormone (FSH), insulin like growth factor-I (IGF-I), insulin, transferrin and selenium (ITS), hydro-cortisone, hypoxanthine and dbcAMP supported the *in vitro* growth of caprine pubertal PFs (50–100 μm) in agar gel up to antrum formation (Huanmin and Yong, 2000). Later on, Zhou and Zhang (2006) reported the extrusion of 1st polar bodies after *in vitro* maturation of oocytes collected from cultured prepubertal caprine PFs. Also, FSH, epidermal growth factor (EGF), IGF-I, IGF-II and transforming growth factor -β were shown to induce the growth of PFs collected from ovaries of adult goats (Rajarajan et al., 2006). A dynamic sequential culture medium was developed with the supplementation of FSH, luteinizing hormone (LH), EGF and growth hormone (GH) for *in vitro* culture of adult caprine PFs, subsequent maturation and development of oocytes into embryo (Saraiva et al., 2010; Magalhaes et al., 2011). Recently, supplementation of culture medium with phytohemagglutinin (10 μg/mL) was found to maintain the follicular viability and ultrastructure, and to promote the formation of antral cavity after 6 days of culture *in vitro* (Cunha et al., 2013). The fetal ovaries also contain a large pool of PFs which undergo further growth into ovulatory follicles under the influence of gonadotropins, but the majority of these follicles undergo atresia by an apoptotic mechanism before birth (Markstrom et al., 2002). Efficient culture methods for *in vitro* growth of fetal PFs would be an added advantage to the existing PF culture systems, in order to yield more competent oocytes for IVP of embryos. Also, use of fetal oocytes from cultured PFs could considerably shorten the generation interval (Betteridge et al., 1989). In addition, *in vitro* culture of PFs from fetal ovaries would be an appropriate model to understand the basic biology of the folliculogenesis. Studies on the isolation and culture of PFs from fetal ovaries have been undertaken in cattle (Figueiredo et al., 1993; Hulshof et al., 1994; Wandji et al., 1996), human (Wu et al., 1998), hamster (Yu and Roy, 1999), mouse (Shen et al., 2006) and buffalo (Santos et al., 2006). However, *in vitro* development of fetal PFs up to blastocyst stage was achieved only in mouse (Shen et al., 2006). In the current study, the influence of thyroxin (T_4), FSH, GH, EGF and IGF-I, individually as well as in combinations, on *in vitro* development of goat fetal PFs was investigated.

2. Materials and methods

Unless otherwise stated, all culture media, hormones, growth factors, FBS and chemicals were purchased from Sigma (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). All media were incubated at 39 °C under a humidified atmosphere of 5% CO_2 in air for 2 h prior to use. Phosphate buffered saline (PBS), collection medium for PFs (HEPES-buffered tissue culture medium 199 supplemented with 0.5% bovine serum albumin (BSA), 50 μg/mL gentamicin sulphate, 0.23 mM of sodium pyruvate, 2 mM L-Glutamine and 25 IU/mL heparin), handling medium (collection medium without heparin), stock solutions of EGF and FSH were all prepared as described by Tamilmani et al. (2005). The preparations of thyroxin, GH and IGF-I solutions were made as per Arunakumari et al. (2007, 2010). Bicarbonate buffered tissue culture medium 199 supplemented with 50 μg/mL gentamicin sulphate was used as control medium.

2.1. Collection of goat fetal ovaries, isolation, selection and culture of preantral follicles

Goat fetuses were collected from a local slaughter house. The age of the fetuses was determined by fetal age prediction equation, $X = 2.1\ (Y + 17)$, where X is the developmental age in days and Y the crown rump length in centimeters (Nwaogu and Ezeasor, 2008). Fetal crown-rump length was measured from the frontal eminence to the sacro-coccygeal junction (Osuagwuh and Aire, 1979). Ovaries were collected from 50 fetuses, aged greater than 125 days. Procedures for processing of ovaries, isolation, selection, classification and culture of the PFs were exactly as described earlier (Arunakumari et al., 2007, 2010). Briefly, each ovary was cut into two halves along its longitudinal axis. Then, each half of the ovarian cortex was dissected into thin slices using a 26 gauge needle and sterile surgical blade. Under a stereo zoom microscope (Nikon, Japan), these cortical slices were subjected to microdissection in collection medium for isolation of the PFs, in the size range of 150–300 μm. The diameter of PFs was measured using Scopetek software, China. Care was taken to leave a small amount of stromal tissue attached to the basement membrane of the follicles. PFs having a centrally placed spherical oocyte with an intact basement membrane were chosen for culture. The selected PFs were washed thrice in handling and culture medium and placed individually in 20 μL droplet of culture medium in 35 mm tissue culture dishes. The micro droplets were overlaid with autoclaved pre-equilibrated mineral oil and cultured for 6 days in 5% CO_2 incubator at 39 °C. Half of the culture medium was replaced with fresh culture medium every 48 h.

2.2. Morphological evaluation of cultured preantral follicles

Morphological evaluation was done as described by Arunakumari et al. (2007). Each follicle was evaluated every 24 h during culture, using an inverted microscope (Olympus, Japan), for increase in diameter, antrum formation and extrusion of oocyte from the follicle.

2.3. Experimental design

Two different experiments were conducted. In the first experiment, influence of the individual growth factors and hormones on *in vitro* development of goat fetal PFs was investigated by supplementing the control medium with T_4 (0.5, 1, and 2 μg/mL), FSH (1, 2, and 4 μg/mL), GH (1, 2, and 4 mIU/mL), EGF (10, 25, and 50 ng/mL) and IGF-I (10, 15 and 20 ng/mL). After determining the best concentration for each of the above five growth factors and hormones (Table 1), the effect of using them in different combinations (C_1–C_8, Table 2) was examined in the second experiment