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Domestic Animal Endocrinology 41 (2011) 195–206

DOMESTIC ANIMAL ENDOCRINOLOGY

www.domesticanimalendo.com

Characterization of serum-free buffalo granulosa cell culture and analysis of genes involved in terminal differentiation from FSH- to LH-responsive phenotype

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Received 17 April 2011; received in revised form 5 July 2011; accepted 25 July 2011

Abstract

In the present study, buffalo granulosa cells were cultured under serum-free conditions and characterized to study the changes in gene expression associated with the transition of granulosa cells from estrogen- to progesterone-secreting phenotype. The cells were cultured in vitro under completely serum-free conditions for 8 d. Gene expression and hormone analysis showed that on day 4 granulosa cells exhibit FSH responsiveness with preovulatory phenotype having highest *CYP19* gene expression and 17β-estradiol production, whereas a significant increase in transcript abundance of *STAR*, *CYP11,* and *HSD3B* genes accompanied with an increase in progesterone production was observed on day 8. Cells treated with LH on day 4 followed by gene expression analysis at 1, 2, 4, 6, 18, and 24 h showed significant increase in transcripts of LH-responsive genes. In conclusion, culture condition used in the present study showed that granulosa cells were FSH responsive and attained attributes of granulosa cells of dominant follicles at day 4 with highest *CYP19* and *LHR* gene expression beyond which they acquired the ability to luteinize and thus were more LH responsive. In addition, after LH treatment, analysis of early LH-responsive genes (*EGR2, RUNX1,* and *NR4A1)* on day 4 showed that granulosa cells at this stage in culture exhibits phenotype similar to that of preovulatory follicles before LH surge in vivo and corresponds to the in vivo transition of well-orchestrated gene expression profile after LH surge. The characterized culture conditions represent a suitable in vitro model for analysis of genes involved in terminal differentiation of granulosa cells from FSH- to LH-responsive phenotype during folliculogenesis in buffalo.

Keywords: qPCR; Transcript abundance; 17β-Estradiol; Progesterone; Gonadotropins

1. Introduction

The LH surge triggers ovulation and induces in granulosa cells of preovulatory follicles the rapid and transient expression of certain genes in a species-specific manner that have been shown to be critical for ovulation [1]. A coordinated up-regulation of certain genes with the consequent reduction in the expression of others is required for the conversion of the 17β-estradiol–producing follicle to progesterone-secreting postovulatory structure. *CYP19* gene, encoding the enzyme aromatase cytochrome P450, catalyzes the final rate-limiting step in the biosynthesis of estrogens from androgens [2]. Earlier studies in bovine granulosa cells suggest that mRNA encoding *CYP19* is more labile than other granulosa cell steroidogenic en-

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doi:10.1016/j.domaniend.2011.07.001

zymes [3]; thus, the maintenance of *CYP19* gene expression in cultured cells in itself is a task. Previous attempts to establish a serum-free culture for buffalo granulosa cells [4] have used coatings of fibronectin to attach the cells in the absence of serum but with the inability of the system to maintain aromatase gene expression. However, the cells under these conditions, independent of gonadotropins, are known to luteinize [5,6] and loose their granulosa cell phenotype along with *CYP19* gene activity [7]. Studies in cow have optimized the serum-free cell culture system [8–10], and the system was shown to maintain *CYP19* gene expression and 17β-estradiol production. Although cows are closely related to buffaloes, the results from bovine granulosa cell culture are not directly translatable to buffaloes because there are several marked physiological differences between cow and buffalo. The fertility in water buffalo (*Bubalus bubalis*) is much lower than that in cattle (*Bos taurus*). Poor breeding efficiency is attributed to late onset of puberty, seasonality, poor estrus expression, and long calving intervals [11]. The ovaries of mature water buffalo are smaller than in the bovine [12]. In addition, Terzano [13] has described that physiologically the buffalo ovary shows insufficient reproductive potentiality because the number of follicles (primary and antral) is less than those found in cattle. In buffalo, there is nearly a complete lack of information on the factors that control the selection of the dominant follicle, the period of functional dominance, and the effects of environmental factors, such as climate and nutrition, on follicular dynamics [14]. Studies in relation to change in gene expression profile in the in vitro systems have not yet been accomplished, particularly in ruminants, because of a lack of an appropriate in vitro culture model that can respond to gonadotropins (FSH and LH) with desired phenotype of in vivo. In view of these factors, the objective of the present study was to develop and validate a system of buffalo granulosa cell culture under completely serum-free conditions that maintains *CYP19* gene expression and 17β-estradiol production without the cells losing their phenotypic characteristics. The overall aim was to establish a culture system for gene expression analysis during the transition of the FSH-responsive granulosa cells to LH-responsive luteal cells in vitro.

2. Materials and methods

2.1. Collection of ovaries

Buffalo ovaries (approximately 200) were collected from commercial abattoir, Delhi. Ovaries were placed in chilled normal saline (0.9% NaCl) containing penicillin (100 U/mL) and streptomycin (100 μg/mL) within 10 to 20 min after slaughtering and were transported to the laboratory rapidly (within approximately 4 h). Ovaries were washed at least five times in saline, disinfected once in 70% ethanol for 30 s, and then washed again with saline and processed immediately.

2.2. Isolation, culture, and treatment of granulosa cells

Healthy, developing follicles were assessed by the presence of vascularized theca externa and clear amber follicular fluid with no debris. The follicular fluid was aspirated from small and medium antral follicles ($\leq$8 mm) with the use of an 18-gauze needle and sterile, nontoxic, nonpyrogenic monoinjected brand syringes (Dispovan, Faridabad, Haryana, India; 2 mL). The fluid was collected in PBS that contained penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (1.25 μg/mL) in a 15-mL centrifuge tube under sterile conditions while continuously maintaining the cells on ice. The granulosa cells were finally separated by centrifugation at low speed (1500 rpm) for 4 to 6 min to pellet out the cells. Cell number and viability were estimated in a hemocytometer with the use of the trypan blue exclusion method.

All the culture reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) unless otherwise stated. Cells were seeded in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 2×10^5 viable cells in 1 mL of Dulbecco modified Eagle medium. The composition of serum-free culture medium was as follows: L-glutamine (3 mM/mL), protease-free BSA (1 mg/mL), sodium selenite (4 ng/mL), transferrin (2.5 μg/mL), androstenedione (2 μM), bovine insulin (10 ng/mL), nonessential amino acid mix (1.1 mM), ovine FSH (1 ng/mL), human rIGF1 (1 ng/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cultures were maintained at 37°C in 5% CO_2, 95% air for 8 d, with 700 μL of medium being replaced every 48 h (2 d). After the first 48 h, cells were either left untreated (control) or treated with different doses of FSH or IGF1 or a combination of both. For LH responsiveness, cells were treated with LH on day 4 of culture. Before LH treatment, the cells were treated with optimized doses of FSH and IGF1 that served as zero (0) h control. At the end of the treatment period, spent medium was collected at different time intervals and stored at -20°C until assayed for hormonal content. For gene expression analysis, cells were lysed for RNA extraction.