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Luteinizing Hormone effect on the GDF-9 and BMPR-1a Expression of Bovine Granulosa Cells culture

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Abstract. Growth Differentiation Factor-9 (GDF-9) and Bone Morphogenetic Protein Receptor 1a (BMPR-1a) was a member of the transforming growth factor-β (TGF- β) superfamily known to regulated ovarian functions in mammals. In addition, Luteinizing Hormone (LH) also has an important role in ovarian function. The objectives of this research are to observe effect of LH on the GDF-9 and BMPR-1a expression in granulosa cells (GCs) culture. Bovine ovaries were obtained from a slaughterhouse and carried to the laboratory within 30 min in a container kept at 37 ^{0}C in saline containing gentamicin and amphotericin. The ovaries were washed with pre-warmed PBS supplemented with gentamicin and amphotericin. Granulosa cells were collected from follicles by aspiration using a syringe with needle (20G). The cells were centrifuged for 1 min at 800g at room temperature and washed twice in DMEM/F12 medium and filtered through a stainless steel filter (100 um) to get the GCs then seeded in 6 well culture plates. The cells were cultured at 37 $^{\circ}$C in a 5% CO_2 atmosphere for 24 h and then the wells were washed with PBS to remove unattached cells. The culture medium was replaced with serum-free medium supplemented with LH at 100 ng/mL and cultured for 24 h. Total RNA was extracted from GCs using TRIsure. The RNA quality and quantity were estimated by using spectrophotometer at 260/280 nm. Aliquots (1 ug) of total RNA from each pool of GCs were independently reverse-transcribed to cDNA. The mRNA expression of GDF-9 and BMPR-1a was estimated by the quantitative real-time PCR method. The results showed that LH added to the culture medium can increase the expression of GDF9 and BMPR-1a of granulosa cells.

1. Introduction

Conventionally, the control of follicular development focus on endocrine regulation (LH and FSH) and local regulation (insulin-like growth factor-I and activin/inhibin/follistatin) systems. Recently study, the bone morphogenetic proteins (BMPs) and growth differentiation factor 9 (GDF-9) are key regulators of follicular development [1]. GDF-9 is a member of the transforming growth factor-β (TGF- β) superfamily known to regulated ovarian functions in mammals, including in primordial follicle recruitment [2], granulosa cell proliferation [3] and steroid hormone synthesis [4]. GDF-9

expressed in rat oocytes [4], bovine granulosa cells [3], human granulosa cells [5]. Luteinizing hormone (LH) is a hormone synthesized by pituitary, which plays an important role in the regulation of ovarian function. Increased LH concentration induces the expression of cyclooxygenase-2 (COX-2), which can induce expansion of cumulus cells [6].

BMPs have been found to interact with type I and type II receptors, namely, BMPR-1a (receptor bone morphogenetic protein receptor type 1a), BMPR-1b (receptor bone morphogenetic protein receptor type 1b), and BMPR-2 (receptor bone morphogenetic protein receptor type 2) for their activity [1]. Using immunohistochemistry shown that BMP receptor (BMPR-1a, BMPR-1b and BMPR-2) have been strongly expressed in granulosa cell layer of follicles from primary to late antral stages of development [7].

2. Materials & Methods

2.1 Isolation and culture of Granulosa cells

The ovaries were washed with pre-warmed PBS supplemented with gentamicin and amphotericin. Granulosa cells (GCs) were collected from small follicles (SFs) and large follicles (LFs) by aspiration using a syringe with needle (20G). The cells were centrifuged for 1 min at 800 g at room temperature and washed twice in DMEM/F12 medium and filtered through a stainless steel filter (100 um) to get the GCs then seeded in 6 well culture plates. The cells were cultured for 24 h at 37 ^{0}C in a 5% CO_2 atmosphere and then the wells were washed with PBS to remove unattached cells. The culture medium was replaced with serum-free medium supplemented with LH at 100 ng/mL and cultured until confluence.

2.2 RNA Extraction and cDNA synthesis

Total RNA was extracted from GCs using TRI sure reagent. After aqueous phase separation, RNA was precipitated in isopropanol and washed in 75% (v/v) ethanol, and the RNA pellet was re-suspended in 30 ul nuclease-free water. The RNA quantity and quality were evaluated by spectrophotometry at 260/280 nm. The samples were stored at -80°C. Single-strand cDNA was reverse transcribed from 1 ug of RNA template using 1st strand cDNA Synthesis Kit.

2.3 RT-PCR of Bovine GDF-9 and BMPR1a

Expression for *GDF-9* and *BMPR-1a* genes were quantified by Real Time PCR analyses using commercial kit (SYBR Green assays). The primers were design using Primer-3 based on the Gen Bank data base. GDF-9 primer: Forward GCTAGGACTGCGTTGGAATC and Reverse GTTCCCTGTGCCCATCATAC. BMPR-1a primer: Forward GATATGCGTGAGGTGGTGT and Reverse AGTCTGGAGGCTGGATTGT.

2.4 Network Construction

Network analysis was used to understanding the effect of Luteinizing Hormone (LH). The network analyzing LH with protein was constructed using App Cytoscape 3.6.0.8 [8]. LH and protein interaction was established with STICH proteins/compound network. In the network graphic, LH and proteins were presented as nodes, while LH-proteins and proteins-proteins interaction were presented as edges.

2.5 Statistical analysis

The Independent-Sample T-test was used to compare mean values within one experimental group. The experiment were carried out in at least four replicates. The result quantifying copy numbers of GDF-9 and BMPR-1a mRNA are expressed as the mean of the ratio of GDF-9 or BMPR-1a mRNA to GAPDH. A P value of <0.05 was considered as statistically significant

3. Results & Discussion

3.1 Quantity analysis of mRNA expression

Based on the results mRNA expression and cDNA synthesis, the quantity analysis of mRNA expression are shown in table 1.

Table 1. Quantity analysis of mRNA of bovine granulosa cells

No	RNA (ng/ul)	
	Without LH	With LH
1	314.00	476.40
2	802.00	856.00
3	696.40	786.00
4	570.00	736.00
5	316.00	506.40
6	254.00	450.50
7	566.24	512.40

This study describe, that the mRNA total with LH treatment was significantly higher than without LH. The increase of mRNA expression is probably due to the influence of LH on the activity of the PKA pathway in granulosa cells. The bond between LH and LHR on granulosa cell membranes activates a number of cellular signaling cascade, including the cyclic AMP / protein kinase A (cAMP / PKA) pathway [9]. This cAMP/PKA pathway will induce gene expression that causes phenotypic and physiological changes in cells [10, 11]. Carletti and Christenson [11] found that one hour after LH administration can increase the expression of several genes in mice granulosa cells.

3.2 GDF-9 and *BMPR-1a expression*

To study the LH effect on the expression of GDF-9 and BMPR-1a in bovine granulose cells, Real Time PCR were carried out. The result showed that LH significantly increasing expression of GDF-9 and BMPR-1a (Fig 1). In our study, increasing of expression BMPR-1a were compared to the previous study [12] showed that LH treatment caused a significant increase expression of BMPR-1a in porcine granulosa cells. LH plays an important role for ovulation and for luteinisation of granulosa cells. Luteinization is under the control of oocyte derived factors, including BMP-6, BMP-15, and GDF-9 [12]. LH can affect opening and closing gap junction. This type of cell communication will affect paracrine factor, suc as GDF 9, are secreted by the oocytes. secretion by oocytes [13]. In addition, LH can also activates the cAMP/PKA pathway and will induce gene expression [10] including GDF-9 and BMPR-1a.

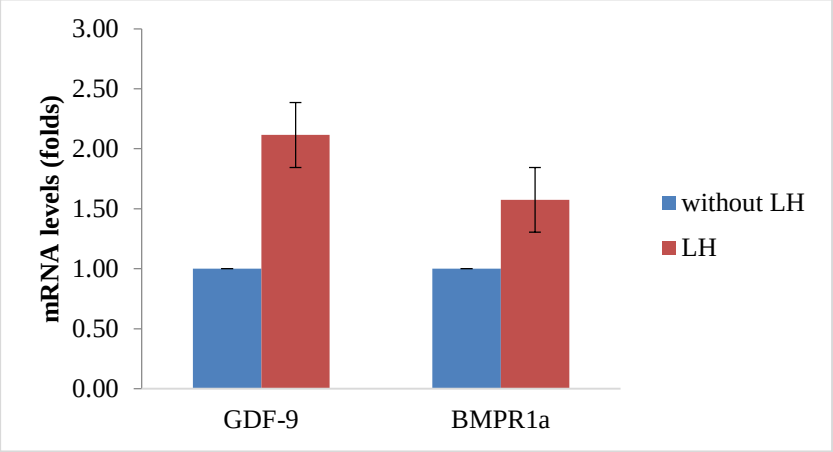

Figure 1. Expression level of GDF-9 and BMPR-1a

3.3 Network analysis

The interaction between LH and proteins target has been constructed (Figure 2). The network constructed is shown that some of protein has direct interaction with LH like KISS-1, KISS-1R, GnRH1, GnRHR and other proteins have indirect interaction like GnRH2, PROK2, TAC3, TRHR. KISS-1 has a role in the metabolic control of fertility, expression of KISS-1 gene at the hypothalamus increase circulating gonadotropin levels [8]. Expression of KISS-1 hormonally regulated and potently stimulate LH secretion [14]. KISS-1 was G-protein coupled with receptor KISS-1R their operates as a central conduit for conveying metabolic information onto the centers governing reproductive function, through a putative leptin kisspeptin GnRH pathway [8].

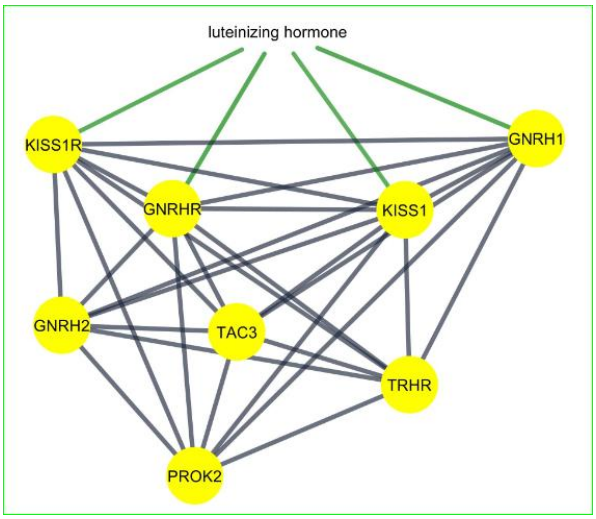

Figure 2. LH-Protein network. The yellow rectangular represents protein. The green line represents LH-protein interaction. The grey line represents protein-protein interaction.

Two types of GnRH, GnRH1 and GnRH2 are play a role in the reproduction. Receptor for gonadotropin releasing hormone (GnRHR) that mediates the action of GnRH to stimulate the secretion of the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [15]. GnRH complexes with its cell surface receptor on the anterior pituitary gonadotropin cells, where it

stimulates the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and FSH [16]. High frequency GnRH were preferentially decreased GDF9 gene transcription and expression. GnRH regulation of GDF9 was concentration dependent and involved ERK and PKA[17], gonadotropin synthesis and fertility is dispensable by BMPR1A [18]. Protein like TAC3 whereas indirect interact with LH that play a role a central regulator of gonadal function [19].

4. Conclusion

Based on the results of the study, it can be concluded that luteinizing hormone increase the GDF-9 and BMPR-1a Expression of Bovine Granulosa Cells culture. Some of protein like GNRH, KISS1R, KISS1, GnRH1 has direct interaction with LH.

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