Theriogenology 88 (2017) 124–130

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effect of alpha lipoic acid on *in vitro* development of bovine secondary preantral follicles

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ARTICLE INFO

Article history:
Received 17 January 2016
Received in revised form 5 September 2016
Accepted 6 September 2016

Keywords:
Preantral follicle
Bovine
Alpha lipoic acid
Ovulation

ABSTRACT

The present study aimed to evaluate the *in vitro* effect of alpha lipoic acid (ALA) addition to the culture medium on the development of the bovine secondary preantral follicles. Bovine secondary preantral follicles were collected and divided into two groups depending on their size (80–100 μm and 100–110 μm). They were cultured *in vitro* for 15 days (D) using different media including at three different doses of ALA. The genes expression levels of follicle-stimulating hormone beta-subunit (FSHR), insulin-like growth factor (IGF-1), Activin, luteinizing hormone/choriogonadotropin receptor, bone morphogenetic protein receptor type IA, transforming growth factor beta receptor 1, growth differentiation factor 9, BCL2-associated X protein (BAX), and C-Myc were studied using real-time polymerase chain reaction. The protein expression levels of FSHR, IGF-1, and BAX were measured using Western blot analysis. The results of the present work revealed that *in vitro* addition of ALA-induced significant increase in the growth and development of secondary preantral follicles throughout the culture period as compared to control. The FSHR, IGF-1, luteinizing hormone/choriogonadotropin receptor, bone morphogenetic protein receptor type IA, transforming growth factor beta receptor 1, growth differentiation factor 9, and Activin A genes were upregulated in ALA-treated follicles as compared to the control. On contrary, preapoptotic genes BAX and C-Myc were downregulated in treated follicles compared to control ones. The protein levels of FSHR and IGF-1 were highly expressed in treated follicles compared to control; however, BAX protein was downregulated in the treated follicles groups. The addition of ALA to the culture medium enhances secondary preantral follicles development and growth.

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1. Introduction

The total number of mammalian ovarian follicles is determined early in life, and the depletion of this pool leads in turn to reproductive senescence [1]. Dynamics of ovarian

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0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.09.013

follicle development is an interesting scope to endocrinologists and developmental biologists [1]. Therefore, study of follicle development *in vivo* and *in vitro* has gained clinical relevance [1,2]. Follicles development is usually regulated by reproductive cell signals, especially follicle-stimulating hormone receptor (FSHR), inhibin and other signals [3]. Moreover, several growth factors including FGF-2, IGF-I, bone morphogenetic protein 15 (BMP15), growth differentiation factor 9 (GDF9) were expressed during follicles development [4–6]. Particularly, oocyte-specific growth factors, bone BMP15, and GDF9 play crucial roles in development and fertility of granulosa cells [6].

In addition, BMP15 and GDF9 are candidates of superfamily called "the transforming growth factor h" [4,7,8]. Activities of GDF9 linked with secondary to tertiary transitions during the process of the follicular development [9]. Growth differentiation factor 9 and BMP15 as oocyte-derived factors are elaborated in mediating follicle creation and growth [10–12]. Therefore, these factors are extremely vital for follicular formation and development [13]. Furthermore, oxidative stress is another factor affecting oocytes maturation and fertilization; excess free radicals production may cause damaging effect on oocyte fertilization and embryo development [4,14]. As well, free radicals may cause oocyte meiotic arrest in the germinal vesicle; induce embryonic developmental arrest and cell death [4,14]. Therefore, study on the effect of potential roles of antioxidants on development of bovine secondary preantral follicles is an interesting subject.

Alpha lipoic acid (ALA) plays an important role in regulation of mitochondrial function, ALA in its reduced form acts as potent antioxidants and scavenges free radicals [15]. Moreover, ALA maintains thiol groups on intracellular proteins such as ATPases and other vital proteins [16–19]. Several studies reported that ALA supplementation reduced apoptosis, however, it upregulates growth and antioxidant enzymes in cumulus-oocyte complexes [16–19]. In this context, ALA supplementation has been implicated in several diseases including infertility [20,21]. Despite several studies, address the effect of ALA on follicles maturation. Yet, no enough data are available about the expression of follicles regulating genes and apoptotic-induced genes under the effect of ALA, further studies are required.

It is important to mention that growth factors are expressed at different stage during the follicle development. For example, FGF-2 has been shown to promote activation of primordial follicles [19], whereas IGF-I transcripts were low during the primary follicular stage but increased to a maximum in the late preantral and early antral stages [20]. In other words, no prediction could be suggested on the effect of ALA on primordial follicle, and we think that a serious study should be done on this context.

The secondary preantral follicles are a good cellular model which give means for the study of the biology of follicular development, ovulation, and for studying factors that affect secondary preantral follicles development *in vitro* [14]. Based on aforementioned data addition of ALA may enhance the development, maturation of the bovine secondary preantral follicles. On contrary, ALA may reduce their apoptosis secondary preantral follicles. In this study, effect of ALA of addition on the expressions of follicles development regulating genes (FSHR, luteinizing hormone/choriogonadotropin receptor [LHCGR], IGF-1, BMPR1a, transforming growth factor beta receptor 1 [TGFβR1], TGFβ1, ActRIIB, GDF9, and Activin A) and apoptotic-induced genes (BCL2-associated X protein [BAX] and C-Myc) were estimated *in vitro* using bovine secondary preantral follicles.

2. Materials and methods

Medium 199 with Earle's salts (M199), Dulbeccos's phosphate-buffered saline (PBS), and trypsin with EDTA solution were obtained from Gibco (Grand Island, NY, USA). The present reagents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Isolation of preantral follicles

Six ovaries of adult bovines, were placed in isotonic sodium chloride solution (0.9% NaCl) to which penicillin (200 IU/mL) and streptomycin (200 μg/mL) have been added at 30 °C to 35 °C. Sections of the ovarian cortex were obtained using a scalpel and placed into a tissue chopper adjusted to produce 200-μm sections and then were put in Dulbecco's phosphate-buffered solution supplemented with 36-μg/mL sodium pyruvate, 1-μg/mL glucose, and 3 mg/mL BSA. After several washings and repeated pipetting, the samples were filtered first through a 150-μm filter to remove all large fragments and debris, then through a 80-μm filter to exclude blood cells, small follicles, and other minute particles. The tissue remaining on the 80-μm filter was washed and collected in an embryo searching dish (100 × 20 mm, Falcon) to collect different size of the follicles.

2.2. Culture of isolated preantral follicles

The freshly isolated follicles were examined microscopically; the normal follicles were selected (healthy appearing, spherical with one or more compact layers of granulosa cells around the oocyte with an intact basal membrane and no apparent sign of necrosis and no antrum). Secondary preantral follicles with diameters of 80 to 110 μm were collected and divided into two categories according to the size, the first set started the cultures by 80 to 100 μm and the second by 100 to 110 μm. The follicles were cultured *in vitro* using tissue culture medium of the following system:

The isolated follicles were cultured individually in droplets of TCM-199 (20 μL) plus FSH (100 ng/mL) and EGF (100 ng/mL). Then, it was supplemented with, 10% NCS (Newborn Calf Serum, Gibco), 0.23-mM sodium pyruvate, 1% ITS (Insulin, Transferring, Selenium; Gibco), 100 mIU/mL of penicillin, 75 mg/mL of streptomycin, and 2.2-g/L sodium bicarbonate, overlaid sterile mineral oil in falcon culture dishes at 38.5 °C, 100% humidity and 5% CO_2 for up to 15 days. ALA stock solution was prepared and diluted appropriately, and then 0, 100, 250, or 500 μM of ALA were added to culture medium according to the doses selected in the study of Talebi et al. [14].

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