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Follicle-stimulating hormone–induced rescue of cumulus cell apoptosis and enhanced development ability of buffalo oocytes

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ABSTRACT

The effect of follicle-stimulating hormone (FSH) on apoptotic status of cumulus cells, expression of proapoptotic and antiapoptotic genes, and development rate of in vitro fertilization–produced buffalo embryos were investigated. FSH supplementation in in vitro maturation–medium resulted in a dose-dependent reduction in the expression of proapoptotic genes namely, BCL2-associated X protein (*BAX*), cytochrome c, and caspase-3 and increase in the expression of antiapoptotic genes such as B-cell lymphoma 2 (*BCL2*) and X-linked inhibitor of apoptosis protein (*XIAP*) in cumulus cells of mature oocyte. Cumulus expansion, oocyte maturation, cleavage, and blastocyst development rates were significantly higher ($P < 0.05$) in 5 and 10-μg/mL FSH-supplemented groups as compared with control. Significant increase in the expression of FSH receptor messenger RNA was also found with 5 and 10-μg/mL FSH ($P < 0.05$). Terminal deoxynucleotidyl transferase dUTP nick end labeling assay confirmed that the population of apoptotic cumulus cells of matured oocytes was reduced in the FSH-treated groups as compared with control ($P < 0.05$). In conclusion, our data suggest that FSH may attenuate apoptosis in cumulus cells via mitochondria-dependent apoptotic pathway by increasing *XIAP* expression, resulting in a more favorable ratio of *BCL2*/*BAX* expression and decreasing the cytochrome c and caspase-3 expression, eventually contributing to developmental competence of oocytes. The information generated will help in improving the in vitro embryo production program in buffalo.

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1. Introduction

Follicular atresia is an integral part of the natural folliculogenesis process in mammalian ovaries, and it happens at any stage of follicular development [1]. Popular schools of thought represent that the apoptotic state of the somatic cells surrounding the oocytes eventually decides their development fate. The exact sequence of molecular events that lead to follicular atresia, however, remains unclear [2]. Growing evidence indicates that the death of granulosa cells mediated by the active process of programmed cell death or apoptosis is associated with the rate of follicular atresia in porcine [3], bovine [4,5], and rodent ovaries [6].

Apoptosis is characterized by cytoplasmic and nuclear fragmentation, chromatin condensation, DNA fragmentation and phagocytosis [7,8]. It is generally accepted that the events during programmed cell death are the consequence of activation of the caspase cascade [9]. Apoptosis, in mammalian cells can be initiated by 2 distinct pathways namely the extrinsic pathway, which is triggered by

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ligation of death receptors and subsequent caspase-8 activation [10,11], and/or the intrinsic pathway, which is initiated by cellular stress level and relies on a cell's ability to sense changes in the ratio between members of the B-cell lymphoma 2 (BCL2) family of proteins followed by activation of caspase-9 [12,13]. These 2 pathways converge to a common execution phase that requires the activation of caspase-3 and -7 from their inactive zymogen to active forms [14–16]. A shift in equilibrium toward proapoptotic (eg BCL2-associated X protein [BAX]) vs antiapoptotic (eg BCL2, BCL-xL) proteins makes the mitochondrial membrane permeable, releasing cytochrome c into the cytosol [17–19], which subsequently binds to the cytosolic apoptotic protease activating factor-1 (Apaf-1) and procaspase-9 [20] to form the apoptosome complex [21]. Eventually, this results in activation of the main executor caspase-3, thus, arriving at a "point of no return" [18,22,23].

Vulnerability of oocytes and associated cumulus cells to apoptotic stimuli has been studied by several workers by measuring the ratio of proapoptotic and antiapoptotic members of *BCL-2* family [24–26]. The levels of apoptosis in cumulus cell have been found to be correlated with developmental potential of in vitro fertilization (IVF)–produced human [27] and bovine embryos [28]. Follicle-stimulating hormone (FSH) alone or in combination with other growth factors has been reported to suppress follicular cell apoptosis in murine preantral, antral, and preovulatory follicles [29], and in cultured granulosa cells of bovine and porcine systems [3,30]. In bovine in vitro granulosa cell culture, supplementation of FSH has been demonstrated to increase the ratio of *BCL2* to *BAX* and improved cell survivability [31]. Similarly, FSH-mediated suppression of apoptosis in granulosa cells by stimulated X-linked inhibitor of apoptosis protein (*XIAP*) expression [32,33] and inhibited caspase-3 expression have been reported [34].

As an aid to augment reproduction rate in buffalo, numerous assisted reproduction technologies have been tried but with limited success [35–37]. Some peculiarities of this species such as a limited stock of primordial follicles, higher rate of atresia, reduced number of good quality oocytes, and so forth call for a strategy to augment the developmental competence of oocytes.

The present study was conducted to investigate how FSH supplementation affects the cumulus cell health and developmental competence of buffalo oocytes.

2. Materials and methods

All media and chemicals were procured from Sigma-Aldrich, St. Louis, MO, USA unless otherwise indicated. Disposable plastic wares used were from Falcon NJ, USA and Nunc, Denmark. Fetal bovine serum used was from Hyclone, Canada.

2.1. Production of buffalo embryos

Buffalo ovaries were collected from an abattoir and transported within 3 to 4 h to the laboratory in phosphate-buffered saline (PBS) containing streptopenicillin (0.05 mg/mL). Ovaries were washed several times in normal saline, and cumulus-oocyte complexes (COCs) were aspirated from ovarian follicles in N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid-buffered hamster embryo culture medium [38]. Only excellent grade oocytes with more than 5 compact layers of cumulus cells and homogenous ooplasm were used for in vitro maturation (IVM) and IVF. A group of 25 COCs were kept in each drops of 100-μL IVM medium (tissue culture media-199 N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid modified with 0.005% streptomycin, 0.01% sodium pyruvate and 0.005% glutamine, and 0.01% polyvinyl alcohol). The IVM medium was supplemented with different concentrations of porcine FSH (0, 1.0, 5.0, and 10.0 μg/mL) and cultured for 24 h at 38.5°C and 5% CO_2. IVF and culture of embryos were carried out as described earlier [39]. Briefly, the oocytes after maturation were washed 3 times in modified synthetic oviductal fluid (mSOF)-based IVF medium and transferred to 50-μL fertilization drops in groups of 20–25. Frozen semen of a buffalo bull was thawed at 37°C and washed 2 times, and 1×10^6 sperm were transferred to fertilization drops containing matured oocytes and incubated in CO_2 incubator for 14 h at 38.5°C and 5% CO_2. Throughout the experiment, semen from a single bull was used. Presumptive zygotes were collected from the fertilization droplets, whereas the adhered cumulus cells and supernumerary spermatozoa were mechanically removed by repeated pipetting and washed 4 times in mSOF-based in vitro culture (IVC) media. After washing, 15–20 presumptive zygotes were cocultured with monolayers of cumulus cells in 100-μL droplets of mSOF-based IVC medium at 38.5°C and 5% CO_2. Thirty-six hours after insemination, presumptive zygotes were evaluated for cleavage, and after 72 h, all the cleaved embryos were transferred to IVC-II medium (mSOF supplemented with 10% fetal bovine serum, 5.6-mM glucose, 0.33-mM pyruvate, 3.3-mM lactate, 1-mM glutamine, 1x minimum essential medium essential amino acid, 1x nonessential amino acid and 50-μg/mL gentamicin) and maintained for 7 d at 5% CO2 and 38.5°C with replacement of medium at every 48 h. Each experiment was conducted with at least 120 immature oocytes as starting material, and all IVM, IVF and IVC experiments were repeated at least 4 times for each experimental group.

2.2. Observations made on developing oocytes and embryos

After 24 h of IVM, the cumulus expansion index (CEI) was calculated on a scale of 0 to 4 as described earlier [40]. Attainment of metaphase-II (M-II) was determined by denuding the oocytes and staining with Hoechst 33342 dye after the method of Smith [41] with slight modification. Briefly, denuded oocytes from all experimental groups were fixed in 4% (wt/vol) paraformaldehyde solution (in PBS, pH 7.4) for 1 h at room temperature. After fixing and washing, groups of 50 oocytes were transferred to 200-μL drops of 10-μg/mL Hoechst 33342 dye solution for 20 min in the dark. Stained oocytes were washed 3 times in PBS-polyvinylpyrrolidone (PVP) solution and placed on glass slides and mounted with proLong mounting medium (Invitrogen, USA) and observed under a fluorescent microscope with UV filter (Olympus, Japan). Oocytes with intact nucleus and a polar body were considered as mature (M-II). The maturation percentages were calculated for each IVM group involving at least 250 oocytes in 4

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