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Quantitative expression of antiapoptotic and proapoptotic genes in sheep ovarian follicles grown *in vivo* or cultured *in vitro*

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ABSTRACT

To test the hypothesis that the poor development of the oocytes in cultured ovarian follicles of mammals is due to aberrant expression of developmentally important genes, quantitative expression patterns of *Bcl2* (B-cell leukemia/lymphoma 2; antiapoptotic) and *Bax* (Bcl2-associated X protein; proapoptotic) genes in preantral, early antral, antral, large antral follicles, and cumulus–oocyte complexes (COCs) grown *in vivo* or cultured *in vitro* were studied. The level and pattern of expression of Bcl2 in the cumulus cells isolated from different development stages of *in vivo*– and *in vitro*–grown ovarian follicles were similar suggesting that *in vitro* culture did not alter the expression of this antiapoptotic gene in the cumulus cells. However, between the *in vivo*– and *in vitro*–grown ovarian follicles (1) Bcl2 expression levels in the oocytes from antral follicles (2.21 ± 0.14 vs. 0.87 ± 0.19), large antral follicles (0 ± 0.35 vs. 1.56 ± 0.13), and COCs (0.45 ± 0.31 vs. 2.69 ± 0.15), Bax expression levels in the (2) cumulus cells from early antral (2.09 ± 0.11 vs. 0.98 ± 0.13) and large antral follicle (0.63 ± 0.44 vs. 0 ± 0.21), and (3) oocytes from antral follicles (1.65 ± 0.20 vs. 0.97 ± 0.15), large antral follicles (0.93 ± 0.18 vs. 2.08 ± 0.11), and COCs (1.03 ± 0.17 vs. 2.09 ± 0.11) were significantly different ($P \leq 0.05$). Similarly, Bcl2 to Bax ratios were also significantly different between some but not all stages of *in vivo* and *in vitro* development. From the present results, it is concluded that imbalance in the expression of proapoptotic and antiapoptotic genes may be an important cause for the compromised development potential of the oocytes in cultured ovarian follicles of sheep.

1. Introduction

Apoptosis or programmed cell death, known as atresia among the ovarian follicles, is a normal physiological process controlled by the hormones and growth factors [1]. Apoptosis occurs during prenatal and postnatal development of the ovarian follicles in mammals [2]. Apoptosis could be triggered by both internal and external signals involving several genes such as tumor necrosis factor family, B-cell lymphoma/leukemia 2 (Bcl2) family, small mitochondria-derived activator of caspases and, apoptotic protease activating factor 1 [3–6]. The Bcl2 family consists of both apoptosis promoting (e.g., *Bax*) and inhibiting genes (e.g., *Bcl2*) [6–9]. Although Bcl2 to Bax ratio reportedly drives the apoptosis in the ovarian follicles [10], systematic studies in mammals on the expression of *Bcl2* and *Bax* genes in different development stages of ovarian follicles were not reported.

In the mouse [11], the buffalo [12], the sheep, [13] and the goat [14], it is now possible, although to a limited extent, to overcome the postnatal apoptosis of the ovarian

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follicles by *in vitro* culture of primordial/preantral follicles (PFs) and *in vitro* fertilization of the oocytes from these cultured PFs. However, in the sheep [13] and the other species of mammals [11,12,14], the development competence of the oocytes in cultured PFs was poor relative to the oocytes in the *in vivo*–grown follicles. It was therefore hypothesized that aberrant expression of developmentally important genes in the cultured PFs may be responsible for the compromised development of these oocytes [13]. In this connection studies on quantitative expression patterns of developmentally important genes in *in vivo*– and *in vitro*–grown ovarian follicles are expected to improve the comprehension of ovarian folliculogenesis and hence explain the poor development of oocytes in the cultured follicles. A recent report from the laboratory on the pattern of expression of aromatase gene P450 in different development stages of *in vivo*– and *in vitro*–grown ovarian follicles in sheep is in accordance with this presumption [15]. Because apoptosis is the major route of destruction of the ovarian follicles [1], imbalance in the expression of apoptosis inducing and preventing genes in cultured ovarian follicles might be another important cause for the poor development of oocytes in cultured ovarian follicles.

In view of the previously mentioned information, the present study compared for the first time the quantitative expression pattern of *Bcl2* and *Bax* genes in the cumulus cells and oocytes at different development stages of *in vivo*–grown and cultured ovarian follicles in sheep.

2. Materials and methods

The present study was undertaken as per the guidelines of the institutional research and ethics committee. All the materials and methods used in this study were as standardized in the laboratory [13,15,16]. However, a brief description is provided hereunder.

Unless otherwise stated, culture media, hormones, growth factors, fetal calf serum, and all the other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and plastics from Nunclon (Roskilde, Denmark). All the hormones and growth factors used were cell culture tested and endotoxin free.

2.1. Collection and processing of ovaries

On each day of the study, 5 to 10 pairs of ovaries from sheep recovered at a local slaughter house were transported to the laboratory in sterile, warm (37 °C) PBS. The ovaries were trimmed off adherent tissues and ligaments and washed twice in PBS supplemented with 50 μg/mL gentamycin sulfate. All the subsequent procedures were carried out in a laminar flow [13,15–19].

2.2. Isolation of different stages of ovarian follicles

Ovaries were cut into two halves, and the medulla was scooped out. After the removal of medulla, the ovaries were placed in 35-mm plastic culture dishes (153066; Nalge Nunc, Denmark) containing a handling medium (HEPES-buffered tissue culture medium 199 supplemented with 0.23 mM of sodium pyruvate, 2-mM L-glutamine, and 50 μg/mL gentamycin sulfate) and cut into thin slices using a sterile surgical blade. From these thin ovarian slices, intact PFs, early antral, antral, and large antral follicles were mechanically isolated (Fig. 1A, C, E, G) by microdissection under a stereoscopic zoom microscope (SMZ 2T; Nikon Corporation, Japan) using two 26-gauge needles fitted to 1 mL syringe barrels and a surgical blade. To avoid damage to the basement membrane, a small amount of stromal tissue was left attached (Fig. 1A) [13,15–19]. From each ovary, 5 to 10 preantral, 5 to 10 early antral, 3 to 5 antral, and 1 to 2 large antral follicles could be routinely isolated. Accordingly for each of the three replicates of the present study, 60 to 70 ovaries collected from 30 to 35 ewes were used.

2.3. Selection and culture of PFs

Intact PFs in the size range of 250 to 400 μm having centrally placed spherical oocytes with no signs of atresia and with intact basement membrane were selected for the culture (Fig. 1A). Bicarbonate-buffered tissue culture medium 199 containing 50 μg/mL gentamycin sulfate (Sigma G1272), 1 μg/mL Thyroxin (T_4; Sigma T1775), 2.5-μg/mL FSH (Sigma F2293), 10 ng/mL insulinlike growth factor 1 (Sigma I8779), and 1 mIU/mL of growth hormone (Sigma S8648), which supported the best development *in vitro* of PFs and maturation of oocytes to metaphase II stage earlier [13] was used in this study to culture the PFs. Culture medium was pre-incubated for 1 hour at 39 °C under humidified atmosphere of 5% CO_2 in air. The selected follicles were washed thrice in the culture medium and subsequently placed individually in 20 μL droplets of the culture medium in 35-mm plastic culture dishes (Nunc, 15066). To avoid evaporation of the medium, the microdroplets were overlaid with autoclaved lightweight mineral oil (Sigma M8410) preequilibrated with the medium overnight at 39 °C in 5% CO_2 in air. These culture dishes were incubated at 39 °C under humidified atmosphere in 5% CO_2 in air for up to 6 days. The culture procedure was found to be optimum earlier [13,15–19]. The day on which the PFs were placed in culture was designated as Day 0 and the subsequent days as Day 1, 2, and so on. Half the medium was replaced by an equal volume of fresh medium every 48 hours. Each follicle was morphologically evaluated every 24 hours during culture period using an inverted microscope (Leica, DMIRB, Germany).

In vivo– and *in vitro*–grown ovarian follicles at morphologically corresponding development stages (Fig. 1) were carefully opened using two 26-gauge needles attached to 1-mL syringe barrels, to release the cumulus–oocytes complexes (COCs) (Fig. 1I and J). The oocytes were denuded of adhering cumulus cells by repeated pipetting through a marrow bore glass pipette. However, some COCs from 6-day cultured follicles and those from *in vivo*–grown large antral follicles were matured *in vitro* for additional 24 hours has described before isolation of cumulus cells and oocytes.

2.4. IVM of oocytes obtained from the in vivo–grown and cultured large antral follicles

The COCs from the PFs cultured for 6 days (Fig. 1J) and *in vivo*–grown large antral follicles (Fig. 1I) were subjected