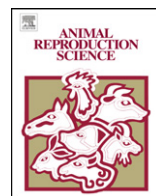




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# Early apoptosis is associated with improved developmental potential in bovine oocytes

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### ABSTRACT

The poor quality of oocytes may be the main reason for the low efficiency of the current *in vitro* embryo production. However, efforts are required to understand the mechanisms of oocyte development, which is believed to be largely regulated by apoptosis *in vivo*. The aim of this study was to investigate the levels of apoptosis in bovine immature oocytes with different developmental potentials and to determine whether early apoptosis in bovine oocytes is correlated with their subsequent development. Cumulus–oocyte complexes (COCs) were selected and classified into four groups according to oocyte cytoplasm and cumulus status. Early and late stages of apoptosis were detected by Annexin-V and TUNEL staining, respectively. Developmental competence was evaluated by nuclear maturation (MII) after *in vitro* maturation and development rates in different stages following *in vitro* fertilization. Meanwhile, the transcripts of Bcl-2 and Bax genes were carried out in immature oocytes by real-time RT-PCR. Results indicated that Annexin-V-positive oocytes were detected in various groups at different percentages, and Group III showed the highest positive ratio. No TUNEL-positive oocytes were found in any immature COCs. Group III oocytes demonstrated the highest nuclear maturation, cleavage, blastocyst, and hatching blastocyst rates. Meanwhile, Group III oocytes exhibited the highest Bax (initiating apoptosis) transcriptional level and the lowest Bcl-2 (preventing apoptosis) transcriptional level. Taken together,

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Annexin-V and quantitative PCR results indicated that early apoptosis was beneficial for developmental competence, while TUNEL staining showed that none of the immature oocytes were undergoing late-stage apoptosis. This is the first time that Bax and Bcl-2 transcripts were characterized in the immature bovine oocyte, and results indicated that the genes are good markers of early apoptosis and embryo development. This research overthrows the traditional view that oocytes undergoing apoptosis have poor developmental competence, and the findings will facilitate oocyte selection and improvement of *in vitro* embryo production.

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## 1. Introduction

It is generally accepted that the quality of embryos produced *in vitro* is significantly lower than that of their *in vivo*-derived counterparts (Sirard, 1989; Cognie et al., 2003; Peterson and Lee, 2003). In terms of efficiency, approximately 30–40% of bovine oocytes retrieved from abattoir ovaries develop to the blastocyst stage (Lonergan et al., 2003), which could partially be due to the use of inferior-quality oocytes (Rizos et al., 2002a,b, 2003). Therefore, evaluation of oocyte quality is the most important and challenging task during *in vitro* embryo production (IVEP).

On a routine basis, oocyte quality is assessed immediately after recovery by using several non-invasive, visual assessment parameters such as the morphology of COCs (Wasielak and Bogacki, 2007) and oocyte and follicle diameter (Acosta, 2007; Miyano and Manabe, 2007). Oocytes with a compact cumulus composed of several layers of cells and a homogeneous cytoplasm are considered healthy; these oocytes are then selected for *in vitro* maturation and *in vitro* fertilization. Interestingly, studies examining the developmental competence of bovine oocytes on the basis of their visual appearance have revealed that COCs showing early signs of atresia (e.g., slight expansion of the cumulus and slight granulation of the cytoplasm) have a higher developmental potential than those considered to be morphologically healthy (Blondin and Sirard, 1995; de Wit and Kruij, 2001; Bilodeau-Goeseels and Panich, 2002). Similarly, the percentage of blastocysts increased with increasing signs of follicular atresia, except in the case of highly atretic follicles (de Wit et al., 2000). To date, the levels of apoptosis in groups of oocytes with different developmental potential have never been completely characterized. In this study, apoptosis levels were characterized using three techniques in order to study the early and late events of apoptosis. Furthermore, we measured the levels of apoptosis in oocytes with different developmental competencies.

Apoptosis is defined as self-destruction of cells under physiological control (Ameisen, 2002). Typical features of apoptotic cells include cell shrinkage, translocation of phosphatidyl-serine to the outer cytoplasmic membrane, DNA fragmentation, and segmentation of the cell into apoptotic bodies. Members of the Bcl-2 gene family, such as Bax and Bcl-2, play key roles in regulating apoptosis (Yang and Rajamahendran, 2002). The role of the Bcl-2 family members in apoptosis in female gonads has been extensively studied by Kim and Tilly (2004), who suggested that Bax and Bcl-2, respectively, were 2 key factors in initiating or preventing apoptosis in female germ cells. In this study, real-time RT-PCR was carried out to determine whether the expression levels of Bax and Bcl-2 genes on the RNA correlated with apoptosis in immature oocytes.

Many reports have examined apoptosis in bovine ovarian follicles (Isobe and Yoshimura, 2007) and cumulus cells (Luciano et al., 2000), but information on apoptosis in bovine oocytes is limited and inconsistent. For example, Matwee et al. (2000) demonstrated that apoptosis both in mature and immature oocytes by using TUNEL staining, while Yuan et al. (2005), who used the same technology, detected no apoptotic oocytes before or after maturation.

The objective of the present study is to investigate whether or not bovine oocytes in COCs undergo apoptosis. If apoptosis occurs, the relationship between the frequency of apoptosis in oocytes and their developmental competence needs to be determined. In order to address these issues, we assessed the developmental competence of COCs by nuclear maturation (MII) after *in vitro* maturation and the development rates in the cleavage, blastocyst, and hatching blastocyst stages after *in vitro* fertilization.

Early (change in cytoplasmic membrane) and late (DNA fragmentation) markers of apoptosis were detected by Annexin-V and TUNEL staining. Furthermore, transcript analysis of Bax and Bcl-2 genes with pro- and anti-apoptotic functions were carried out on immature oocytes by real-time RT-PCR.

## 2. Materials and methods

### 2.1. Chemicals

Unless otherwise stated, all chemicals were purchased from the Sigma Chemical Co. (St Louis, MO, USA).

### 2.2. Collection and classification of COCs

Cow ovaries were obtained from a local abattoir and transported to the laboratory in a sterilized saline solution containing 100 IU/ml penicillin and 0.05 mg/ml streptomycin; transport took place within 4 h of slaughter and the temperature was maintained at 30–35 °C. Immature oocytes were collected from 2- to 8-mm follicles by an 18-gauge needle. The COCs were isolated from the follicular fluid and washed 3 times with TCM-199 media (Gibco, Grand Island, NY, USA) supplemented with 0.05% (w/v) polyvinyl alcohol and 10 mM Hepes.

According to the appearance of their cumulus and cytoplasm, COCs were divided into four groups: GI COCs, with granular cytoplasm and more than five layers of cumulus cells; GII COCs, with homogeneous cytoplasm and more than five layers of cumulus cells; GIII COCs, with granular cytoplasm and between three and five layers of cumulus cells; and GIV COCs, with homogeneous cytoplasm and three to five layers of cumulus cells (Fig. 1). COCs exhibiting characteristics other than the abovementioned criteria were excluded. Half the COCs from each group were denuded in the TCM-199 medium and supplemented with 300 mg/ml hyaluronidase at the collection time with the intent of performing Annexin-V staining and TUNEL assay before maturation. The remaining COCs went through an *in vitro* maturation procedure.

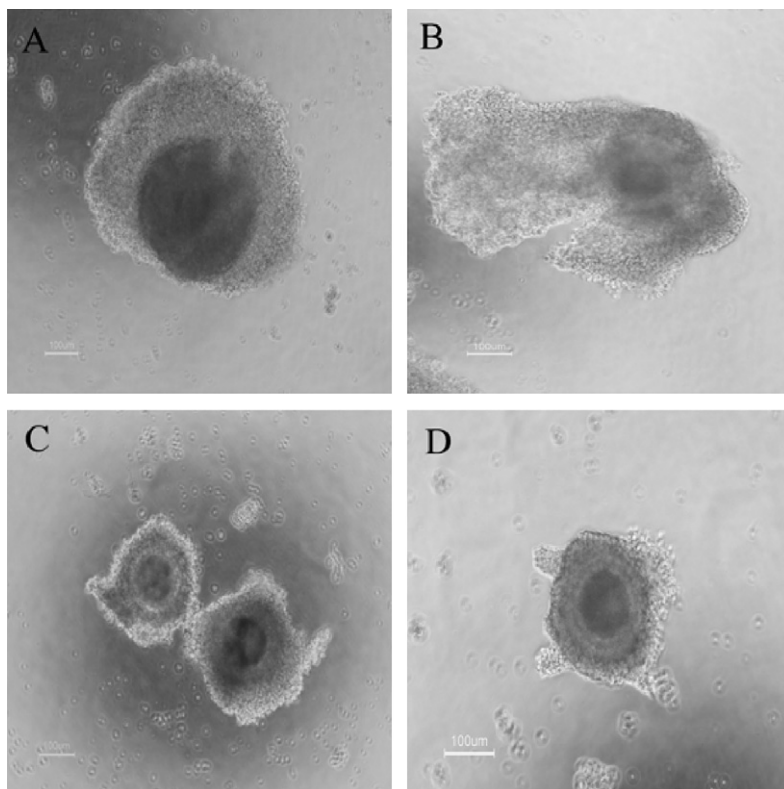
### 2.3. TUNEL assay in immature oocytes

All samples were washed three times in PVP (1 mg/ml polyvinyl-pyrrolidone in PBS) and fixed in 4% paraformaldehyde for 1 h at room temperature. Then, samples were rinsed with PVP and incubated in Triton X-100 sodium citrate (0.5% Triton X-100 plus 0.1% sodium citrate) for 1 h at room temperature. Finally, TUNEL staining was performed upon these samples. Positive and negative control samples were transferred to 0.1 U/ml DNase for 1 h at 37 °C, while the rest of the samples were kept in PVP. After DNase treatment, TUNEL staining was performed as instructed by the kit manufacturer (Roche, 1684795), followed by 1 h of RNase (Sigma) restriction at room temperature. The negative control was incubated without the terminal deoxynucleotidyl transferase enzyme. Subsequent samples were treated with 6.25 mg/ml of propidium iodide (PI) for 15 min before mounting them on slides for observation under a laser confocal scanning microscope (Bio-Rad MRC 1024ES). PI stained the nuclei from live cells red, while fragmented nuclei were stained yellow-green by fluorescein of the TUNEL assay.

### 2.4. Annexin-V staining in immature oocytes

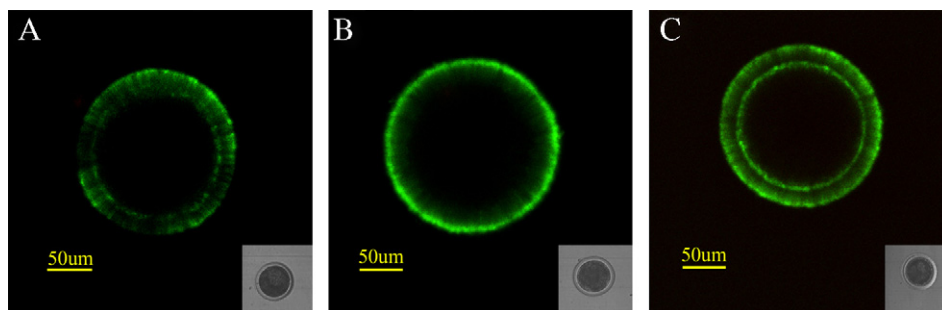
Staining was performed with an Annexin-V kit according to the manufacturer's instructions (BioSea Biotech Co., Beijing, China). Samples were stained with Annexin-V, a phospholipid-binding protein that detects the translocation of phospholipid phosphatidylserine (PS) from the inner to the outer cytoplasmic membrane, which is known to occur during the early stages of apoptosis.

At the same time, samples were also stained with PI to distinguish live cells from dead cells. Briefly, denuded immature oocytes were washed twice in PBS and stained with 200 µl of binding buffer containing Annexin-V- FITC plus PI for 30 min at 4 °C in the dark. Then, 300 µl of binding buffer was added. Following this, samples were mounted on siliconized slides and observed under a laser confocal scanning microscope (Bio-Rad MRC 1024ES).



**Fig. 1.** Morphology of various Cumulus–oocyte complexes (COCs).

According to the previous description (Anguita et al., 2007), the denuded oocytes were classified into the following three groups: (1) necrotic oocytes with PI-positive red nuclei, which is indicative of membrane damage. Oocytes with a discontinuous green signal originating from the remnant portions of the membrane of the cumulus-cell projections (Van Blerkom and Davis, 1998) were considered to be viable non-apoptotic oocytes (see Fig. 2A); (2) viable oocytes that were negative for annexin staining (see Fig. 2B); and (3) early apoptotic oocytes with a homogeneous annexin-positive signal in the membrane (see Fig. 2C).



**Fig. 2.** Oocyte classification by Annexin-V staining.

### 2.5. *In vitro* maturation

The immature COCs were cultured in a maturation medium (TCM-199 supplemented with 10%(v/v) heat-inactivated fetal calf serum (FCS), 0.2 IU/ml of follicle-stimulating hormones (FSH), 1 mg/ml of 17 $\beta$ -estradiol, and 25 mM of Hepes) within four-well plates (Nunclon, Roskilde, Denmark) containing 0.5 ml of maturation medium per well. The plates were incubated at 38.5 °C in 5% CO<sub>2</sub> humidified air for 22 h. Matured COCs from each group were divided into two fractions: one set was freed from cumulus cells for examination of nuclear maturation and the other for *in vitro* fertilization (IVF) and *in vitro* culture (IVC).

### 2.6. Evaluation of nuclear maturation in oocytes

After maturation, COCs were washed twice in PBS and vortexed to free the cumulus cells in PBS, supplemented with 0.3% hyaluronidase. Extrusion of the first polar body was assessed under a microscope. After this, the denuded oocytes were mounted on slides, submerged into the fixing solution (dehydrated ethanol:glacial acetic acid = 3:1) for 24–28 h, stained with orcein (LOBA CHEMIE PVT. LTD, India) for 15 min, and decolorized in washing solution (glycerin:glacial acetic acid = 1:3). Finally, COCs were evaluated for nuclei maturation under a phase contrast microscope (Nikon, Tokyo, Japan).

### 2.7. *In vitro* fertilization and embryo culture

Mature COCs were subsequently fertilized *in vitro* over a 6-h period. In brief, frozen bovine semen was thawed and washed twice with BO-1 media (BO + 10 mM caffeine), suspended at a concentration of 1  $\times$  10<sup>7</sup> ml<sup>-1</sup> in BO-2 (BO + 20 mg/ml of bovine serum albumin (BSA) + 2  $\mu$ l heparin/ml), and prepared as a 100- $\mu$ l suspension (Brackett and Oliphant, 1975). Then, the matured COCs were transferred into the sperm suspension to incubate at 38.5 °C in 5% CO<sub>2</sub> for 6 h. After that, all samples were cultured in synthetic oviduct fluid, supplemented with amino acids and fetal calf serum (SOFaa + FCS) medium. For the cultured embryos, the rates of cleavage and eight-cell stage were assessed at 48 h post-insemination and then again on day 7/8 and day 9/10 to assess rates of blastocyst and hatched blastocyst formation (the day of fertilization = day 0).

### 2.8. Quantification of pro- and anti-apoptotic molecules BAX and BCL-2 mRNA

COCs were recovered from cow ovaries and divided into four grades (Fig. 1). After cumulus cells were denuded from the COCs, immature oocytes were washed five times in PBS treated with diethyl pyrocarbamate (DEPC) and 50 oocytes from each group were stored in 15  $\mu$ l PBS within 1.5 ml tubes (Eppendorf, California, USA) at -80 °C to await RNA extraction. Total RNA extraction from pools of 50 oocytes was performed using the RNeasy kit (Fastagen, Shanghai, China) and treated with RNase-free DNase I to remove any possible DNA contamination. RT and qPCR, run in two separate steps, amplified the targets. However, RNA yield from each sample was so low that it could not be accurately quantified by spectrophotometry (Eppendorf, Hamburg, Germany); therefore, 6.5  $\mu$ l aliquots of RNA were reverse transcribed with PrimerScript<sup>TM</sup> RTase (TaKaRa, Inc. Dalian, China). RNA was converted to cDNA, again, according to the manufacturer's directions, in the following manner: a 20- $\mu$ l reaction for 15 min at 37 °C, followed by 5 s at 84 °C to inactivate the reverse transcriptase.

The following primers were used: Bax (GeneBank accession no. NM173894): forward 5'-TTT GCT TCA GGG TTT CAT C-3' and reverse 5'-CAG CTG CGA TCA TCC TCT-3' with an annealing temperature of 57 °C to amplify a 173 bp fragment; Bcl-2 (GeneBank accession no. XM586976): forward 5'-CTG CAC CTG ACG CCC TTC AC-3' and reverse 5'-GCG TCC GAC CCT CCG TTG T-3' with an annealing temperature of 64 °C to amplify a 236 bp fragment;  $\beta$ -Actin primers (GeneBank accession no. BT030480): forward 5'-GTC ACC AAC TGG GAC GAC A-3' and reverse 5'-AGG CGT ACA GGT GAC AGC A-3' with two annealing temperatures of 57 °C (with Bax) and 64 °C (with Bcl-2) to amplify a 208 bp fragment.

Real-time PCR was preformed in a 20  $\mu$ l reaction buffer containing 10- $\mu$ l of SYBR<sup>®</sup> Premix Ex TaqTM (TaKaRa, Inc. Dalian, China) (2 $\times$ ), 0.8  $\mu$ l of 200 nM each, of forward and reverse primers 2  $\mu$ l cDNA and 7.2  $\mu$ l H<sub>2</sub>O. PCR was performed using a DNA Engine Opticon2 two-color real-time PCR Detection

**Table 1**  
Nuclear maturation and developmental competence of oocytes.

COCs classes	No. of IVM oocytes (replicates)	Nuclear maturation rates (% ± S.E.M.)	No. of IVM/IVF Oocytes (replicates)	Cleavage rates (% ± S.E.M.)	Blastocyst rates (% ± S.E.M.)	Hatched blastocyst rates (% ± S.E.M.)
I	71(4)	71.2 ± 3.5ab	119(4)	66.7 ± 4.1bc	22.5 ± 1.8bc	5.8 ± 0.7bc
II	64(4)	57.6 ± 6.9b	127(4)	56.4 ± 5.2c	15.6 ± 0.5c	3.7 ± 1.3c
III	57(4)	85.3 ± 2.2a	113(4)	84.1 ± 2.8a	39.6 ± 2.2a	16.4 ± 2.4a
IV	65(4)	70.9 ± 7.ab	140(4)	75.0 ± 4.7ab	27.5 ± 3.2b	9.0 ± 1.6b

Values in the same column with different superscripts (a, b, and c) differ significantly ( $P < 0.05$ ).  $a > b > c$ .

System (Bio-Rad laboratories, Inc. USA). The reaction protocol was as follows: 45 cycles of 5 s at 95 °C for denaturation, 15 s at 57 °C/64 °C for annealing, and 10 s at 72 °C for extension. Gene expression was presented using a modification of the  $2^{-\Delta\Delta CT}$  method, first described by K. Livak in PE Biosystems Sequence Detector User Bulletin 2 (Schmittgen et al., 2000). The expression of each gene was presented as  $2^{-\Delta CT}$ , where  $\Delta CT = CT$  of the sample –  $CT$  of the internal control. Each group was replicated six times. Amplicon specificity and size were confirmed using a melting curve analysis and 2% agarose gel electrophoresis; amplicon sequences were confirmed by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

2.9. Statistical analysis

The statistical analyses of data were performed using SPSS11 for Windows (Analytical Software, Chicago, IL). The rates of oocyte maturation (MII) and embryo development, Annexin-V positive cells, and the relative expression of the difference in Bax and Bcl-2 mRNA among oocytes in the different COC groups were analyzed by one-way ANOVA. The least significant difference (L.S.D.) test was applied for the post hoc multiple comparison test. Meanwhile, relative mRNA levels were expressed as the mean ± S.E.M. ( $n = 6/\text{group}$ ). Differences were considered significant if  $P < 0.05$ .

3. Results

3.1. Nuclear maturation and development potential of oocytes

The rates of nuclear maturation and development of bovine oocytes in four morphological groups subsequent to *in vitro* maturation (IVM) and IVF are shown in Table 1. Most oocytes (>70%) in GI, GIII, and GIV achieved nuclear maturation at 22 h after IVM, while oocytes in GII matured at a slower rate (<60%). The highest rates of cleavage, blastocyst, and hatching blastocyst were observed for GIII-COCs, and the lowest rates were found in GII-COCs. Statistically significant differences were observed between GII- and GIII-COCs ( $P < 0.01$ ).

3.2. Annexin-V and TUNEL staining of the immature oocytes

The results of Annexin-V staining in bovine immature oocytes are represented in Table 2. Many oocytes were positively stained by Annexin-V in different groups (ranging from 14.4% to 46.7%; Fig. 2).

**Table 2**  
Annexin-V staining in different groups.

COCs classes	No. of immature oocytes (replicates)	Early apoptosis rates (% ± S.E.M.)
I	98(4)	28.7 ± 7.5b
II	116(4)	14.4 ± 3.5b
III	69(4)	46.7 ± 4.8a
IV	92(4)	25.6 ± 5.9b

Values with different superscripts (a and b) differ significantly ( $P < 0.05$ ).  $a > b$ .



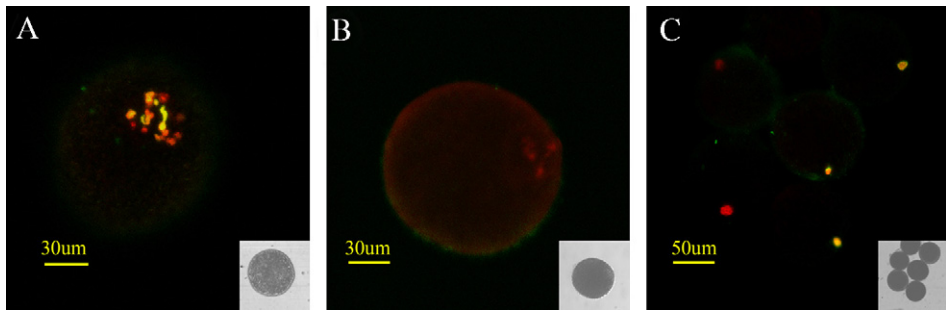


Fig. 3. Oocytes stained by TUNEL.

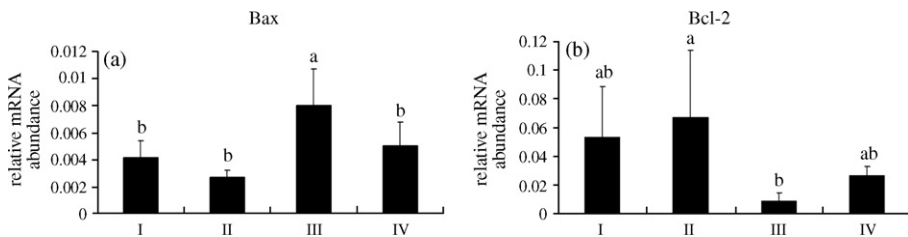


Fig. 4. Relative abundance of Bax and Bcl-2 transcripts in immature bovine oocytes.

The oocytes in COCs of GIII displayed a significantly higher ratio of positive cells than those in the other three groups ( $P < 0.05$ ). The lowest early apoptosis ratio existed in GII-COCs.

Compared with the positive control (Fig. 3A), TUNEL positive oocytes were not detected in any immature COCs (Fig. 3B). However, after 72 h of maturation, the majority of oocytes in all groups showed TUNEL-positive fluorescence (Fig. 3C).

### 3.3. BAX and BCL-2 transcript relative quantification

Relative quantification of Bax and Bcl-2 genes was performed to detect the differential expression in the immature oocytes of the four groups. Statistical analysis of the data demonstrated that bovine Bax and Bcl-2 mRNA expression in oocytes of GII- and GIII-COCs showed a distinct reverse pattern, exhibiting high expression levels of Bax with low expression of Bcl-2 (Fig. 4). Oocytes from GIII-COCs exhibited the highest expression level of Bax, while the lowest expression levels were observed in GII-COCs ( $P < 0.05$ ). The expression levels of oocytes in GI- and GIV-COCs were higher than those in GII-COCs, but this difference was not significant ( $P > 0.05$ ).

## 4. Discussion

Previous studies have shown that COCs showing early signs of atresia (e.g., slight expansion of the cumulus and slight granulation of the cytoplasm) had higher developmental potential than those considered to be morphologically healthy (Blondin and Sirard, 1995; de Wit and Kruip, 2001; Bilodeau-Goeseels and Panich, 2002). These findings increased our knowledge about the relationship between COC morphology and developmental competence; however, it remains unclear whether the oocytes with early signs of atresia undergo apoptosis to some degree. Furthermore, the question still remains as to whether apoptosis affects the developmental competencies of immature oocytes. In this study, the apoptotic changes and their possible effects on the developmental potentials of oocytes in different groups were investigated.

In the present study, oocytes with granulating ooplasm (GI and GIII) possessed numerically higher nuclear maturation, cleavage, blastocyst, and hatching blastocyst rates than those with homogeneous

cytoplasm (GII and GIV), in terms of the same cumulus cell layers. These results agreed with previous findings (de Loos et al., 1992; de Wit and Kruip, 2001; Bilodeau-Goeseels and Panich, 2002), suggesting that COCs showing early signs of atresia (e.g., slight expansion of the cumulus and slight granulation of the cytoplasm) had higher nuclear maturation and development potential than those considered to be morphologically healthy. Similarly, the study comparing oocytes with homogeneous and heterogeneous ooplasm (Nagano et al., 1999) also revealed that oocytes with granulations had a lower incidence of polyspermia and higher cleavage rates. Previous reports have concluded that COCs with good morphology tend to develop better *in vitro*, while other studies illustrate that COCs with good morphology could originate from the late atretic follicles and develop more poorly (Jewgenow et al., 1999; de Wit et al., 2000). Meanwhile, regardless of the ooplasm conditions, oocytes with three to five layers of cumulus cells (GIII and GIV) displayed a higher nuclear maturation and developmental capacity than those with more than five layers of cumulus cells (GI and GII). It is known that cumulus cells are involved in oocyte growth and maturation, but whether oocytes with more cumulus cells, like those from GI and GII, might be from the late atretic follicles needs to be investigated further. Taken together, GIII-COCs exhibited the highest nuclear maturation, cleavage, blastocyst, and hatching blastocyst rates, while GII-COCs demonstrated the lowest nuclear maturation and developmental competence. This result indicates that atresia may influence the developmental potential of COCs.

Studies on the incidence of apoptosis have produced contradictory results: Matwee et al. (2000) observed apoptosis in bovine oocytes before maturation, while Yuan et al. (2005) did not. In this study, TUNEL-positive oocytes were not detected in the immature COCs of any group, which is consistent with the findings of studies in cows (Yuan et al., 2005), humans, and mice (Van Blerkom and Davis, 1998). Differing from TUNEL results, many immature oocytes were positively stained by Annexin-V in all groups (ranging from 14.4% to 46.7%). It is known that the oocyte is the last compartment to be affected by apoptosis during atresia of antral follicles (de Wit et al., 2000). Therefore, those immature oocytes may be at earlier stages of apoptosis before DNA breakdown. However, it is currently unknown whether the earlier apoptosis affects the developmental capacity of oocytes.

In many well-known models, apoptosis can be broken down into four general stages—stimuli, signals, regulators, and effectors. Before cells enter into the latter stages of regulation, apoptosis is a reversible process (Morita and Tilly, 1999). Oberhammer et al. (1994) stated that the PS exposure analyzed in the Annexin-V assay is a very early phenomenon during apoptosis, preceding nuclear condensation and breakdown of the intracellular cytoskeletal and nuclear matrix constituents. In some studies, Annexin-V staining has been performed in oocytes because it is an earlier marker of apoptosis than TUNEL (Anguita et al., 2007; Lobascio et al., 2007; De Felici et al., 2008). In addition, Bax and Bcl-2 are thought to be 2 of the key regulators in the third stage, effectively dividing the ultimate destiny of cells (Morita and Tilly, 1999). Previous studies performed on rat (Vitale et al., 2002), human (Kugu et al., 1998), and monkey (Uma et al., 2003) ovaries showed that increased Bax expression at the mRNA level has consistently been correlated with granulosa cell demise and follicular atresia. In addition, targeted (transgenic) expression of Bcl-2 suppresses apoptosis in mouse oocytes (Flaws et al., 2001), indicating that Bcl-2 is fully capable of promoting germ cell survival in the female, irrespective of the developmental status of the oocyte or the ovary. In this study, both Annexin-V staining and real time RT-PCR of expression monitoring in Bax and Bcl-2 mRNA were chosen as the early apoptosis detection methods in order to clarify the relation between early apoptosis in immature bovine oocytes and their developmental potential.

In this study, many oocytes from immature COCs demonstrated the characteristics of early apoptosis. The oocytes in GIII- and GI-COCs displayed a higher ratio of Annexin-V-positive cells than those in GIV and GII-COCs, in terms of the same cumulus cell layers. This was confirmed by the results of Bax and Bcl-2 transcript-relative quantification that showed that the dynamic changes in the transcriptional profile of Bax corresponded with the early apoptosis and development of oocytes, while the transcriptional pattern of Bcl-2 exhibited a contrasting pattern. Our findings are consistent with those of Bilodeau-Goeseels and Panich (2002), indicating that oocytes with early signs of atresia have good developmental potential. Many previous reports suggest that a low level of atresia in follicles tends to improve the *in vitro* competence of oocytes (Blondin and Sirard, 1995; Moor et al., 1996; Hagemann et al., 1999; Feng et al., 2007). The results of this study may provide direct proof supporting the aforementioned studies.



Sirard (1989) and Assey et al. (1994) proposed that, to promote the final maturation, oocytes are required to go through some changes in the cytoplasmic ultrastructure (such as location variation of golgi complexes, endoplasmic reticulum, and cortical granules) that were considered to correspond with cytoplasmic maturation. The similarities of structural changes occurring during oocyte degeneration in subordinate follicles and in the oocyte of the dominant follicle prior to the LH surge also support this finding (Assey et al., 1994; Fair et al., 1997). Hendriksen et al. (2000) demonstrated that oocytes from follicles showing signs of atresia also undergo similar maturing processes. In this study, early apoptosis appears to be positively correlated to the *in vitro* competence of oocytes, which might be related to cytoplasmic maturation. However, this remains to be further studied. As we know, apoptosis is a sequential, but reversible, process of cell death, with ultimate committance being made before cells enter into the latter stages of regulation (Morita and Tilly, 1999). It has been indicated that the occurrence of early apoptosis in oocytes does not mean that they must develop into late apoptosis, which decreases their developmental competence (Anguita et al., 2007; Jaroudi and SenGupta, 2007).

## 5. Conclusion

In the present study, early apoptosis in the immature oocyte is beneficial for developmental competence of the developing embryo at post fertilization, while none of the immature oocytes undergo late-stage apoptosis. This is the first time that Bax and Bcl-2 transcripts were characterized in the immature bovine oocyte, and the results indicate that Bax and Bcl-2 can be used as good markers of early apoptosis and embryo development. This research may be useful in modifying the currently held views on immature oocytes with different developmental competencies and will facilitate the selection of the best-quality oocytes during IVEP.

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