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ORIGINAL ARTICLE

Embryo apoptosis identification: Oocyte grade or cleavage stage?



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Abstract Apoptosis is a programmed cell death that is vital for tissue homeostasis. However, embryo apoptosis had been known to be related to embryo fragmentation which should be avoided in in vitro fertilization (IVF). The purpose of this study was to evaluate the relationship of embryo apoptosis with the grade of immature oocytes and cleavage stage of in vitro produced (IVP) cattle embryos. This study consisted of 345 oocytes collected through ovary slicing. Immature oocytes were graded as A, B and C. This grading was based on cumulus cell thickness and compactness. All oocytes then underwent an in vitro maturation (IVM) procedure. An IVF was done 24 h after IVM culture. Prior to staining, stage of cleaved embryos was determined and classified as either 2, 4, 8 or >8-cell embryo stage. Apoptosis status of cleaved IVP embryos was determined by using annexin V-FITC staining technique at 48 and 72 h post insemination (hpi). Apoptosis status for

Abbreviations: ART, assisted reproductive technologies; BO, Brackett and Oliphant; BSA, bovine serum albumin; CaI, calcium ionophore; CC, cumulus cells; COC, cumulus–oocyte complex; CO₂, carbon dioxide; CR1aa, Charles Rosenkran's 1 amino acid; DNA, deoxyribonucleic acid; DO, denuded oocyte; EA, early apoptosis; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FSH, follicle stimulating hormone; GSH, glutathione; hpi, hours post insemination; IVC, in vitro culture; IVF, in vitro fertilization; IVM, in vitro maturation; IVP, in vitro produced; LA, late apoptosis; LH, luteinizing hormone; PBS, phosphate buffered saline; PI, propidium iodide; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transfer-mediated dUTP nick end-labeling.

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each embryo was classified as either early or late. The result showed that there was no significant difference ($p > 0.05$) of apoptosis status among grade A, B and C embryos. All grades of oocytes showed embryo apoptosis where 1.5% late apoptosis for grade A, 4.5% and 10.4% of early and late apoptosis for grade B and grade C. Early apoptosis was not seen in grade A embryo. We also noted no significant difference ($p > 0.05$) of apoptosis status between 2, 4, 8 and > 8 -cell embryo stage. Early apoptosis was also not seen in > 8 -cell stage. Even though there were no differences in apoptosis expression between the three classes, the cleavage rate of grade A oocytes was significantly higher ($p < 0.01$) than grade B and grade C. In conclusion, the apoptosis expression in the embryo can occur regardless of the oocyte quality and the cleavage stage of the embryo produced.

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1. Introduction

Success in assisted reproductive technologies (ART) is defined as the live birth of an ART cycle without regard of the number of delivered infants (Hogue, 2002). Embryo produced must be viable in order to achieve successful ART. Embryo morphology evaluation is a non-invasive method that had been widely used in clinical and research purposes to predict embryo viability. Pronuclear scoring (Scott et al., 2000; Tesarik and Greco, 1999), embryo fragmentation (Dennis et al., 2006; Ebner et al., 2001), morula compaction (Ebner et al., 2009; Ivec et al., 2011) are examples of embryo morphology evaluation that had been much discussed in the past years and been widely used in the clinic and in research.

Embryo apoptosis is one of the few embryo quality assessment methods that is used in embryology research. Apoptosis is a programmed cell death, which triggered cells to commit suicide without the induction of an inflammation reaction (Gosden and Spears, 1997). The presence of phosphatidylserine (PS) on the outer leaflet of the membrane lipid bilayer of a cell is the earliest event of the apoptosis process. In healthy cells, PS is found exclusively on the inner layer of the membrane cell (Hanshaw and Smith, 2005). The externalization of PS had been observed by Levy et al. (1998) and Mateusen et al. (2005) in all stages of apoptotic preimplantation embryo (2-cell stage embryo until blastocyst stage). Another common staining method that was reported to be used in detecting embryo apoptosis is terminal deoxynucleotidyl transfer-mediated dUTP nick end-labeling (TUNEL) assay. TUNEL assay was used to detect the fragmented DNA where the fluorescence assay labels the 3' end of oligonucleosome fragments (Hardy, 1999). The event of DNA fragmentation was reported to have occurred only at a later stage of a preimplantation embryo as the genome in early stages (less than 8-cell) was said to be inactive (Antunes et al., 2010). For this reason, we had used annexin-V FITC to observe apoptosis in this study.

The occurrence of apoptosis was closely associated with embryo morphology fragmentation and arrested embryo. A study showed that all fragmented embryos were positive for the apoptosis marker. However, not all early cleaved embryos that were arrested possess fragmented morphology (Mateusen et al., 2005). Another study proved that only 30% of the arrested embryos were TUNEL assay positive. In contrast, annexin V-FITC fluorochrome was observed at all stages of the arrested and fragmented embryo (Levy et al., 1998). To our knowledge, no study had been done to associate immature oocyte grading and early stage of preimplantation embryo apoptosis.

It is well-known that embryo quality is key to successful ART. However, oocyte quality must not be underestimated. Studies showed that embryo apoptosis can also be affected by the female gamete. We suspect that the relationship of oocyte morphology and embryo apoptosis is closely related to the early embryo morphology. The previous study had demonstrated that a small size oocyte ($< 110 \mu\text{m}$) will produce an embryo with high apoptotic cell ratio compared to a large oocyte ($> 120 \mu\text{m}$) (Vandaele and Van Soom, 2011). Contrary to oocyte size and its outcome, the influence of cumulus cell (CC) thickness toward embryo apoptosis still remains unexplored. Interestingly, the influence of CC toward maturation rate and cleavage rate is well documented. Past studies had conclusively shown that maturation rate and cleavage rate of the oocyte in an enclosed CC will grow much better than a denuded oocyte (DO) (Godard et al., 2009; Kakkassery et al., 2010; Lasienė et al., 2009).

Thus, based on the above, we believed that immature oocyte grading might contribute to the quality of the embryo. For this reason, we had investigated the influence of immature oocyte grade toward embryo apoptosis. Cumulus thickness that enclosed the oocyte had been used to grade the immature oocyte and DO was not included in this study. This study had also further investigated the influence of cleavage stage toward its apoptosis status.

2. Materials and methods

All chemicals were from Sigma–Aldrich unless otherwise indicated.

2.1. Cumulus–oocytes complex collection and in vitro maturation

Ovaries were collected from Banting, Selangor slaughterhouse and transported to the laboratory in phosphate buffered saline (PBS) solution supplemented with 0.1% v/v 10,000 U penicillin–streptomycin (pen-strep). The buffer temperature was maintained between 30 °C and 38 °C and transported to the laboratory within 3 h. Ovaries were sliced immediately in a slicing solution. Slicing solution was the transport buffer supplemented with 10% prepared bovine serum. Cumulus–oocytes complex (COC) were picked from the slicing solution and washed in in vitro maturation (IVM) medium containing TCM-199 supplemented with 10% v/v fetal bovine serum (FBS), 0.5% of 14.6 mg/ml L-glutamine, 0.8% of 2.2 mg/ml sodium pyruvate, 0.1% 10,000 U v/v pen-strep, 0.1% v/v of 0.5 µg/ml follicle stimulating hormone (FSH), 0.1% v/v of

0.5 µg/ml luteinizing hormone (LH) and 0.1% v/v of 1 mg/ml β-estradiol hormone. During the first wash, COCs were classified into 3 groups according to its cumulus thickness and compactness. Grade A COC consisted of oocytes that were surrounded by five or more layers of compact cumulus cells. Grade B is classified as oocytes surrounded by two to four layers of cumulus cells which was less compact. Grade C on the other hand, consisted of either oocytes surrounded by less than two cumulus cells layers or half naked oocytes. After the second washing, the COCs were transferred into droplets of IVM medium according to the group mentioned previously. The droplets were coated with mineral oil and kept for 24 h in 38.5 °C at 5% CO₂ with maximum humidity.

2.2. In vitro fertilization (IVF) and embryo culture (IVC)

Sperm preparation was done by using a swim up procedure. Approximately 500 µl of cryopreserved sperm was thawed in the 37 °C water bath for 30 s. Thawed sperm was transferred into a centrifuge tube and 7 ml of Brackett and Oliphant (BO) medium was slowly added into the tube. The mixture was centrifuged at 300×g for 10 min. Later, the supernatant was removed. The pellet was placed gently at the bottom of 1.5 ml of sperm swim solution which contained BO medium supplemented with calcium ionophore (CaI), 10 mg/ml bovine serum albumin (BSA) and 0.01% v/v of 0.02 mg/ml heparin (Tocris Bioscience, UK). The sperm was allowed to swim up in the media at 38.5 °C. After 45 min to 1 h, fertilization droplets were made from the middle part of the incubated sperm swim solution. The prepared droplets were incubated at 38.5 °C under 5% CO₂ with maximum humidity, while oocytes washing procedure commenced. Oocytes were washed twice in BO medium supplemented with 10 mg/ml BSA according to the aforementioned groups. After the washing procedure, oocytes were then placed into the incubated sperm droplets for fertilization. After an incubation of 16–20 h, presumptive embryos were washed in oocyte washing medium. Subsequent to the second wash with washing media, the embryos were introduced to Charles Rosenkran's 1 amino acid (CR1aa). After that, the embryos were cultured in the CR1aa droplets according to the initial COC grading. The cultures were incubated at 38.5 °C under 5% CO₂ with maximum humidity.

2.3. Cleavage rate and embryo apoptosis evaluation

The presumptive embryos were maintained in the culture media from 24 to 72 h post insemination (hpi). Observation was done under an inverted microscope (BMS74575; Breukhoven b.v., Netherland) for every 24 h. During 48 hpi observation, the cleaved embryos were isolated. The type of cleavage stage was determined and classified 2, 4, 8 and >8-cell embryo stage.

Later, the classified cleaved embryos were transferred into a new CR1aa droplet for apoptosis staining. The embryos were stained by using annexin-V FITC apoptosis kit (Calbiochem®, Germany). After staining, the cleaved embryos were immediately observed under a fluorescent microscope (CyScope® HP; Sysmex, Germany) in order to avoid fluorescent bleaching. The same procedure was also conducted to cleaved embryos at 72 hpi. Results of apoptosis staining were then divided into three categories which are no apoptosis (nil), early apoptosis (EA) and late apoptosis (LA). Emitted fluorescent

light by FITC was an indicator of early apoptosis while fluorescent light emitted by propidium iodide (PI) indicated late apoptosis. Absence of no fluorescent signal indicated that the embryos were intact.

The cleavage rate of cleaved embryos at 48–72 hpi was determined using the equation shown below. Cleavage rate results were distributed according to the initial COC grading – grade A, grade B and grade C. The sum of the cleaved embryos from the same droplet (same grade) was then later divided by the total number of COC cultured in the same droplet (Appendix A).

2.4. Statistical analysis

All statistical analyses were performed by using IBM SPSS Statistic version 20.0. The cleavage rate assessment was tested by using ANOVA. Chi-square was used to determine the influence of COC grading and cleavage stages toward apoptosis. A *p* value of less than 0.05 was considered significant for each test.

3. Results

3.1. Cleavage rate assessment according to the cumulus oocyte complex grades

A total of 345 cumulus–oocytes complex (COC) have been used in 14 cycles of IVF. Grade A consisted of 60 COCs where 35% developed into embryos. Grade B showed 18.2% of embryo cleaved from 121 COCs while 14.63% embryos derived from 164 grade C COCs. Table 1 showed that the cleavage rate of grade A embryo was significantly higher (*p* < 0.01) compared to grade B and grade C. There was no significant difference between the cleavage rate of grade B and grade C. However, the mean cleavage rate of grade C was lower than grade B.

3.2. Embryo apoptosis assessment between group and cleavage stage

In this experiment, the embryos were grouped according to the initial COC grading. A total of 67 embryos were stained in this experiment where 16.4% of the total embryo showed apoptosis signals. From 67 embryos cleaved, 29.9% of grade A, 28.4% of grade B and 25.4% of grade C were also intact and showed no apoptosis signals. For early apoptosis, 3.0% of grade B and 7.5% of grade C embryo showed a positive FITC signal. There was no grade A embryo showed early apoptosis signal.

Table 1 Number of embryos and cleavage rate, according to classified cumulus–oocyte complex (*N* = 345).

Cumulus–oocyte complex group	No. of cumulus–oocyte-complex	No. of cleaved embryo	Cleavage rate (mean % ± SEM)
Grade A	60	21	37.07 ± 5.58 ^a
Grade B	121	22	18.69 ± 2.29 ^b
Grade C	164	24	14.37 ± 1.91 ^b

^{a,b} Values without a common letter in their superscripts in the same column differ (*p* < 0.01).

Table 2 Apoptosis status of each cleaved embryo which was classified according to the initial cumulus–oocyte complex classification (COC). There was no significant difference for the status of apoptosis between the embryos of classified COC, $\chi(4) = 0.5890$, $p = 0.208$ where $N = 67$.

Group	Grade A	Grade B	Grade C
<i>Apoptosis status</i>			
Nil	20	19	17
Early apoptosis	0	2	5
Late apoptosis	1	1	2
Total embryo	21	22	24

Table 3 Number of embryos produced classified according to the embryo stage from 48 to 72 h post insemination (hpi). Embryos were stained with annexin-V FITC kit for apoptosis status determination. There was no significant difference among the group; $\chi(6) = 3.918$ ($p = 0.688$) where $N = 67$.

Apoptosis status	No apoptosis (Nil)	Early apoptosis (EA)	Late apoptosis (LA)
<i>Cleavage stage</i>			
2-Cell	28	4	1
4-Cell	19	2	1
8-Cell	5	1	1
> 8-Cell	4	0	1
Total embryo	56	7	4

However, 1.5% of grade A embryo showed late apoptosis signals. Grade C showed the highest number of positive PI signals. As per grade A, 1.5% of total cleaved embryos showed late apoptosis in grade B. The differences seen between the grades and the apoptosis status were not significantly different (Table 2).

During the cleaved embryo isolation for embryo fluorescent staining, the cleavage stage of each embryo had been determined. From 67 embryos, 49.3% developed into 2-cell stage, 32.8% development showed in 4-cell embryo stage, 10.4% for 8-cell stage and 7.5% for > 8-cell stage. As for the apoptosis status, 83.6% showed no apoptosis where the 2-cell stage showed the highest percentage (41.8%) followed by 4-cell stage (28.4%), 8-cell stage (7.5%) and the lowest was > 8-cell stage (6.0%). A total of 10.4% of developed embryo showed the early apoptosis signal where 2-cell showed 6.0%, 3.0% shown by the 4-cell stage and 1.5% shown by the 8-cell stage. None of > 8-cell stage showed early apoptosis signals in this experiment. As for the late apoptosis, all cleavage stages showed 1.5% of the embryo developed into late apoptosis (Table 3).

4. Discussion

In this study, the quality of each oocyte was evaluated by observing the thickness of cumulus cells (CC) surrounding it. Hence, the slicing method was chosen in order to retrieve COC. Studies showed that the slicing method was able to retrieve higher oocytes with cumulus compared to other methods (Wang et al., 2007a,b; Zeinoaldini et al., 2013). As a comparison to the aspiration methods, slicing method can reduce mechanical

damage during the harvesting process. This is due to the non-usage of the needle and suction unit which reduces the damage to COC especially to the CC (Neglia et al., 2003).

Based on Table 1, the result showed that the number of grade A COC retrieved was the lowest. Surprisingly, the mean cleavage rate percentage of grade A was twofold than grade B and grade C. Similar results had been reported, but in different species (Wood and Wildt, 1997). This might be due to the fact that CC is very important in regulating metabolites and signaling molecules for resuming meiotic arrest of an oocyte, oocyte maturation and fertilization process. We speculate that in this study, thick CC enclosed grade A oocyte gives a more suitable microenvironment for the oocyte to be matured and fertilized. This is in agreement with Warriach (2004) study where the finding was the oocyte with more than three CC layers showed higher maturation rates compared to oocytes with partially denuded or completely denuded oocytes (Warriach, 2004). Furthermore, a similar study had also shown that progesterone was produced by layers of CC even if the COC was cultured in Tissue Culture Media 199 (TCM 199) alone, without supplementation of any hormone (Salhab et al., 2011). Progesterone that is produced from CC was proven to be a stable source of chemoattractants toward sperm compared to progesterone from the oocyte itself (Guidobaldi et al., 2008). Thus, we postulate that the thicker the CC that surrounds an oocyte, the higher the chances of the oocyte being developed into an embryo. Table 1 also presents that the cleavage rate of grade B and grade C was not significantly different. Hence, we suggest that group B and C COC can be grouped together.

Even though there was significant difference in the cleavage rate among COC groups, the apoptosis status in developing embryos was not significantly different (Table 2). However, we can see a trend where the number of apoptotic embryos increased in the lower grade of COC for both early apoptosis and late apoptosis. Apoptosis occurrence can be prevented and be reduced by antioxidant treatment. A study proved that CC has the capability to produce glutathione (GSH) (Mazangi et al., 2015). GSH is a small antioxidant molecule that plays a major role in protecting gametes against oxidative damage (Luberda, 2005). Intracellular GSH was also proven to be correlated to the male pronuclear formation after IVF and blastocyst rate. Partially removed CC around the oocyte showed lower, but not significant percentage of male pronuclear and blastocyst rate than intact COC. However, both parameters were significantly lower in DO (Maedomari et al., 2007). As we did not measure the GSH level, we cannot confirm the effect of GSH in this experiment. However, based on the previous study, we believed that CC thickness differences among the COC group might give effect to the GSH production and thus differences in apoptosis status occurred.

There was also no significant difference showed in comparison of apoptosis incidence among cleavage stage. Our result was consistent with previous findings where the early cleavage embryo stage (2-cell to 8-cell stage) showed no signs of apoptosis due to the genome that was inactive (Antunes et al., 2010; Gawecka et al., 2013). DNA fragmentation was not observed in early stage embryos (less than 8-cell stage) yet was reported to be observed in the oocyte, morula and blastocyst (Matwee et al., 1999). We speculate that the inactive genome might also affect the externalization process of PS.

Most of the previous study had used terminal deoxynucleotidyl transfer-mediated dUTP nick end-labeling (TUNEL)

assay which stained fragmented DNA. TUNEL assay will only recognize fragmented DNA where it can only occur at the later stage of the apoptosis process. Meanwhile, the use of annexin-V FITC in this study was meant to detect PS on the outer leaflet of the bilayer plasma membrane. The PS that flips from inner to outer leaflet is the first event of the apoptosis process. As for PI, it can only be permeable to the cell when the membranes of the nucleus and plasma are disrupted. The permeability of membranes during apoptosis allowed the PI to bind to the DNA. This might explain the reason why some embryos in this study showed positive annexin-V FITC signal.

5. Conclusions

As a conclusion, although the cleavage rates were clearly influenced by the oocyte quality, the apoptosis occurrence in the embryo might happen regardless of the oocyte quality and the cleavage stage of an embryo produced. However, choosing a good quality COC might contribute a little to reducing the apoptosis possibility. This is due to CC that has the capability to produce factors to improve embryo development.

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Appendix A

$$\text{Cleavage rate} = \frac{\text{Total number of embryo (48 hpi + 72 hpi)}}{\text{Total number of COC cultured in the same droplet}} \times 100$$

Appendix A: formula for cleavage rate calculation.

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