Saudi Journal of Biological Sciences (2016) 23, S50–S55

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Embryo apoptosis identification: Oocyte grade or cleavage stage?

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Received 27 June 2015; revised 29 October 2015; accepted 29 October 2015
Available online 10 November 2015

KEYWORDS

Apoptosis;
Oocyte grading;
Cleavage stage

Abstract Apoptosis is a programed 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; CO_2, 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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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2015.10.023

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.

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 programed 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 posses 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 μm) will produce an embryo with high apoptotic cell ratio compared to a large oocyte (>120 μm) (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; Lasienë 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