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Apoptosis in Bali Cattle Embryo Cells Produced *In Vitro*

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ABSTRACT

In vitro production of Bali cattle embryos still needs in-depth investigations to produce embryos suitable for transfer. The current study aimed to examine the level of cell apoptosis in Bali cattle embryos produced *in vitro* and at three stage of oocyte maturation, fertilization, and embryo culture. A total of 107 pairs of ovaries derived from slaughterhouses of Indonesia were collected. The used oocytes were grades A and B (Grade A had compact cumulus oocyte complex (COC) cells surrounded by five or more layers of cumulus cells, and grade B had a non-compact COC and a dark cytoplasm with complements from the complete radiata corona but surrounded by no more than five layers of cumulus cells). Fertilization of oocytes was done using the semen of a Bali bull. Bali cattle semen was frozen in straw semen for 5 minutes at 1500 rpm twice, then the supernatant and spermatozoa were separated and equilibrated for 30 minutes. Fertilization lasted for 5-6 hours in the incubator. Then, oocyte culture was carried out using CR1aa media and evaluated at 48 hours post-insemination (hpi). The result of the current study showed that the development of Bali cattle embryos produced *in vitro* after 48 hours of culture included 2 cells (31.91%), 4 cells (32.97%), 8 cells (24.46%), and 16 cells (10.63%). The percentage of embryos containing at least one nucleus exhibiting Terminal dUTP nick-end labeling (TUNEL) characteristics of apoptosis entailed 28.33% (2 cells), 41.93% (4 cells), 43.48% (8 cells), and 50% (16 cells). The division ability of embryos aged 48 hpi consisted of 2, 4, 8, and 16 cells. In conclusion, apoptosis in Bali cattle began to be detected in the two-cells stage. The sooner a cell undergoes apoptosis, the lower the level of the cell's ability to develop further.

Keywords: Apoptosis, Bali cattle, Embryo, *In vitro*, cell cleavage

INTRODUCTION

Bali cattle are local cattle native to Indonesia and are widely developed in community farms. Bali cattle have eminent traits including adaptability to high temperatures (24-35°C) and feed use efficiency (Baco et al., 2013; Putra et al., 2019). One way to maintain the quality and quantity of Bali cattle is by applying reproductive technology assisted by *in vitro* embryo production. *In vitro* embryo production technology can be applied by utilizing the ovaries of cattle livestock from slaughterhouses. The technology can provide information on livestock infertility (Karja et al., 2010). The challenge for in vitro production laboratories can increase the number of embryos produced with high-quality in each round of in vitro culture (Lonergan et al., 2004). The success of *in vitro* embryo production is usually determined by the number of embryos that reach the morula or blastocyst (Oliveira et al., 2019). Morula and blastocyst stages are resistant to freezing and can be of worthy transfer to the recipient (Bó and Mapletoft, 2013; Hansen, 2020). *In vitro* embryo production consists of three main stages, namely, maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). Each stage plays a crucial role in supporting the success of embryo production (Kharche et al., 2011). Some factors, such as the genotypic effect, the quality of oocytes, the microenvironment, the conditions of in vitro production, lipids, and other molecules, determine the embryo's competence (Marsico et al., 2019). Overall, the efficiency of this biotechnology process is still low because many processes can still lead to the failure of embryo production *in vitro*, including culture conditions that are not under *in vivo* conditions of female reproduction (Smith et al., 2012).

Fast-developing embryos are of higher quality than slow-developing embryos (Velker et al., 2012). During *in vitro* culture, stressful conditions affect the embryos' quality and survival ability (Ramos-Ibeas et al., 2020). The development of the zygote to the blastocyst stage varies greatly, and the individual outcome is uncertain (Leidenfrost et al., 2011). In embryonic development, mainly at the stages of morula and blastocysts, apoptosis mediates the elimination of certain cells (Ramos-Ibeas et al., 2020). Abnormal embryos produced *in vitro* trigger aging at the cellular level by entering the cessation of the cell cycle and showing active metabolism and high levels of reactive oxygen species (ROS, Nandi et al., 2019). The protective role of aging and apoptosis is to ensure that unhealthy cells and early embryos do not develop, avoiding long-term adverse effects (Galluzzi et al., 2018). Cell death during this process has been reported with unclear interpretations ranging from pathological phenomena to an integral part of normal blastocyst development (Betts and King, 2001). Preliminary studies on the embryonic development of Bali cattle cultured *in vitro* have different developments on the same culture day (Hasbi et al., 2020). In order to find out the differences, it is necessary to measure

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the fragmentation of DNA in embryonic cells. DNA fragmentation indicates the occurrence of apoptosis or cell death (Hadi, 2011). Studies on DNA fragmentation have been reported in zebu cows (*Bos Indicus*) (Garcia et al., 2015), buffalo oocytes (Gustina et al., 2019), and humans (Hardy et al., 2003). Apoptosis is considered the cause of death, resulting in the embryo's failure to undergo subsequent division. Given the importance of this issue, there is a dearth of research on apoptosis (DNA fragmentation) in Bali cattle. Therefore, this study was conducted to test the level of apoptosis of cattle embryos produced *in vitro*.

MATERIALS AND METHODS

Ethical approval

This study has been approved by the Animal Ethics Commission, Hasanuddin University, Makassar, Indonesia, number 404/UN4.6.4.5.31/PP36/2022.

Collection and selection of oocytes

The 107 pairs of ovaries of Bali cattle from the slaughterhouse in Makassar city, Indonesia, were taken to the laboratory *in vitro* production at Hasanuddin University, Makassar, by transport media (0.9% NaCl solution plus antibiotic gentamycin 100 µ/mL, Sigma-Aldrich, USA). The oocytes were collected using the slicing method (Hasbi et al., 2017). Oocytes grades A and B were selected using a microscope (Olympus, Japan). Grade A had compact cumulus oocyte complex (COC) cells surrounded by five or more layers of cumulus cells, and grade B had a non-compact COC and a dark cytoplasm with complements from the complete radiata corona but surrounded by no more than five layers of cumulus cells (Kakkassery et al., 2010; Bakri et al., 2016). Of 838 total oocytes, 505 were selected. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Company (USA) unless otherwise indicated.

Oocyte maturation

The selected oocytes were washed three times using collection media by phosphate buffered saline (PBS, Gibco by life technologies, USA) to which 0.2% bovine serum albumin (BSA, Sigma-Aldrich, USA) and 50 µg/mL gentamycin (Sigma-Aldrich, USA) were added. Then, they were matured in a maturation media consisting of M199 (Gibco by Life Technologies, USA) through the addition of 0.3% BSA, 10 IU/mL pregnant mare serum gonadotrophin (PMSG, Intergonan, Intervet Deutschland GmbH, Netherlands), 10 IU/mL human chorionic gonadotrophin (HCG, Chorulon, Intervet International BV Boxmeer-Holland, European Union), and 50 µg/mL gentamycin (Sigma-Aldrich, USA). Maturation was carried out in the form of a drop (80 µL/drop) with an oocyte count of 10-15/drop and covered with mineral oil (Sigma-Aldrich, USA). Maturation was performed in a 5% CO_2 incubator with a temperature of 38.5°C for 24 hours (Hasbi et al., 2017).

In vitro fertilization

Bali cattle semen was frozen in straw by centrifuging the 0.25 mL semen for 5 minutes at 1500 rpm twice, then the supernatant and spermatozoa were separated (Hasbi et al., 2020). The semen was added with fertilization media (Suzuki et al., 2000) so that the final concentration of spermatozoa was 1.5 x 10^6 cells/mL (Hasbi et al., 2020). Then, four drops (80 µL/drop) were placed into a Petri dish covered with mineral oil (Sigma-Aldrich, USA), and equilibrated for 30 minutes. The maturated oocytes were then put into the equilibrated drop and stored in the incubator for 5-6 hours.

In vitro culture

After fertilization for 5-6 hours, oocytes were washed twice using CR1aa culture media, transferred into an 80-µL drop of CR1aa culture media following modification by Sagirkaya et al. (2006) and Somfai et al. (2010) with some modifications. In the next step, 5 mg/mL BSA and 2.5% FBS were added, oocytes were covered with mineral oil (Sigma Chemical Company, USA), and cultured in a 5% CO_2 incubator at the temperature of 38.5°C for 48 hours (Hasbi et al., 2020). On the second day of culture, classification was carried out based on the stages of division. The 2, 4, 8, and 16 cells of the embryo were then transferred to the culture media. Embryo evaluation and medium utilization were carried out every 48 hours and cultured for 96 hours.

DNA fragmentation

The cell fragmentation of each division group was analyzed using a combined technique for nucleic staining and Terminal dUTP nick-end labeling (TUNEL, *in situ* cell death detection system, USA, procedure modified by Gustina et al., 2019). Embryos were fixed overnight at 4°C in 3.7% (weight/volume) paraformaldehyde diluted in PBS. After overnight fixation, the embryos were washed four times in PBS containing 3% (w/v) of polyvinyl alcohol (PVA) and permeabilized in 0.5% (v/v) Triton-X100 for 1 hour and then incubated in a blocking solution (PBS + 10 mg/mL BSA) overnight at 4°C. After washing them in PBS-PVA, the positive control and all treated embryos were incubated in

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fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT, TUNEL reagents) at 38.5 °C for 1 hour in dark. As a positive control, one to two embryos per TUNEL analysis were incubated in 1000 IU/mL of deoxyribonuclease I (DNase I, Sigma-aldrich, USA) for 20 minutes. Meanwhile, the negative controls were incubated in fluorescein dUTP without TdT. After TUNEL, embryos were washed three times in PBS-PVA, and later stained with 50 ug/mL propidium iodide (PI) for 20 minutes to label all nuclei. The embryo was extensively washed in the blocking solution, placed on a glass slide, and covered with a glass cover. The embryo was examined under a fluorescence microscope (Zeiss Axio Imager A2, Germany) using excitation at wavelengths of 488 nm and 568 nm to detect the TUNEL and PI reactions. The pictures were taken with a digital camera (Zeiss AxioCam HRc, Germany, Loo, 2011).

Statistical analysis

The data of cell division were analyzed descriptively. The Pearson correlation coefficient was run for the total number of cells and the apoptosis index using SPSS software (version 20). The significance was defined at $p \leq 0.05$.

RESULTS AND DISCUSSION

The results showed that 48 hours post-insemination, 40.52% of embryos could be divided into 2, 4, 8, and 16 cells (Table 1). Meanwhile, the percentage of embryos which could develop into 2, 4, 8, and 16 cells were 31.91%, 32.97%, 24.46%, and 10.63%, respectively.

Cells showing signs of apoptosis can be seen in Figure 1. The percentages of embryos with at least one nucleus displaying the TUNEL characteristics of apoptosis were in 2, 4, 8, and 16 cells were 28.33%, 41.93%, 43.48%, and 50%, respectively (Figure 2).

The correlation between the total-number of cells and the apoptosis rate in Bali cattle embryos produced *in vitro* is indicated in Figure 3. There was no significant correlation between the apoptosis rate and the total-number of cells observed in embryos produced *in vitro* $R^2 = 0.1355$ and $p > 0.05$.

Table 1. Cell cleavage of Bali cattle embryos 48 hours post insemination

Oocyte	Cultured	Cleaved		Cell cleavage (%)			
Number	Number	Number	Percentage	2 cells	4 cells	8 cells	16 cells
505	464	188	40.52	60 (31.91)	62 (32.97)	46 (24.46)	20 (10.63)

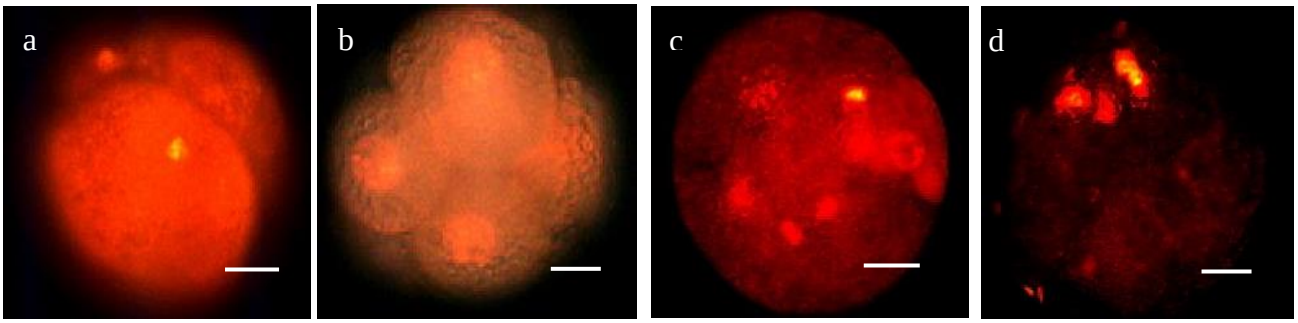

Figure 1. Detection of apoptotic and all nuclei in cattle embryos by TUNEL (fluorescein isothiocyanate-conjugated dUTP; green channel) and propidium iodide (red channel), **a:** 2 cells, **b:** 4 cells, **c:** 8 cells, **d:** 16 cells. Scale bars represent (abcd) 40 µm.

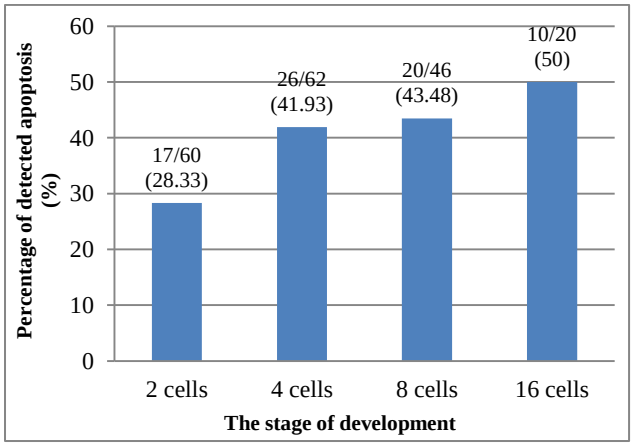

Figure 2. Percentage of embryos in Bali cattle containing at least one nucleus showing characteristics (TUNEL) of core apoptosis.

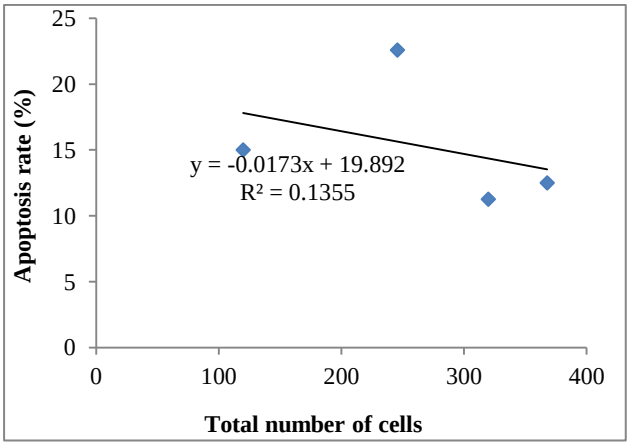

Figure 3. The relationship between the number of cells and apoptosis rate in Bali cattle embryos produced *in vitro*. R: the correlation coefficient for the observations made.

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Oocytes fertilized in the study were oocytes that experienced suitable cumulus expansion after maturation. The oocytes undergoing expansion have reached metaphase II. The quality of oocytes is an early predictor of the development of the embryo's potential (Goovaerts et al., 2010). The oocytes maturated *in vitro* will undergo cumulus cell expansion, and changes occur in the perivitelline space with the formation of the polar body I (Hassa et al., 2014). This indicates the meiosis stage and the success of the metaphase II (MII) stage (Lv et al., 2010; Zafar et al., 2021).

The embryo division ability after 48 hours post insemination (hpi) in Bali cattle *in vitro* reached up to 16 cell divisions. Several factors can cause fertilization failure, including imperfect maturation process of the nucleus and cytoplasm due to poor quality of oocytes (Swain and Pool, 2008), spermatozoa failure to carry out capacitation and acrosome reactions, disabling spermatozoa to fertilize oocytes (Fujihara et al., 2020), and spermatozoa failure to condense in the oocyte cytoplasm, leading to a failure in male pronucleus formation (Zafar et al., 2021). *In vitro* embryo production derived from oocytes surrounded by multiple cumulus cells or bare oocytes results in the lower formation of the blastocyst, compared to oocytes surrounded by a dense layer of cumulus cells (Merton et al., 2012). The selection of oocytes based on the COC was carried out on donors. Oocytes and embryos cultured separately per donor obtained an average blastocyst rate of 16-18% (Machado et al., 2006; Merton et al., 2012). Small-group embryos showed lower total cell number and higher apoptosis rate than large-group cultured embryos (24.17% vs. 12.14%, Cebrian-Serrano et al., 2013).

Bali cattle embryos produced *in vitro* exhibited signs of apoptosis, which was undergoing DNA fragmentation starting from stage 2 cells which continued to increase to the subsequent division (4, 8, and 16 cells). Apoptosis occurs during the pre-implantation development of bovine embryos produced *in vivo* and *in vitro* (Gjørret et al., 2003). Apoptosis has been confirmed from the stage of 6 cells *in vitro* and the stage of 21 cells *in vivo*. In case an error occurs in embryo production (both *in vitro* and *in vivo*), the failure of the first division will directly lead to the death of the embryo or cause the subsequent development to deviate (Burruel et al., 2014). The failure of such cleavages will be the primary source of heterogeneity development (Shi et al., 2015). At the blastosis stage, if there is substantial cell death in the inner cell mass then cell death develops faster (Morris et al., 2010). The main causes of cell death at the beginning of the development of bovine embryos are not mediated by caspase (Leidenfrost et al., 2011).

The first signs of apoptosis commonly occur in slow-cell groups than in fast-cell groups (Morris et al., 2010). Apoptotic cells detected at 48 hpi revealed a possible mechanism of programmatic cell death activation before genome activation occurred. Cell apoptosis observed in slow-developing embryos showed a link between the pro-cell-death and the kinetics of embryonic development in zebu *in vitro* produced embryos (Garcia et al., 2015). Brad et al. (2007) reported that failure of caspase-9 activation could be the cause of the resistance of the two-cells embryos to experience apoptosis. Somfai et al. (2010) used time-lapse cinematography, describing oocytes that underwent direct division from one cell into three or four blastomeres, a phenomenon associated with high frequencies of chromosomal abnormalities. Cells that experience apoptosis will round and shrink, fragmented chromatin nuclei and organelles containing cytoplasm will shrink (Voss and Strasser, 2020).

The TUNEL staining will detect all types of DNA damage and analyze the morphological features of apoptosis and necrosis observed in target cells (Rodríguez et al., 2006). It is used with other apoptosis-specific test combinations (Loo, 2011). The apoptosis rate and the total number of detected cells did not have a strong relationship in Bali cattle embryos produced *in vitro* (r=-0.368). Fragmentation of the core condensed by karyorrhexis is another important component of apoptosis likely affected by *in vitro* production (Betts and King, 2001). Apoptosis features were not observed before the morula stage *in vivo* but were more quickly observed in 9 to 16 cells *in vitro* (Gjørret et al., 2003). DNA fragmentation occurs on the second day of culture in human embryos, for which fragmentation above 25% is considered bad (Hardy et al., 2003). According to Gustina et al. (2019), DNA fragmentation in buffalo oocytes was around 15%, and using 0.05% sericin could reduce DNA fragmentation by up to about 7%.

CONCLUSION

The division ability of an embryo aged 48 hours post-insemination varies by 2, 4, 8, and 16 cells. The incidence of apoptosis in Bali cattle happens in the two-cell stage. The sooner a cell experiences apoptosis, the lower the level of cell ability to develop further. It is, therefore, recommended to conduct future research on apoptosis in Bali cattle embryos *in vivo*.

DECLARATIONS

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Authors' contribution

Erni Damayanti collected the sample, drafted the manuscript, and formatted it, Herry Sonjaya and Sudirman Baco were responsible for the data analysis, Hasbi Hasbi was responsible for designing the study. All authors approved the final manuscript

Competing interests

The authors declared that they did not have any conflict of interest.

Ethical considerations

The research had all credibility and trust and did not plagiarise or copy from any other papers or ideas. The present findings did not have any fabrication or falsification. The authors consent to publish only in World's Veterinary Journal and did not submit this article or any part of the present scientific results in any other journals.

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