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Developmental Ability and Concentration of Hydrogen Peroxide in Bali Cattle Embryos Cultured *In Vitro*

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ED contributed to the conception and design of the study, data collection, statistical analysis, interpretation of data and drafting the manuscript. HS contributed data collection, statistical analysis and interpretation of data. SD and HH were involved in conception and design of study, and critical revision of manuscript. All authors have read and approved the finalized manuscript.

Key words
Bali Cattle, Embryo development, Hydrogen peroxide, *In-vitro* fertilization

ABSTRACT

One of the efforts to support the productivity of Bali cattle is to utilize ovaries from the slaughterhouse by using assisted technology, namely *in vitro* embryo production. This study aims to determine the developmental ability and to measure the concentration of hydrogen peroxide (H_2O_2) in Bali cattle embryos produced *in vitro*. 542 oocytes were matured in TCM 199 medium, were fertilized and were cultured using CR1aa medium for 48 h. After 48 h of culture, the embryo developed into 2, 4, 8 and 16 cells. Furthermore, it was grouped based on the stage of development, each of which was re-cultured for 48 h. After that, its development was re-evaluated and H_2O_2 measurements were taken. The 2-cell embryo was not able to reach the morula, while stage 4-, 8- and 16-cell were able to develop to the morula stage as much as 6(9.83%), 5(12.83%) and 4(25%), respectively. The concentration of H_2O_2 in each group of cells did not differ significantly (P>0.05). However, the results of the correlation test between the H_2O_2 concentration and the embryo's ability to reach the morula stage were negatively correlated. The lower the concentration of H_2O_2, the higher the chances of the embryo reaching the morula stage. The conclusion is that the high H_2O_2 can inhibit the development of Bali cattle embryos that are cultured *in vitro*.

INTRODUCTION

Bali cattle are native Indonesian cattle widely developed by the farming community in South Sulawesi. One way to continue supporting the productivity of Bali cattle is to utilize ovaries from slaughterhouses by using assisted reproductive technology, namely *in vitro* embryo production. In general, studies on embryo production have been widely conducted, but those carried out on Bali cattle are still limited in number. *In vitro* embryo production is still being improved to support the success of embryos that are worthy of transfer (Marsico *et al*., 2019). The initial development embryo often experiences obstacles and becomes a critical problem for embryo production, resulting in obstacles in the production of embryos *in vitro* (Medina *et al*., 2014). Several factors can affect the successful production of embryos *in vitro* including donors, sperm, heredity and the environment (Ahmed *et al*., 2015). In addition, different culturalsystems can also affect embryonic development (Abd El-Aziz *et al*., 2016). *In vitro* production of Bali cattle embryos resulted in zygote cultures which show varying rates of embryonic cell division indicated by the occurrence of 2-, 4-, 8- and 16-cell embryos on the second day or 48 h after culture (Sonjaya and Hasbi, 2019; Hasbi *et al*., 2020). The *in vitro* embryo culture system has not been able to be properly conditioned to resemble *in vivo* so that it can lead to the increase of reactive oxygen species (Agarwal *et al*., 2006).

Reactive oxygen species (ROS) are oxygen-derived molecules that act as strong oxidants. ROS produces oxygen free radicals such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and superoxide anion radicals (O_2), and is a by-product of cell metabolism (Takahashi, 2012). Small amounts of ROS are needed in some physiological processes such as maintaining embryonic development (Sies and Jones, 2020), and a small amount of H_2O_2 can maintain the balance of cell mass (Qian *et al*., 2016), spermatozoa capacity and acrosome reactions (Du Plessis *et al*., 2015). An increase in ROS at the intracellular level can cause developmental disorders, apoptosis and embryo

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death (Giotto *et al*. 2015; Hu *et al*., 2015). Oocytes that experience oxidative stress can cause problems during meiosis (Perkins *et al*., 2016) and increase fragmentation in the embryo (Arias *et al*., 2012). ROS is also an obstacle to the glycolysis process so the embryo does not optimally utilize glucose as an energy source (Eriani *et al*., 1994).

The success of *in vitro* embryo production can be judged by the number of embryos that are able to develop after the fertilization process (Asimaki *et al*., 2022). The success of embryo culture *in vitro* is thought to be related to H_2O_2 concentrations, high H_2O_2 concentrations can inhibit embryo development. Information regarding the measurement of H_2O_2 in Bali cattle embryos produced *in vitro* does not yet exist. This study focuses on measuring the concentration of H_2O_2 in Bali cattle embryos based on cultures with the same number of cells.

MATERIALS AND METHODS

Oocyte collection and selection

This study used 138 pairs of ovaries with 542 oocytes obtained from slaughterhouse. The cattle ovaries were obtained from slaughterhouse and brought to the laboratory by transport media (0.9% NaCl solution). The oocytes collection was carried out using the slicing method and then were selected using the Olympus SZ51 Japan microscope (only Grade A and B oocytes were used). Grade A has a compact cumulus-oocyte complex (COC) cell surrounded by at least 4-5 layers of cumulus cells and homogeneous cytoplasm. Meanwhile, grade B has a COC surrounded by 2-3 layers of cumulus cells attached to the zona pellucida and homogeneous cytoplasm (Bakri *et al*., 2016).

Oocyte maturation

The selected oocytes were then washed with phosphate buffered saline (PBS) (Gibco by life technologies, USA) added with 0.2% bovine serum albumin (BSA) (Sigma-Aldrich, USA) matured in a maturation medium consisting of medium 199 (Gibco by life technologies, USA) added with 0.3% BSA, 10 IU/ml Pregnant Mare Serum Gonadotropin (PMSG) (Intergonan, Intervet Deutschland GmbH), 10 IU/ml human chorionic gonadotropin (hCG) (Chorulon, Intervet international BV Boxmeer-Holland), and 50 µg/ml gentamycin (Sigma-Aldrich, USA). Maturation was conducted in the form of drops (80 µl / drop) containing 10-15 per drop and covered with mineral oil (Sigma Chemical Co. St. Louis MO, USA). Maturation was carried out in a 5% CO_2 incubator with a temperature of 38.5 °C for 24 h (Hasbi *et al*., 2017). In this study, oocytes that experienced an expansion of cumulus cells or activation of cumulus cells, were used as an indication of oocyte maturity (Uhde *et al*., 2018) and continued for fertilization.

In vitro *fertilization*

The semen used was obtained from the same bull. It was thawed for 20 seconds at 37 °C, was centrifuged for 5 min at 1500 rpm 2 times, split the supernatant and spermatozoa, and then semen was added with fertilization media so that the final concentration of spermatozoa was 1.5×10^6 cells/ ml (Hall and Glaze, 2014; Hasbi *et al*., 2020). After that, prepared four drops on a petri dish (80 µL/drop) covered by mineral oil (Sigma Chemical Co. St. Louis MO, USA), equilibrated for 30 min. The matured oocytes were washed 2 times, put into IVF media (Suzuki *et al*., 2000) and stored in a 38.5°C incubator and a 5% CO_2 concentration for 5-6 h.

In vitro *culture*

Oocytes after 5-6 h of fertilization, the oocytes were washed twice using CR1aa culture medium, then transferred in a drop of 80 µl CR1aa culture medium (modification by (Somfai *et al*., 2010; Sagirkaya *et al*., 2006) added with 5 mg/ml of BSA, 2.5% FBS and covered by mineral oil (Sigma Chemical Co. St. Louis MO, USA), which then was cultured in an incubator of 5% CO2 at 38.5°C (Meo *et al*., 2005). The evaluation of embryo division ability is carried out 48 h after culture, then the embryos that develop are re-cultured and as a control, namely embryos that develop are cultured without grouping the number of cells and grouped based on the stages of division, namely embryo 2, 4, 8, and 16-cells. Then the embryo is re-cultured for 48 h and re-evaluated.

Hydrogen peroxide (H_2O_2) measurements

Embryos that have been re-cultured for 48 h, namely control and embryos that have been grouped based on the stages of division, then concentration measurements are carried out H_2O_2 concentration was measured using 2',7'-dichlorodihydro fluorescein diacetate (DCHFDA) (Sigma) as described by Gustina *et al*. (2019) with minor modifications. Embryos in each group were incubated for 15 min with a culture medium containing 10 µM of DCHFDA, were washed in a fresh medium before placed on a glass slide and were covered by a cover glass. Fluorescence emission was recorded with a digital camera (Zeiss AxioCam HRc, Germany) which was attached to a fluorescence microscope (Zeiss Axio Imager A2) after excitation at 480 nm and emissions of 510 nm. Fluorescent images were converted into TIFF files using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA), then were analyzed using ImageJ 1.47 software (Sun Microsystems, Inc., California, USA). Fluorescent

imagery was measured by counting the number of pixels after color reversal. Fluorescence intensity represents intracellular H_2O_2 concentration.

Data analysis

The data of embryo developmental ability were analysed descriptively, the H_2O_2 concentrations were analysed by one-way analysis of variance (ANOVA) and the correlation test was conducted using SPSS 20.00 and significance level of 0.05.

RESULTS

The developmental ability of Bali cattle embryos 48 h after culture varies, namely 2-,4-,8- and 16-cell. Once grouped by the cleavage stages and re-cultured for 48 h, the embryos of Bali cattle that can develop further also vary, as presented in Figure 1. The total number of embryos cultured was 228 embryos consisting of control or no grouping (48 embryos), 2-cells (64 embryos), 4-cells (61 embryos), 8-cells (39 embryos) and 16-cells (16 embryos). The group of embryos cultured of 2-cell was able to develop up to 16-cell by 3.27%. Embryos cultured back from 4-cell 8-cell and 16-cell for 48 h were able to reach the morula. The number of embryos that reached the morula was 15 embryos. Overall, the data of embryo development cultured from a different number of cells for 48 h and continued to be re-cultured for 48 h is presented in Figure 1.

Evaluation of H_2O_2 concentration was carried out by measuring the intensity of H_2O_2 concentration in Bali cattle embryos (Fig. 2). The brighter the green color, the higher the concentration of H_2O_2 in Bali cattle embryos. The results showed that there was no significant influence ($P>0.05$) between each group of embryonic cells which was re-cultured based on its cell count group and the H_2O_2 concentration levels in Bali cattle embryos. The concentration of H_2O_2 in the control group was 168.08±58.77, while the concentration in the 2-cell embryo was 167.88±50.37, 4-cell embryo was 167.20±44.88, 8-cell embryo was 160.68± 45.78 and 16-cell embryo was 150.05±48.75 (Fig. 3).

The testing result on the relationship between the H_2O_2 concentration and the ability of the embryo to reach the morula stage are determined based on the results of the correlation analysis (Fig. 4). The H_2O_2 concentration and embryo developmental ability to reach the morula stage were negatively correlated (-0.916) and had a very strong relationship. The H_2O_2 relationship forms a negative trend, indicating that the lower the concentration of H_2O_2 in the embryo, the higher the embryo's ability to reach the morula (Fig. 4).

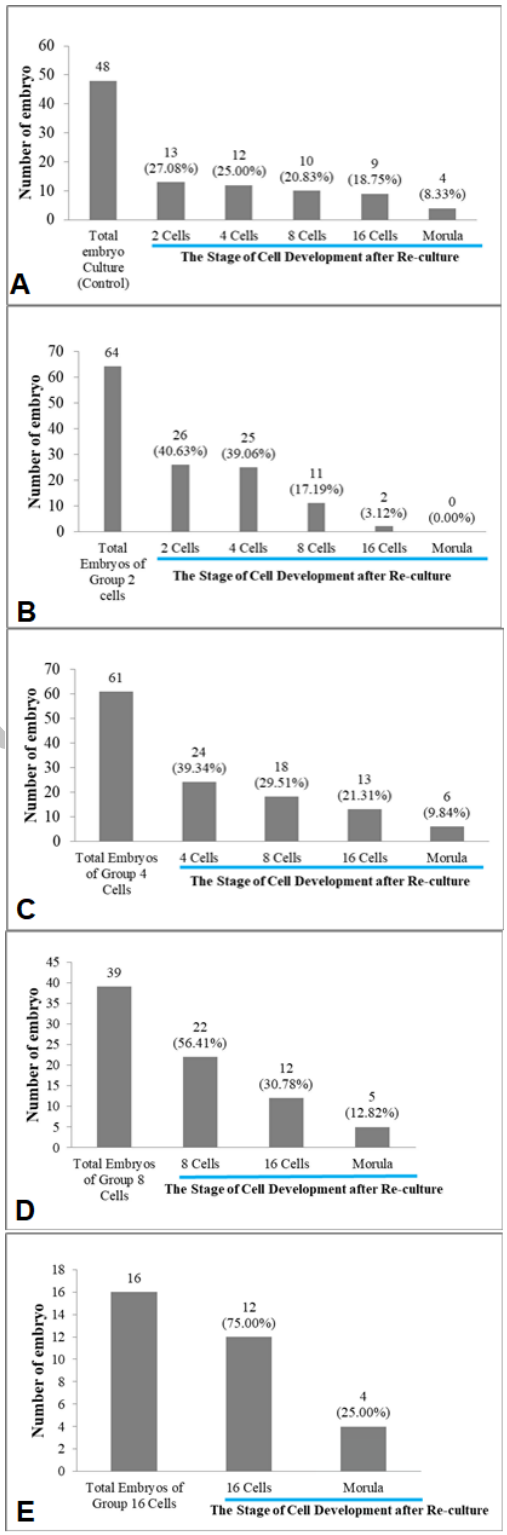

Fig. 1. The cleavage of Bali cattle embryo after re-culture for 48 h; A, Grup of control; B, 2-cell stage (%); C, 4-cell stage (%); D, 8-cell stage (%); E, 16-cell stage (%).

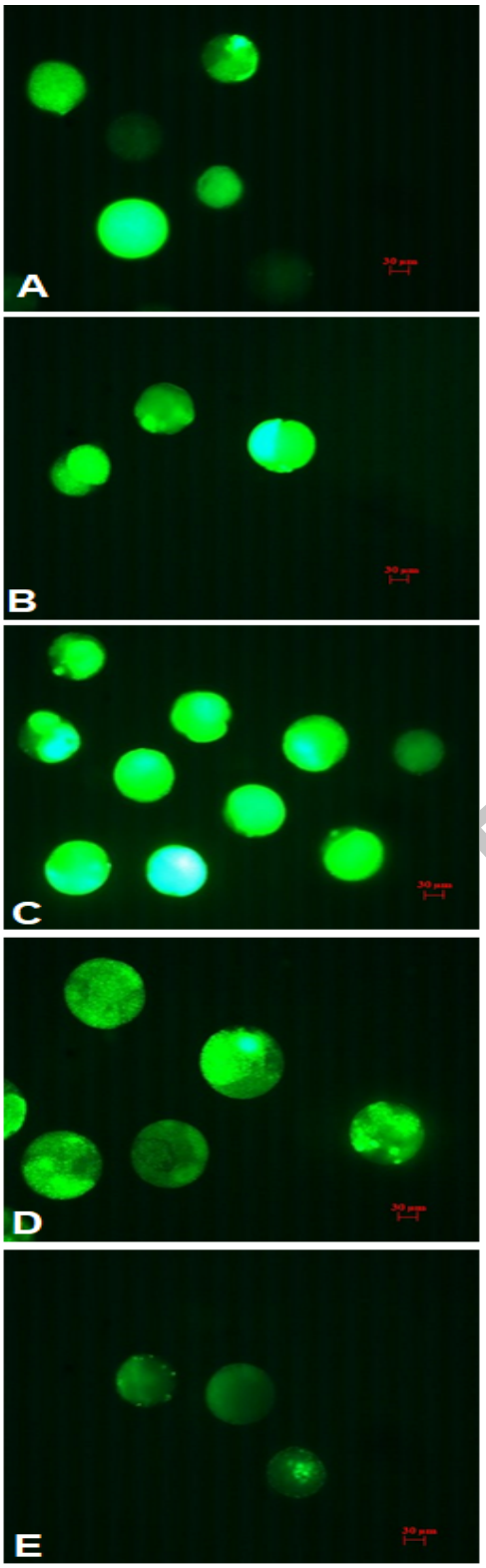

Fig. 2. Fluorescent photomicrographs, 200× magnification. A, group of control; B, 2-cell; C, 4-cell; D, 8-cell; E, 16-cell.

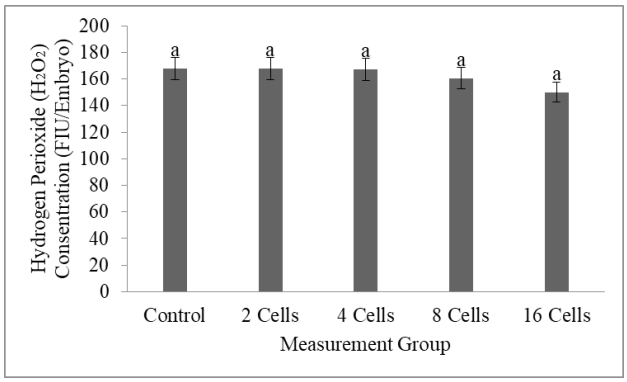

Fig. 3. H_2O_2 concentration of Bali cattle embryos after re-culture for 48 h. FIU values represent fluorescence intensity units.

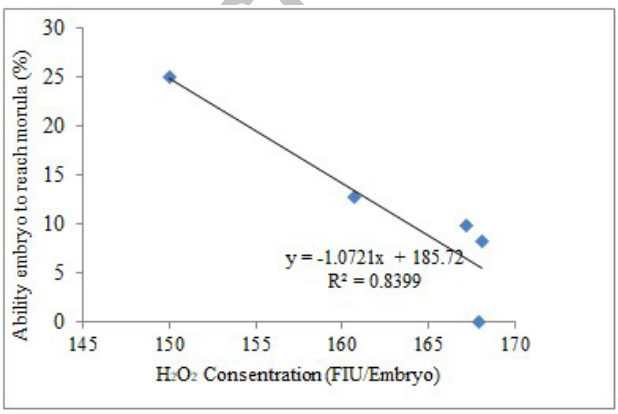

Fig. 4. The correlation between H_2O_2 concentration and embryo developmental ability to reach morula stage after re-culture for 48 h (r=0.916).

DISCUSSION

In the initial development of Bali cattle embryos cultured for 48 h, there were 2-, 4-, 8 and 16-cell cleavage and 42% of which developed from 542 oocytes. The development of Bali cattle embryos cultured *in vitro* is different from that reported by Martins *et al*. (2018) who stated that the stage of cattle embryo cell development on the second day only reaches 4-cell division. Marsico *et al*. (2019) stated that the initial stage of embryonic development is difficult to be carried out because they involve processes that are molecularly dynamically arranged and undergo complex structural changes. The results of grouping after reculturing for 48 h showed that the 2-cell group was unable to develop until morula (Fig. 1A). This indicates that post-fertilization embryo culture is a critical period that determines the rate of division (Mizobe *et al*., 2017; Medina *et al*., 2014) and embryonic

quality (Milewski *et al*., 2018). It is further explained by Milewski *et al*. (2018) that most failures of *in vitro* embryo development occur between the 2-cell and the blastocyst stage. One of the causes of Bali cattle embryos not being able to develop is the high concentration of H_2O_2. It is part of ROS that can lead to lipid peroxidation and enzyme inactivation, resulting in cell damage by encouraging the formation of hydroxyl radicals (Sharma *et al*., 2012). H_2O_2 contained in Bali cattle embryo cells is the cause of the lack of division in cells. Undeveloped embryo cells occur due to ROS production that exceeds cellular defences resulting in oxidative damage to biomolecular substances such as lipids, proteins, carbohydrates and nucleic acids, consequently inducing structural and functional changes such as lipid hydroperoxide, protein carbonylation and DNA with oxidized bases (7, 8 dihydro-8-oxoguanine) (Torres-Osorio *et al*., 2019). Mitochondrial DNA (mtDNA) is also known to become more sensitive to oxidative stress (Nissanka and Moraes, 2018). Oxidative damage to mtDNA can cause mutations and alter mitochondrial function and integrity (Han *et al*., 2013). The results of the study of Tatemoto *et al* (2000) on pig oocytes cultured without cumulus cells are particularly susceptible to oxidative stress and the incidence of degeneration, the duration of DNA migration, and caspase-3 activity increased significantly in DO (denuned oocyte) exposed to ROS compared to DO cultured without XOD (xanthine oxidase).

The 4-, 8- and 16-cell group showed their ability to reach the morula (Fig. 1B, C, D). Poor embryonic development at the beginning of the culture identifies that the embryo is difficult in reaching the next stage of development. Lee *et al*. (2012) stated that the speed of early embryo division can be used as an indicator to assess the quality of embryos. It is further believed that cattle embryos undergo genome activation starting at stages 8-16 cell (Krisher, 2004) and 8-cell (Milazzotto *et al*., 2020), so embryo development up to stage 8-cell depends on the maternal genome carried by oocytes. The process of degeneration in the early development of the embryo is likely due to drastic changes in the synthesis of embryonic proteins, internal signals that turn off maternal control in the process of embryo division and growth control, which may cause the embryo unable to be saved and eventually experience death (Consensus Group, 2020). The development of embryo *in vitro* 2-cell stage that reaches the blastocyst is influenced by the outside environment and suboptimum cultural conditions to support its development (Xie *et al*., 2016). Culture media has a great contribution in the early embryonic development, the quality of blastocyst and the number of embryo cells (Medina *et al*., 2014).

The concentration of H_2O_2 formed in the embryo depends on high oxygen exposure to the cell. Proper concentration of H_2O_2 is needed to keep sperm in cattle normally functioning (Rahman *et al*., 2012). However, high levels of H_2O_2 can lead to sperm dysfunction (Xiao *et al*., 2012). Specifically, ROS serves as an intracellular signalling molecule in embryo metabolism (Yang *et al*., 2018). ROS induces lipid peroxidation in membranes, DNA damage to oocytes and is thought to cause damage to cell division, metabolite transport as well as mitochondrial function (Tamura, 2012).

H_2O_2 concentrations have a very strong correlation (Fig. 4) with the embryo's ability to develop to the morula stage. The higher the H_2O_2 content in Bali cattle embryos, the lesser the embryo's ability to develop further. Oxidative stress, a cellular condition caused by the accumulation of H_2O_2, is thought to contribute significantly to the development of damaged embryos (Loren *et al*., 2017; Tian *et al*., 2017). Excessive levels of ROS or H_2O_2 are one of the main determinants of the quality of embryos produced *in vivo* and *in vitro*, caused by exposure to high oxygen concentrations, light and increased concentrations of metabolites and substrates (Mata-Campuzano *et al*., 2012). Oxidative stress caused by the high level of ROS production results in a reduction in ATP production and not only damages the embryo's development but also triggers apoptotic degeneration (Abd El-Aziz *et al*., 2016). Excessive oxidative stress or insufficient antioxidant protection in human oocytes and embryos can cause adverse effects on male and female reproduction (Lin and Wang, 2021). Oocytes with H_2O_2 of more than 90 ng/ oocytes undergo apoptosis (Tripati *et al*., 2009). Additional 30 µM H_2O_2 to mouse culture medium to induce oxidative stress may reduce mitochondrial membrane potential and decrease mitochondrial activity by up to 40% (Qian *et al*., 2016). An increase in H_2O_2 can be detrimental not only to the success rate of inductive assisted reproduction but also result in epigenetic and genetic changes in the embryo, which have an effect on transgeneration (Hardy *et al*., 2021). Stress tolerance after H_2O_2 exposure is not mediated by the use of antioxidants such as glutathione (Vandaele *et al*., 2010) melantonin (Tamura *et al*., 2012), sericin (Gustina *et al*., 2019) able to block ROS (Cavallari *et al*., 2019). The weakness of this study is that the concentration of H_2O_2 in oocytes after ripening and the concentration of H_2O_2 in Bali cattle sperm have not been measured. It is suggested that it can be a follow-up research for Bali cattle production *in vitro*.

CONCLUSION

The high H_2O_2 can inhibit the development of Bali cattle embryos that are cultured *in vitro*.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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