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Oocytes Population and Development Competence of Bali Cattle Embryo *In Vitro* with Different Ovarian Reproductive Statuses

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ABSTRACT

The present study aims to determine the potential of Bali cattle ovaries as sources of oocytes for *in vitro* embryo production based on different ovarian reproductive statuses. The ovaries were grouped into 4 categories: ovaries with no corpus luteum and dominant follicles (CL-DF-), those with corpus luteum and no dominant follicles (CL+DF-), those without corpus luteum but with dominant follicles (CL-DF+), and those with corpus luteum and dominant follicle (CL+DF+). The oocytes were collected via the slicing technique and grouped into 4 grades (a, b, c, and d). The oocyte's maturation was performed using tissue culture medium 199 basic media. A drop sample (10–15 oocytes/drop) covered with mineral oil was then placed in a 5% CO_2 incubator at a temperature of 38.5 °C for 24 h. Then, the samples were fertilized in 80 µL of fertilization medium with a final spermatozoa concentration of 1.5×10^6 spermatozoa/mL. After 5–6 h of *in vitro* fertilization, the oocytes were washed four times using the Charles Rosenkrans 1aa (CR1aa) medium. Then, the samples were cultured using the CR1aa as a base medium. The results showed no significant difference ($p>0.05$) for the 4 groups based on the oocyte population collected from one pair ovary as well as the number of oocytes that were suitable for maturation. However, group CL-DF+ showed a significant difference ($p<0.05$) in the rate of nuclear maturation (80.00±12.84), fertilization rate (80.00±4.72), and the ability of embryo development (60.19±22.45) when compared to group CL-DF-, CL+DF-, and CL+DF+. This study determines that the oocyte population of Bali cattle ovary pairs and oocytes quantity that are fit for maturation is not influenced by the reproductive status of the ovaries. However, the level of nuclear maturation, fertilization, and the ability of embryo development is higher in the ovaries without corpus luteum but with dominant follicles.

Keywords: Bali cattle; embryo; oocyte population; ovaries; reproduction

INTRODUCTION

In vitro embryo production (IVEP) technology is an assisted reproductive technology (Ferre *et al.*, 2020) that comprises in vitro maturation, in vitro fertilization (IVF), and in vitro culture (Hegab *et al.*, 2009). The successful application of IVEP technology is strongly influenced by the quality of the oocytes used. The excellent quantity and quality of oocytes are influenced by various factors, including ovarian reproductive status. Boediono & Setiadi (2006) reported that the presence of a corpus luteum (CL) in the ovarian pairs has a positive correlation with the number of follicles. Subordinate follicles will continue to grow in the presence of CL.

The CL produces progesterone, which inhibits the growth of the dominant follicle (DF), eliminating the effect of inhibin so that the other follicles can develop (Kor, 2014). Hasbi *et al.* (2017) explained that the presence of CL also greatly affects the presence of growth factor concentrations in the follicular fluid. Mammalian oocytes naturally undergo a maturation process in the follicle surrounded by follicular cells (Arroyo *et al.*, 2020). The growth and development of follicles in the ovaries follow a specific growth cycle. In cattle, there can be several follicular waves in one estrous cycle. Two to three follicular waves generally occur, each of which can produce one or two dominant follicles (DF) (Boer *et al.*, 2011).

The presence of a DF decreases the follicle-stimulating hormone (FSH) concentration due to the inhibin. It causes pressure on the growth of the other follicles that grow simultaneously, thus experiencing regression (Boer *et al.*, 2011; Laird *et al.*, 2019). Furthermore, the DF will ovulate if there is no CL. The remainder of the DF that has been ovulated will form the CL. The CL comprises cells that will produce progesterone and is useful in the process of implantation and maintenance of pregnancy.

The presence of a DF and a CL in the ovary influences follicular development and ovarian status. Presently, the information on the application of IVEP technology to Bali cattle is insufficient, requiring further investigation. This study aimed to determine the potential of Bali cattle ovaries as sources of oocytes for *in vitro* embryo production based on different ovarian reproductive statuses. Through this research, it is hoped that the basic data on the potential of the ovaries of Bali cattle from slaughterhouses with different reproductive statuses as sources of oocytes for *in vitro* embryo production can be applied to the development of native Indonesian animals germplasm.

MATERIALS AND METHODS

Samples Collection

The samples were obtained from Bali cattle ovaries acquired from the Tamangapa slaughterhouse, Makassar City. The frozen semen used in the fertilization was obtained from The Technical Implementation Unit of Artificial Insemination Services and Semen Production, Department of Animal Husbandry and Animal Health, South Sulawesi Province.

Collection and *In Vitro* Maturation of Oocyte

After being collected from the slaughterhouse, the Bali cattle ovaries were brought into the in vitro embryo production laboratory, Faculty of Animal Science, Hasanuddin University. The ovaries were then soaked in a 0.9% sodium chloride solution + 100 IU/mL penicillin and 100 µg/mL streptomycin sulfate. Before being further processed, the ovaries were grouped into 4 categories based on the criteria for each pair of ovaries, both left and right: ovaries without a CL and DFs (CL-DF-), those with a CL and no DFs (CL+DF-), those without a CL but with DF (CL-DF+), and those with a CL and a DF (CL+DF+).

The oocytes were collected using the collection medium (phosphate-buffered saline plus 0.2% bovine serum albumin [BSA] [Sigma, USA]) via the slicing technique and grouped according to grade. There were 4 grades: ((A) the oocytes with a homogeneous cytoplasm and had many compact cumulus cells; (B) the oocytes with a homogeneous cytoplasm surrounded by two to three layers of compact cumulus cells; (C) the oocytes with heterogeneous cytoplasm surrounded by one to two layers of non-compact cumulus cells; and (D) the denudate oocytes), as could be seen in Figure 1. In this study, only the oocytes with grades A and B continued to the maturation process (Abdoon *et al.*, 2014). The selected oocytes (grades A and B) were washed three times using a maturation medium and then matured using tissue culture medium 199 (Sigma, USA). The matured oocytes were added with 0.3% BSA, 10 IU/mL pregnant mare serum gonadotrophin (Intergonan, Intervet Deutschland GmbH), 10 IU/mL human chorionic gonadotrophin (Chorulon, Intervet international BV Boxmeer-Holland), and 50 µg/mL gentamycin (Sigma, USA).

Oocyte maturation was conducted in a Petri dish with Ø 35 mm (Nunclon, Denmark). A sample drop of 100 µL each for 10–15 oocytes and covered with mineral oil (Sigma, USA) in a Thermo Scientific Forma Steri-Cycle incubator (Marietta, OH, USA). with 5% CO_2 and a temperature of 38.5 °C for 24 h (modification from Pereira *et al.*, 2013).

Evaluation of Nuclear Maturation Rate

The level of maturation was assessed based on the chronological changes in meiosis from the germinal vesicle (GV) to the metaphase II (MII) via 2% aceto-orcein staining. GVs are characterized by clearly visible nuclear and nucleolus membranes, and GV breakdown is characterized by the rupture of the nuclear membrane and the nucleus is visibly unclear. Metaphase I (MI) is indicated by the presence of homologous chromosomes lined up in the equator. Anaphase I is indicated by the movement of chromosomes toward the polar. Telophase I is indicated by the chromosomes that have reached two polar regions. MII is indicated by the presence of polar body I and the same chromosome arrangement as the MI (Arroyo *et al.*, 2020). The level of nuclear maturation is assessed based on the percentage of oocytes that successfully reached MII (Figure 2.1) (Gustina *et al.*, 2019).

Staining Procedure

After 24 h of maturation, the cumulus cells surrounding the oocytes were removed using 0.25%

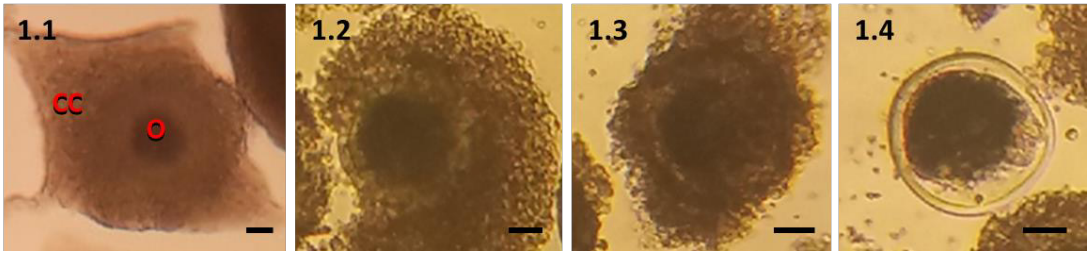

Figure 1. The grades of oocytes. The oocytes with grade A: the oocytes with a homogeneous cytoplasm and have many compact cumulus cells (1.1); grade B: the oocytes with a homogeneous cytoplasm which surrounded by two to three layers of compact cumulus cells (1.2); grade C: the oocytes with heterogeneous cytoplasm surrounded by one to two layers of non-compact cumulus cells (1.3); grade D: the denudate oocytes (1.4). Oocyte (O); Cumulus Cells (CC). Bar= 30 µm.

hyaluronidase enzyme (Sigma, USA) and continued with the removal of cumulus cells (denudation) using a pipette with a diameter of approximately 110–120 µm (according to the size of the oocyte). The oocytes were replaced in a drop of 0.7% potassium chloride on a glass cover with paraffin and vaseline pads on all four corners and then fixed by covering it with a glass object and then reversing the position. The preparations were put in a fixation solution containing acetic acid and ethanol (1:3) for 3 days. The preparations were soaked in an absolute ethanol solution 1 h before staining and then stained with 2% aceto-orcein for 5 min. Then, the dye was cleaned with 25% acetic acid, and the 4 sides of the cover glass were given a clear nail polish solution for subsequent morphological observations using a Zeiss Axio Imager A2 microscope with Zeiss Axiocam HRc (Gottingen, Germany).

In Vitro Fertilization

The fertilization was conducted using frozen semen from the same bull that was used every time the fertilization process occurred in the laboratory. The frozen semen was thawed at 37 °C for 20 s and then centrifuged at 700 g for 5 min. Semen pellets were added with a fertilization medium. The concentration of spermatozoa used was 1.5 × 10^6 spermatozoa/mL, covered with mineral oil, and then incubated in a 5% CO_2 incubator at a temperature of 38.5 °C. The fertilization time was 5-6 hours (modified from Ferre *et al.,* 2020).

Fertilization Rate Evaluation

The fertilization rate was determined based on the pronucleus formation, as seen in Figures 2.2 and 2.3, by performing a staining procedure used to evaluate a nuclear maturation rate.

In Vitro Culture

The oocytes were transferred into a CR1aa medium after 5–6 h of IVF. The culture was carried out in drop form (80 µL/drop), covered with mineral oil (Sigma, USA), and then placed in an incubator with 5% CO_2 at 38.5 °C. The ability of embryo cleavage was observed on days 2 and 4 (Figure 3), whereas the number of embryos that succeeded to be morula and blastocyst was observed on days 6–8 (Hasbi *et al.,* 2017).

Statistical Analysis

The data are in the form of mean value ± standard deviation and analyzed via the analysis of variance. If there is a difference between groups ($p<0.05$), it is

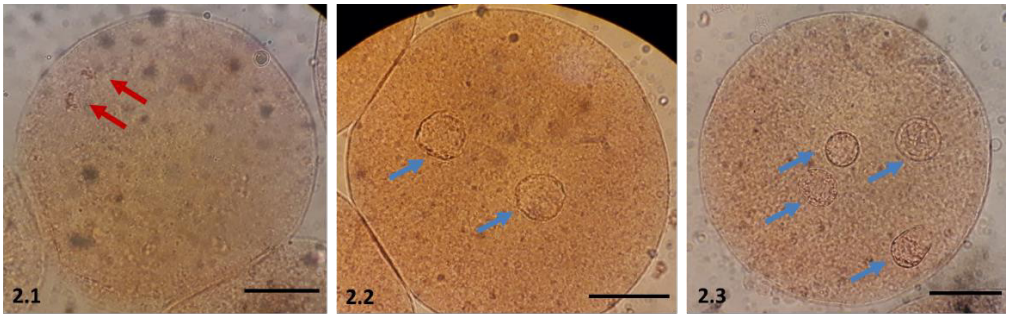

Figure 2. Matured oocyte in metaphase II, which homologous chromosomes marked with red arrows (2.1); fertilized oocyte with 2 pronucleus formation marked with blue arrows (2.2); fertilized oocyte with >2 pronucleus formation marked with blue arrows (2.3). Bar= 30 µm.

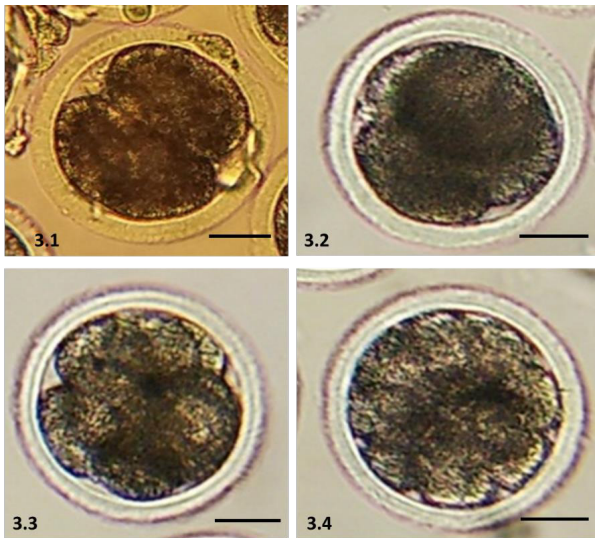

Figure 3. The ability of embryos cleavage. 2 cell (3.1); 4 cell (3.2); 8 cell (3.3); 16 cell (3.4). Bar= 30 µm.

further analyzed via the least significant difference test. The data were processed using IBM SPSS version 21 software (Chicago, Illinois, USA) and MS Office Excel 2007 software (Washington, USA).

RESULTS

Bali Cattle Oocyte Population in Each Ovary Pair

Bali cattle oocyte population from each ovarian pair has a different reproductive status (Table 1). Total oocytes collected at all reproductive statuses of ovaries did not show any significant difference.

Bali Cattle Oocyte Population Based on Grade

The results in Table 2 indicate that the percentage of grade A oocyte was significantly higher ($p<0.05$) in the ovaries without a CL but with DF and in the ovaries without a CL and DFs than in those with a CL and a DF. However, the result was not statistically different from that of the ovary with CL and no DFs.

By contrast, the grade B oocytes were significantly higher ($p<0.05$) in the ovaries with CL and DFs than those without CL and DFs and those with no CL but with DFs. However, the result was not significantly different if there was a CL and no DF in the ovary.

Table 1. Bali cattle oocyte population for each ovary pair from slaughterhouses with different reproductive statuses

Group	Total number of ovarian pairs	Total collected oocytes from each ovarian pair (mean value ± SD)
(CL-DF-)	19	12.32±1.15
(CL+DF-)	26	13.08±1.67
(CL-DF+)	31	13.68±1.81
(CL+DF+)	20	11.30±1.38

Note: The ovaries without corpus luteum and dominant follicle (CL-DF-), the ovaries with corpus luteum and no dominant follicle (CL+DF-), the ovaries without corpus luteum but with dominant follicle (CL-DF+), and the ovaries with corpus luteum and dominant follicle (CL+DF+).

Maturation Rate of the Oocyte Nucleus

This study indicates that most mature oocytes have begun the meiotic process, as indicated by changes in meiotic status reaching the MII stage. Table 3 presents the number of oocytes reaching MII based on the ovarian reproductive status. The percentage of oocytes that reached the MII stage originating from the ovaries without a CL but with DF was significantly higher ($p<0.05$) when compared to the oocytes from the ovaries with no CL and DFs, the ovaries with CL and no DFs, and the ovaries with CL and DFs.

Fertilization Rate

The fertilization rates in this study were determined by calculating the number of oocytes that can form two or more pronucleus (Table 4.). The rate of fertilization of Bali cattle oocytes originating from the ovaries without a CL but with DF was significantly higher ($p<0.05$) than that of oocytes from the ovaries with no CL and DFs, the ovaries with CL and no DFs (61.40±16.88), and the ovaries with CL and DFs.

Embryo Development Capability

After fertilization and culture, the embryonic development capabilities of Bali cattle with different

Table 2. Bali cattle oocyte population based on grade originating from slaughterhouses with different ovarian reproductive statuses

Group	Oocyte total	Oocyte grade (% ± SD)				Oocyte that suitable for maturation (% ± SD)
		A	B	C	D	
(CL-DF-)	234	87 (37.18±17.84)^{b}	64 (27.35±16.46)^{b}	41 (17.52±13.38)	42 (17.95±13.96)	151 (64.53±6.95)
(CL+DF-)	340	116 (34.12±18.73)^{ab}	118 (34.71±21.69)^{ab}	81 (23.82±16.62)	25 (7.35±9.81)	234 (68.83±0.42)
(CL-DF+)	424	179 (42.22±13.26)^{b}	118 (27.83±15.00)^{b}	77 (18.16±11.38)	50 (11.79±9.81)	297 (70.05±10.18)
(CL+DF+)	226	73 (32.30±24.64)^{a}	81 (35.84±21.24)^{a}	53 (23.45±16.52)	19 (8.41±10.95)	154 (68.14±2.50)

Note: The ovaries without corpus luteum and dominant follicle (CL-DF-), the ovaries with corpus luteum and no dominant follicle (CL+DF-), the ovaries without corpus luteum but with dominant follicle (CL-DF+), and the ovaries with corpus luteum and dominant follicle (CL+DF+). Oocytes with a homogeneous cytoplasm and have many compact cumulus cells (grade A); oocytes with a homogeneous cytoplasm which surrounded by two to three layers of compact cumulus cells (grade B); oocytes with heterogeneous cytoplasm surrounded by one to two layers of non-compact cumulus cells (grade C); and denudate oocytes (grade D). ^{ab} Means in the same column with different superscripts differ significantly ($p<0.05$).

Table 3. Maturation rate of Bali cattle oocyte nucleus with different ovarian reproductive statuses

Group	Oocytes total	GV (%±SD)	GVBD (%±SD)	MI (%±SD)	Anaphase/ telophase I (%±SD)	MII (%±SD)
(CL-DF-)	78	8 (10.26±6.34)	6 (7.69±8.97)	9 (11.54±6.81)	3 (3.85±5.30)	52 (66.67±20.16)^{a}
(CL+DF-)	100	10 (10.00±6.08)	8 (8.00±3.80)	9 (9.00±5.41)	8 (8.00±6.50)	64 (64.00±13.18)^{a}
(CL-DF+)	60	1 (1.67±5.10)	3 (5.00±5.74)	7 (11.67±8.64)	1 (1.67±5.10)	48 (80.00±12.84)^{b}
(CL+DF+)	87	5 (5.75±6.61)	7 (8.05±6.25)	15 (17.24±8.54)	5 (5.75±4.58)	55 (63.22±17.93)^{a}

Note: The ovaries without corpus luteum and dominant follicle (CL-DF-), the ovaries with corpus luteum and no dominant follicle (CL+DF-), the ovaries without corpus luteum but with dominant follicle (CL-DF+), and the ovaries with corpus luteum and dominant follicle (CL+DF+). ^{ab} Means in the same column with different superscripts differ significantly ($p<0.05$).

Table 4. The fertilization rate of Bali cattle oocytes with different ovarian reproductive statuses

Group	Oocytes total	1 PN (%±SD)	2 PN (%±SD)	>2 PN (%±SD)	Fertilization rate (%±SD)
(CL-DF-)	53	23 (43.40±10.18)	25 (47.17±8.97)	5 (9.43±6.74)	30 (56.60±10.18)[a]
(CL+DF-)	57	22 (38.60±6.80)	28 (49.12±6.07)	7 (12.28±3.44)	35 (61.40±6.80)[a]
(CL-DF+)	50	10 (20.00±4.72)	37 (74.00±7.58)	3 (6.00±7.43)	40 (80.00±4.72)[b]
(CL+DF+)	51	28 (54.90±8.39)	20 (39.22±12.11)	3 (5.88±7.29)	23 (45.10±8.39)[a]

Note: The ovaries without corpus luteum and dominant follicle (CL-DF-), the ovaries with corpus luteum and no dominant follicle (CL+DF-), the ovaries without corpus luteum but with dominant follicle (CL-DF+), and the ovaries with corpus luteum and dominant follicle (CL+DF+). [ab] Means in the same column with different superscripts differ significantly ($p<0.05$).

Table 5. Embryonic development ability of Bali cattle with different ovarian reproductive statuses

Group	Oocytes total	Embryos development capability (%±SD)				
		2 Cells	4 Cells	8 Cells	16 Cells	Total
(CL-DF-)	61	1 (1.64±0.38)	7 (11.48±0.82)	7 (11.48±1.15)	0 (0.00±0.00)	15 (24.59±11.60)[a]
(CL+DF-)	75	6 (8.00±0.90)	18 (24.00±2.64)	6 (8.00±1.07)	1 (1.33±0.38)	31 (41.33±24.32)[a]
(CL-DF+)	103	5 (4.85±0.96)	25 (24.27±2.23)	27 (26.21±2.79)	5 (4.85±0.76)	62 (60.19±22.45)[b]
(CL+DF+)	73	6 (8.22±1.86)	8 (10.96±0.69)	11 (15.07±1.40)	1 (1.37±0.38)	26 (35.62±10.83)[a]

Note: The ovaries without corpus luteum and dominant follicle (CL-DF-), the ovaries with corpus luteum and no dominant follicle (CL+DF-), the ovaries without corpus luteum but with dominant follicle (CL-DF+), and the ovaries with corpus luteum and dominant follicle (CL+DF+). [ab] Means in the same column with different superscripts differ significantly ($p<0.05$).

ovarian reproductive statuses were assessed (Table 5). The ability percentage in developing embryos originating from the ovaries without a CL but with DF is significantly higher ($p<0.05$) than that of embryos from the ovaries with no CL and DFs, the ovaries with CL and no DFs, and the ovaries with CL and DFs.

DISCUSSION

In the ovary, the dominant follicle (DF) is formed from the small follicles' recruitment and selection process, which then grows and develops. Follicle development is initiated by the Follicle-stimulating hormone (FSH). The DF can produce various hormones such as estrogen, follistatin, activin, and inhibin. However, inhibin secreted from the DF may act as a growth inhibitor for the other follicles (Pirestani *et al.,* 2011; Shabankareh *et al.,* 2015). Other than that, according to Imron *et al.* (2016), in the follicular phase, estradiol with high concentration can suppress LH impulse that leads to progesterone synthesis by the luteal cells, which can influence the function and lifetime of the luteal cells.

After ovulation, the follicle forms a corpus luteum (CL), where cells undergo differentiation to form the luteal cells. The luteal cells in the CL can secrete progesterone, which functions to maintain pregnancy and suppresses the secretion of gonadotropins so that estrus does not occur (Shabankareh *et al.,* 2015). In the luteal phase, the growth of the dominant follicle and estradiol concentration will be suppressed by the production of progesterone by CL, so that the diameter of follicles growing in this phase is smaller than that of the dominant follicle during luteolysis (Imron *et al.,* 2016).

It is found in this study that the reproductive status of the ovaries did not affect the total collected oocytes of Bali cattle (Table 1). This shows that the reproductive status of the ovaries has no impact on the number of oocytes that can be collected in each pair of Bali cattle ovaries. Nagy *et al.* (2018) reported no significant difference in the number of oocytes in the ovaries of sheep with a CL and those that do not have CL, although they showed a higher trend in the ovaries that had a CL. Filippi *et al.* (2020) reported that the presence of the CL and large follicles did not affect follicular growth. Local estrogen levels as well as other factors such as AMH are still able to regulate the recruitment process of primordial follicles in the ovary, even in the presence of DFs and CL. Different things are reported by Amer *et al.* (2008) that the population of buffalo oocytes per ovary was higher in the ovaries without a CL than in those with a CL.

The number of oocytes that were suitable for maturation (grade A and B) did not differ significantly in all ovarian reproductive statuses (Table 2). Abdoo *et al.* (2014) explained that the characteristics of grade A oocytes are surrounded by several layers of compact cumulus cells, and a homogeneous cytoplasm and grade B oocytes are surrounded by two to three layers of cumulus cells and a homogeneous cytoplasm. This indicates that the presence or absence of CL and DFs does not affect the quality of Bali cattle oocytes obtained from the slaughterhouse. According to Shabankareh *et al.* (2015), CL negatively affects the growth and development of oocytes in the small and medium follicles but does not affect the oocytes from the large follicles, where the good quality oocytes come from the tertiary follicles or large follicles. The results of this study differ from those of previous studies by Penitente-Filho *et al.* (2015), who reported that the number of oocytes with good quality is higher in the ovaries with a CL than in those without a CL. A different result has also been reported by Amer *et al.* (2008) and Davachi *et al.* (2011), who stated that the number of oocytes with good quality was less in the ovaries having a CL than in those without a CL. Furthermore, Abdoon (2001) reported the same result that the number of camel oocytes with ≥5 layers of com-

pact cumulus cells was less in the ovaries with a CL than in those without a CL.

The growth and development of follicles in the ovaries are cyclical, i.e., the growth and development of bovine ovarian follicles can occur several times of follicles waves in one estrous cycle. Two to three waves generally occur, each of which can produce one or two DFs (Boer *et al.*, 2011). The presence of a DF in the ovary causes a high concentration of estrogen and inhibin, which will impact the low secretion of FSH (Perera, 2011). Low FSH concentrations cause the inhibition of subordinate follicle development, thereby reducing the number of competent follicles and may also impact the oocyte quality (Boediono & Setiadi, 2006). Price & Estienne (2018) and Regan *et al.* (2018) reported that apoptosis in the cells was higher in the dominant phase than in the growth phase.

The percentage of oocytes that reached the stage of MII was significantly high ($p<0.05$) in the oocytes from the ovaries without a CL but with DF (Table 3). These results indicate that the absence of a CL and the presence of a DF in the ovary affect the ability of Bali cattle oocytes to reach the MII stage. This is different from what has been previously reported by Penitente-Filho *et al.* (2014) that oocyte competence was influenced by the reproductive status of the ovaries, where the number of oocytes with good competence was more in the ovaries without DFs. The presence of a DF in the ovary harms the ability of oocyte development (Cheon, 2012). Furthermore, MCGee & Hsues (2000) and Hajarian *et al.* (2016) explained that the presence of a CL in the ovary, which produces progesterone, causes the inhibition of DF growth, which results in a decrease in the concentration of estrogen and inhibin. The negative effects of estrogen and inhibin that are not there will cause the increased secretion of FSH to produce more and better oocytes (Cheon, 2012).

The meiotic process mechanism in the oocytes is initiated with the activation of G protein, which then activates phospholipase C (He *et al.*, 2021) so that the phosphoinositide is hydrolyzed and then forms inositol triphosphate, which causes the intracellular Ca^{2+} mobilization followed by the extracellular Ca^{2+} entry (Ajduk *et al.*, 2008). Besides inhibiting adenylyl cyclase, which causes exposure to cAMP/PKA, the extracellular Ca^{2+} influx also activates calmodulin-dependent protein kinase (CaM II kinase), which will modify or activate the maturation-promoting factor (Conti *et al.*, 2012). Furthermore, Paulini *et al.* (2014) and He *et al.* (2021) reported that the distribution of organelles in the cytoplasm was closely related to oocyte competence and maturation.

The quality of the oocytes used has a major effect on the success of the IVF process. The oocyte criteria used in this study were homogeneous cytoplasm and surrounded by compact cumulus cells (grades A and B). The oocytes with these criteria come from the tertiary follicles that can initiate the meiosis process. The oocytes from the tertiary follicles have components of reactive oxygen species, antioxidants, hormones, and metabolites (Hennet & Combelles, 2012), glucose, pyruvate, and glycine (Gu *et al.*, 2015), which play essential roles in supporting the fertilization process.

The rate of oocyte fertilization originating from the ovary pair without a CL but with DF was significantly higher ($p<0.05$) than that of other groups (Table 4). These results indicate that the absence of corpus luteum but the presence of DF affect the ability of Bali cattle oocytes to be fertilized. The oocyte ability to be fertilized is greatly influenced by the quality and the source of the oocytes used. The data from the results of this study are different from those previously reported by Penitente-Filho *et al.* (2014) that the competence of oocytes is better in the ovaries that do not have DFs. Cheon (2012) explained that the presence of a DF harms the ability of oocyte development. The results in Table 4 also show that the estrogen and inhibin produced by the DF have no negative effects on the number of fertilized Bali cattle oocytes. Perera (2011) explained that DFs impact high concentrations of estrogen and inhibin; hence, FSH secretion is low. Boediono & Setiadi (2006) reported that low FSH concentrations impacted the oocyte quality.

Embryonic development after fertilization is influenced by the following factors, i.e., the competence of the oocyte to restart the meiotic process, division after fertilization, division to the blastocyst stage, implantation, and the ability to develop properly and healthily (Santella *et al.*, 2020). Furthermore, Coticchio *et al.* (2015) and Xu *et al.* (2020) explained that the intrinsic quality of the oocyte is one of the factors that greatly affect the ability of embryo development to the blastocyst stage after fertilization.

The ability of Bali cattle embryo development was significantly higher ($p<0.05$) in the ovaries without a CL but with DF compared with those of other groups (Table 5). This shows that the ability of embryo development in Bali cattle is better in the embryos developed from the ovaries without CL but with DFs. Kor (2014) explained that the presence of the CL in the ovary would produce progesterone, which will impact the inhibition of the growth of the DF, thereby eliminating the influence of inhibin so that the other follicles can develop. The presence of a DF in an ovary impacts the high concentration of estrogen and inhibin (Perera, 2011). The presence of estrogen in the intrafollicular environment results in better follicular growth and increases cytoplasmic maturation and fertilization rates and embryo division in vitro (Hennet & Combelles, 2012). Besides estrogen, inhibin is produced by the follicular granulosa cells (Laird *et al.*, 2019), a specific glycoprotein that inhibits FSH secretion (Das & Kumar, 2018), causing a decrease in the functional integrity of small follicular growth and cannot grow into DFs.

The limitation of this study was the limited number of available ovaries, and also, the oocyte grading was based only on morphological analysis, not by assessing the intracellular quality. Good oocyte quality is related to the early development and survivability of the embryo. According to Krisher (2004), there are a lot of important processes in the oocyte's cytoplasm to ensure the perfect embryo development, such as metabolism and glycolytic activities, as well as the follicle size that involves the mRNA and protein storage. This study only made the oocyte selection based on the number of cumulus cell layers and the cytoplasm's homogene-

ity without assessing the follicle size, where the oocyte comes from.

Besides, Lee *et al.* (2020) explained that the communication between cumulus cell and oocyte via gap junction also plays a pivotal role in oocyte development, especially related to the oocyte's proliferation and differentiation. The event of cumulus cell apoptosis also affects the development process, even though the oocyte itself did not undergo apoptosis. This study also did not do a detailed assessment of the cumulus cells surrounding the collected oocytes.

CONCLUSION

The different reproductive statuses did not affect the population and the total oocytes that were suitable for maturation in each pair of ovaries of Bali cattle as the samples. However, the rate of nuclear maturation, fertilization, and the ability of embryo development was higher in the ovaries without a CL but with DF. Therefore, in the *in vitro* embryo production program, it is important to select pairs of ovaries as sources of oocytes with these characteristics.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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