The potential of oocytes based on the status of ovary activity to achieve the level of in vitro maturation in Bali cattle

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Abstract. The physiological condition of slaughterhouse bovine ovaries may vary greatly, including the luteal phase, follicular phase, and pre-puberty. However detailed information is scarce on how it affects oocyte maturity in vitro. Therefore, this work aimed to investigate the impacts of ovarian activities on the oocyte maturity of Bali cattle in vitro. Completely randomized designed was arranged, consisting of three ovarian stages (luteal, follicular, and pre-cycle) as a research variable and carried out at four replication. Oocytes were recovered by slicing the follicles from surface-visible ovaries. The oocyte maturation was performed in an incubator containing 5% CO_2 at 38.5°C. Following the removal of the cumulus, the oocyte was prepared for fixation, then observed under a microscope. The research showed that the structure of oocyte grades A (compact cumulus cells and thick cytoplasm) in the follicular and luteal phase was similar, but such condition was significantly different compared to the prepubertal phase. The oocyte maturity of M-II was significantly lower ($P<0.05$) in the pre-cycle phase than in the luteal and follicular phase. Meanwhile, the stages of GV, GVBD, and MI appeared to be identical in all phases. This work concluded that the structure of the oocyte population is closely related to ovarian stages, resulting in higher maturity on M-II oocytes during the luteal and follicular phases.

1 Introduction

Bali cattle (*Bos sondaicus, Bos javanicus*, and Bos/Bibos banteng), a domesticated descendant of the wild Banteng (*B. banteng*) represents approximately 27% of the total cattle population in Indonesia (approximately 11 million heads in 2004). Bali cattle is the most preferred in the smallholding system, because of their rusticity, fertility, and low calf mortality and breeding programs have slowly been built in the country using artificial insemination (AI) with frozen-thawed semen from, largely, phenotypically selected bull sires [1].

The rise of livestock productivity can be achieved through biotechnology. It enables to increase in the utilization of slaughterhouse ovaries as oocyte sources, contributing to in vitro production of bovine embryos. The success of embryo production relies highly on the procedures, and now, chemicals required for that biotechnological procedure are commercially available [2].

The ovary may produce an abundant quantity of oocytes, resulting in a wide opportunity in the in vitro production of the embryo. After collected, maturation of the oocytes is needed. Even though in

vitro maturation is performed in a medium, the embryo can be developed properly similar to in vivo procedure [3].

In this case, ovaries are collected from slaughterhouse Tamangapa, Makassar. The slaughterhouse ovaries may come from cattle with different reproductive backgrounds, including luteal phase, follicular phase, and even recovered from prepubertal cattle. The ovarian dynamics remarkably affect the population of follicles as well as the competence of oocytes [4]. Therefore, this research aimed to evaluate the ovarian dynamics on the competence of Bali cattle oocytes to reach maturity during in vitro procedures. Our present work could be meaningful in providing scientific evidence for further researches on the in vitro production of the embryo.

2. Materials and methods

The research included three stages i.e., oocyte collection, in vitro maturation, and observation of oocyte maturity.

2.1. Oocyte collection

Fresh slaughterhouse ovaries recovered in 0.9% NaCl, 100 IU/ml penicillin, and 100 µ/ml streptomycin sulfate were transported immediately to the laboratory. Oocytes were collected by slicing the follicles from the ovarian surface, which induced the release of follicular fluid. The sliced follicles were flushed using 0.9% NaCl via syringe to ensure aspiration of the oocyte. Observation by microscope was performed to screen the qualified oocytes (homogenous cytoplasm, surrounded by less than three layers of cumulus cells). The oocytes were then collected in a petri dish containing phosphate-buffered saline (PBS; Gibco, Grand Island, NY, USA) supplemented by 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). They were then washed in a collection medium containing PBS and 10% FBS and in a maturation medium every two replications. Maturation of oocytes was carried out in tissue culture medium (TCM) 199 (Sigma, USA) supplemented by 10% FBS, 10 IU/ml of pregnant mare serum gonadotrophin (PMSG) (Intergonan, GmbH Intervet Deutschland), 10 IU/ml of human chorionic gonadotrophin (hCG) (Chorulon, Intervet international B.V. Boxmeer-Holland) and 50 µg/ml of gentamicin (Sigma, USA).

2.2. In vitro maturation procedure of oocytes

Following the previous procedure, oocyte maturation was performed in an equilibrated medium using four drops (50 µL/drop) in a petri dish, then covered by mineral oils (Sigma Chemical Co. St. Louis MO, USA). The covered petri dish was incubated at 38.5°C for 24 h in an incubator with 5% CO_2.

2.3. Evaluation of oocyte maturity

Mature oocytes were denuded by 0.25% of hyaluronidase (Sigma, USA) using multiple pipetting techniques. Denuded oocytes were mounted on glass slides with drops of 0.7% KCl. We used paraffin and vaseline (1:9) to keep the coverslip in contact with the oocytes without extensive pressure. For fixation, the slides were immersed in ethanol and acetic acid (3:1, v/v) for 3–4 h at room temperature. After fixation, the slides were immersed in absolute ethanol for an hour and drained using a tissue. Oocytes were stained in 2% aceto-orcein for five minutes and examined under Microscope Axo Cam following the removal of staining using 25% acetic acid.

2.4. Research parameters

Oocyte performance was evaluated according to quality and quantity. The appearance of oocytes was depicted in figure 1 and evaluated according to criteria as follows:

- Grade A = Oocytes surrounded by thick and compact layers of cumulus oophorus and corona radiata
- Grade B = Oocytes surrounded by compact, but less thick layers of cumulus oophorus and corona radiata
- Grade C = Cumulus oophorus is absent, with thick and not compact layers of corona radiata

- Grade D = Cumulus oophorus is absent, with thin and not compact layers of corona radiata

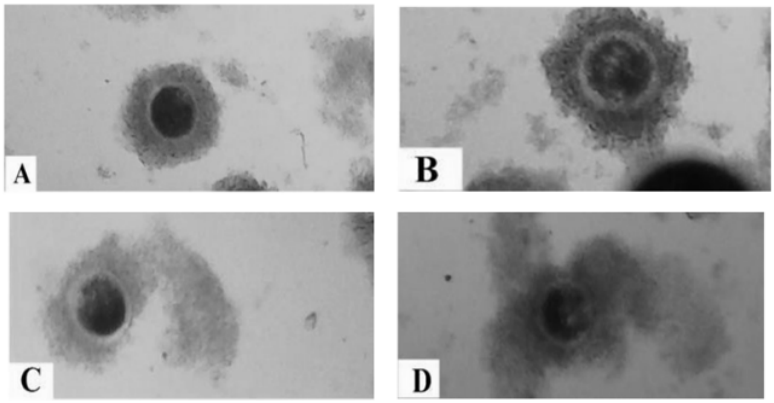

Figure 1. Appearances of the oocyte. **(A)** Homogenous and compact oocyte; **(B)** Compact oocyte, but not homogenous; **(C)** Homogenous oocyte, but not compact; **(D)** Neither compact nor homogenous oocyte.

Figure 1 showed the dynamics of oocytes that represent the level of their maturity. Previously, cell division from oocytes having more than five layers of cumulus cells was higher than oocytes with fewer cumulus cell layers, despite possessing homogenous cytoplasm [5]. The following formula was applied to calculate the quality of oocytes:

$$\text{Oocyte with grade A (\%)} = \frac{\text{total grade A in one cycle}}{\text{total oocyte in one cycle}} \quad (1)$$

In addition, the maturity of oocytes is based on the status of ovarian activity.

1. Germinal Vesicle (GV) indicated by the presence of nuclear membrane and nucleolus
2. Germinal Vesicle Breaking Down (GVBD) indicated by disruption of the nuclear membrane, invisible nucleolus
3. Metaphase–*I* (M-I) indicated by the presence of paired homolog chromosomes and aligned along the equatorial plate.
4. Metaphase-*II* (M-II) is indicated by the presence of pole body I, consisting of chromosomes similar to those in M-1.
5. To enumerate the maturity of oocytes, the following equation was applied:

$$\text{Maturity (\%)} = \frac{\text{total oocyte in one stage}}{\text{total oocyte in one phase/treatment}} \quad (2)$$

2.5. Statistical analysis

The data of oocyte maturity were statistically evaluated using analysis of variance (ANOVA) following the transformation of data for normal distribution. The significant difference between treatments was verified using the least square difference (LSD) test [6]. The relationship between the percentage of population structure and oocyte potential was analyzed using Chi-Square. Data were processed using SPSS 18.0 for windows, using the following mathematical model:

$$Y_{ij} = \mu + \tau i + \varepsilon i$$

i = 1,2,3
j = 1,2,3,4

Where Y_{ij} is oocyte maturity in phases according to ovarian stage i^{th} and replication j^{th} ; μ = means; τi = effects of ovarian stages on oocyte maturity i^{th}; and ε= experimental error in ovarian stage i^{th} and replication j^{th}.

3. Results and discussion

3.1. Oocyte competence to reach maturity

Figure 2 shows the population of oocytes by their quality. The results indicated that the population structure differed greatly between ovarian stages. We found that the distribution of the population in the follicular phase was similar to that in the luteal phase; in this case, the prepuberty phase showed a distinct pattern compared to the follicular and luteal phases. Chi-square analysis exhibited a difference in the distribution of oocyte quality between the ovarian phase. Statistically, the structure of the oocyte population for the follicular phase did not differ with the luteal phase ($P>0.05$), while the structure for the prepubertal phase showed a significant difference ($P<0.05$) compared with the follicular and luteal phase. The similarity of population structure between follicular and luteal phase was associated with the initial ovarian condition, meanwhile, follicular development in the prepuberty phase was very low in comparison with the follicular and luteal phase. This obviously indicates a difference in the in vivo physiological status between the active ovarium and prepuberty phase. In the prepubertal condition, the hormonal pathway of the hypothalamus-hypophysis-anterior is incomplete, Hypophyse anterior gland is unable to produce gonadothropin sufficiently, thus the estrogen cannot be produced optimally by the ovary [7].

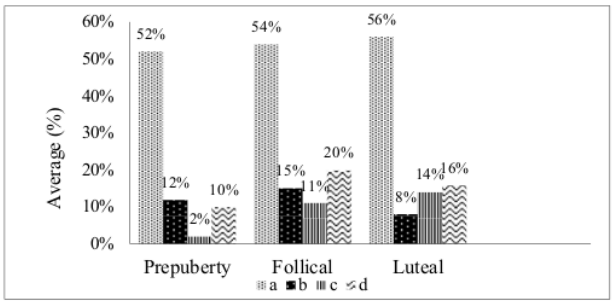

Figure 2. Structures of oocytes population according to their quality in ovarian stages of Bali cattle. (a) grade A; (b) grade B; (c) grade C; (d) grade D.

Oocyte with grade A occurred proportionally in all stages of ovarium, while grade B, C, and D were found to be lower in the prepuberty phase than in the follicular and luteal phase. The difference is caused by the initial stage of ovarium. The luteal phase is characterized by the higher quantity of corpus luteum and better quality of oocyte compared with ovarium without corpus luteum. The corpus luteum which is responsible for the synthesis of progesterone retards the growth of the dominant follicle reaching ovulation, thus reducing adverse effects of the follicle on the growth and development of subordinate follicles [8].

3.2. Oocyte maturity by ovarian stage

Statistical tests using the LSD test showed that maturity of M-II was lower ($P<0.05$) in the prepuberty phase than in the follicular and luteal phases (table 1). The activity of the ovarium in the prepuberty phase was less activated, which caused the reduction of oocyte maturity. [9] argued that the hormonal mechanism involving hypothalamus-hypophysis-anterior in the prepubertal stage was not fully activated; therefore, the hypophysis anterior gland was unable to completely secrete gonadotropin. As

consequence, the ovarium is also incompetent in producing estrogen as an impact of incomplete growth of follicle and oocyte.

Table 1. The average percentage of oocyte maturity based on ovarian stages.

Ovarian Stages	Nuclear Maturity (%)			
	GV	GVBD	MI	MII
Prepuberty	4.54±9.09	15.84±12.75	48.37±16.15	20.81±17.59[a]
Follicular	0.00±0.00	0.00±0.00	46.73±2.43	53.26±2.43[b]
Luteal	1.38±2.78	8.61±13.59	29.26±11.96	60.73±23.67[b]

Similar superscripts following means in the same column showed no significant difference (P>0.05).

Table 1 exhibits that nuclear maturity in the prepubertal phase and luteal phase begins with oocyte maturity at GV, GVBD, MI, and MII; meanwhile, the maturity in the follicular phase begins in MI and MII. This clearly suggests that the difference in the ovarian phase relates to oocyte maturity.

The follicular phase showed a faster maturity, started from oocyte maturity in MI and MII. This relates to the initial condition (in vivo) with the presence of primordial follicle, preantral and antral follicle until reaching preovulatory follicles [10]. The follicle was developed due to the adverse effect removal of progesterone, thereby increasing GnRH. The rise of GnRH leads to the increment of FSH and LH, which enables provoke follicle proliferation. Follicle de Graaf would produce more estrogen. Therefore, in vitro growth of oocytes was faster in MI and MII [4].

In the luteal phase, oocyte maturity started at GV, GVBD, M-I, and M-II. During in vivo conditions, there is a growth of the primordial and preantral follicles, but they did not reach the antral and preovulatory follicle. The retardation of follicle growth was caused by CL (Corpus Luteum) producing progesterone that inhibits follicle growth and preovulatory. Hence, oocytes at the initial stage of in vitro growth started from GV, GVBD, MI, and MII [11].

4. Conclusion

The population structure of oocyte quality closely relates to the ovarian state. The structure of the population in the follicular and luteal phase was dominated by grade A. Percentage of oocyte maturity at MII was found to be higher in the luteal and follicular phase.

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