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Quantitative Histomorphometry of Pre-ovulatory Follicles and Uterine Glands of Ongole-grade Heifer in Response to the Low Doses of PMSG Administration

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ABSTRACT

Injection of female mammalian animals with gonadotropin prior to mating using superovulation dose increases estradiol 17beta (E2) synthesis and secretion and improves the growth and development of the uterus to support prenatal growth with the side effect of increasing litter size. This experiment was designed to study the effects of low doses of PMSG injection in heifers on the growth of granulose cells (GC) of pre-ovulatory follicles, E2 synthesis, and uterine glands growth and development. Nine Ongole-grade heifers were divided into three groups of doses of PMSG injection, i.e., 0 IU/kg BW, 0.5 IU/kg BW, and 1.0 IU/kg BW. The injection of pregnant mare serum gonadotropin (PMSG) was conducted at the early stage of the second follicle wave. Blood samples were collected at estrus (day 0) to measure plasma E2 concentrations. The ovary and uterus were collected for histological observation after slaughtering the animal. The results showed that non-superovulation doses of PMSG injections at the second follicle wave significantly increased the growth of GC that eventually produced a significantly higher plasma E2 concentration by 39.47% and 113.57%, in heifers receiving 0.5 IU/kg BW and 1.0 IU/kg BW ($p<0.05$), respectively. The increase in E2 synthesis and secretion significantly stimulated the growth and development of uterine glands ($p<0.05$), which was reflected in the improved measures of uterine glands. In conclusion, injections of heifers with low doses of PMSG at the second follicle wave could increase the growth of GC to produce a higher secretion of E2 that eventually improves the growth and development of uterine glands in the endometrium without the risk of multiple calving.

Keywords: E2; GC; Heifer; pre-ovulatory follicle; uterine gland

INTRODUCTION

The productivities of mammalian animals are determined by the success of reproduction to produce healthy, vigor, and superior offspring with good survival and growth rates from birth to weaning and adult lives. The main limitation in animal production is the lower birth weight and survival of the offspring during postnatal life. The ideal environments of the uterine and placenta during pregnancy are important to reduce these circumstances. The uterine environment is mostly controlled and regulated by the uterine glands to synthesize and secrete substances and materials required for the survival and development of the embryo, fetus, and associated embryonic membranes (Spencer, 2014) to support the prenatal growth of the offspring. Therefore, improving the uterine environment during estrus and the early phase of pregnancy will improve the prenatal growth and development of embryo and fetus (Sferuzzi-Perri *et al.*, 2013; Reynolds *et al.*, 2019). This process is known as intrauterine programming (Fowden & Forhead, 2004). The aims are to produce good offspring that will grow better with superior phenotype performances during postnatal and adult life.

The growth and development of the uterine glands in the endometrium are started during the increased secretions of estradiol (E2) by the granulosa cells (GC) in the growing pre-ovulatory follicles at the beginning of the estrus cycle and further continued by the progesterone produced by the theca cells of the corpus luteum (Wang *et al.*, 2007; Sugiura *et al.*, 2018). Injection of female mammalian animals with gonadotropin at superovulation doses significantly increases E2 during estrus and progesterone concentrations during pregnancy that eventually increasing fecundity and prolificacy, pregnancy rate, uterine growth, embryonic growth, fetal growth, litter survival rate, and birth weights in polytocous animals such as sheep (Habibizad *et al.*, 2020) and

goat (Andriyanto *et al.*, 2015; Andriyanto *et al.*, 2017). Superovulation using PMSG (2000-3000 IU in cattle) increases the number of ovulating follicles, as indicated by the increase of E2 and progesterone (Abdel-Khalek *et al.*, 2018). There is a greater risk of increasing litter size when gonadotropin injection is applied prior to mating in monotocous animals, especially cattle, with a standard superovulation dose. However, the study in Chinese Holstein showed that using a non-superovulation dose of PMSG did not increase litter size (Fu *et al.*, 2013).

The present experiment was designed to evaluate the use of lower or non-superovulation doses of gonadotropin in bovine to stimulate the growth and development of GCs in a dominant follicle to produce a higher concentration of E2 without recruiting multiple follicles. The growth of GC in response to stimulation by the non-superovulation dose of gonadotropin was evaluated by measuring the histomorphology of GC in the growing dominant follicle and relating it with the synthesis and secretion of E2 by measuring plasma E2 concentrations at the pre-ovulatory stage of the estrus cycle. In addition, the measurement of histomorphology of uterine glands in response to the increased E2 synthesis and secretion is the main objective of the present study to improve the growth and development of uterine glands to support the optimum growth and development of the embryo at the beginning of intrauterine programming of growth and development in mammalian animals to optimize the growth and development of embryo and fetus during prenatal life to support the optimum growth and development of offspring during postnatal life.

MATERIALS AND METHODS

The Animal Ethics Commission of the Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia, approved all procedures involving animals in this experiment with an ethical approval number of 090a/KEH/SKE/XI/2017.

Preparations of Experimental Heifers

Nine (9) Ongole-crossbred heifers, with an average body weight of 265.83±7.26 kg, were selected based on the body condition scores (BCS) of 2.5-3.0 (on a scale of 5 according to Wildman *et al.*, 1982) and the age range of 20-24 months. The reproductive health status was checked and observed in real-time by using a portable ultrasonography (USG) device (ALOKA Co. LTD, SSD-500 series, Tokyo, Japan) with a linear probe 7.5 MHz. The selected heifers were acclimatized in the cattle pens in the Laboratory Animal Maintenance Unit, School of Veterinary Medicine and Biomedical Sciences, IPB University, for one month. Feed was given twice daily in the form of concentrate and grass, as well as drinking water was available *ad libitum*. In addition to feeding, the experimental heifers were given multivitamins and anthelmintics to normalize their health status.

Experimental Design

The experiment was conducted in a completely randomized design with 3 treatments and each treatment used three (3) heifers. Before treatment, the estrus cycles of the experimental heifers were synchronized by injecting $PGF_{2\alpha}$ (Lutalyse®, Zoetis, Dublin-Ireland) intramuscularly at a dose of 25 mg/heifer 2 times with an interval of 12 days. The treatment is the dose of PMSG administration (Folligon®, MSD Animal Health, Intervet BV, The Netherlands), i.e., 0 IU/kg BW (as control by injecting blank PMSG solvent), 0.5 IU/kg BW, and 1.0 IU/kg BW. The protocol of treatment and observation of the experimental heifers are presented in Figure 1. On the day of estrus was detected (day 0), the follicle growths were monitored and observed daily at 06.00 by using the same USG and operator to monitor and observe the beginning of the second follicle wave. The second follicle wave was marked by the appearance of the small follicles group with a diameter range of 0.3-0.5 mm (Adams *et al.*, 2008). At the beginning of the second follicle wave, the experimental heifer was administered with PMSG intramuscularly according to the treatment. Furthermore, after the treatment, prostaglandin F2α (Lutalyse, Zoetis, Dublin-Ireland) were administered 48 hours later.

Blood Samples and Reproductive Tract Collections and Tissue Processing

Collections of blood samples and reproductive tracts were conducted at the estrus phase. Heifers confirmed in the estrus cycle continued with blood sample collections to measure plasma E2 concentration. The reproduction tracts were collected by slaughtering the animal to study the histomorphology of the pre-ovulatory follicles and the uterine glands. Blood samples were collected from the jugular vein at the volume of 5 mL and transferred into a tube containing EDTA. Around 30 minutes after collection, the blood plasma was separated by centrifugation at 1500x *g* for 20 minutes. The collected plasma was stored at -20 ºC until

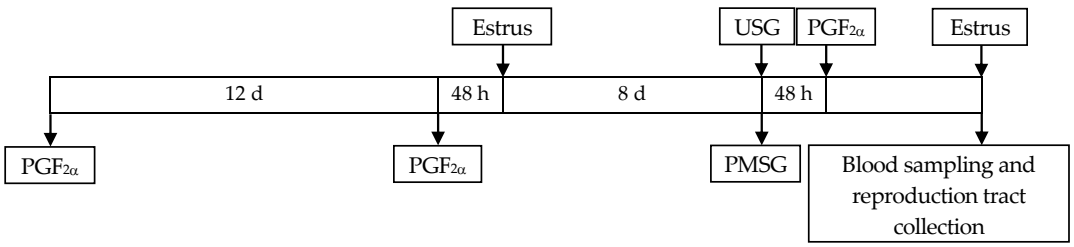

Figure 1. The protocol of estrus synchronization, treatments, and observations

the measurement of plasma E2 concentrations by using ELISA.

The collection of reproductive tracts was conducted on the same day as blood sample collections. After collecting the blood samples, the experimental heifers were sacrificed to collect the ovary and reproductive tracts. The ovary was independently isolated from the mesovarium and the other tissues. The fixation process continued by soaking in Bouin solution (picric acid, formalin, and glacial acetic acid with a ratio of 15:5:1) for 24 hours. Twenty-four (24) hours later, the ovary was transferred into 70% alcohol as a stopping point until the conduction of the further process. The other reproductive tracts were fixated using 10% buffered neutral formalin (BNF) and followed by several slices in the uterine wall six hours later to maximize the fixation processes.

The sections of the ovary and the uterine tissues were further dehydrated and embedded in paraffin. Furthermore, the tissue paraffin blocks were sliced with a thickness of 5 μm to be put in the glass object (Wang *et al.,* 2007). After that, the paraffin of the tissue was removed by soaking in xylene and continued with a rehydrating process using graded ethanol. The Hematoxylin-Eosin (HE) staining was conducted thereafter and then sealed with Entellan® (Merck Millipore, Germany).

Histomorphology Evaluations of the Ovary and Uterus

Each histological preparation of the ovary and uterus was documented using a microscope (Olympus CX31, Japan) connected with a digital camera (Olympus, Japan). Furthermore, each parameter was analyzed using MacBiophotonic ImageJ (NIH, US) application calibrated at microscope enlargements of 10x, 100x, and 400x. The ovary was observed in the pre-ovulatory follicle with two parameters, i.e., the thickness of GC layer (GCL) (μm) and the density of GC (cells/1000 μm^2) of the pre-ovulatory follicle. The thickness of GCL is a distance measured between the basal membrane layer and the outermost GC, while the density of GC was measured by counting the number of GC in each 1000 μm^2 observation field area in the GCL. Both parameters were measured randomly from five (5) observation fields of the follicle, and each observational field area was measured five times at random sites. Histomorphology parameters of GC were cell diameter (μm), nucleus diameter (μm), cell volume (μm^3), cytoplasm volume (μm^3), nucleus volume (μm^3), percentage of cytoplasm volume to the total volume of GC (%) or percentage volume of cytoplasm, percentage volume of the nucleus to the total volume of GC (%) or percentage volume of the nucleus.

The diameters of GC and nucleus were measured using MacBiophotonic ImageJ application in the image observed at 400x magnification. The calibration of size in the MacBiophotonic ImageJ application was conducted by arranging the scale in the '*set scale*' menu that was adjusted to the real size by using *microscope stage micrometer* images with 400x magnification. The diameter was measured in the largest cells among the observed cells to ensure that the cutting was conducted right in the middle of the cells.

The quantitative morphological characteristics of GC were observed by counting the ratio between the volume of the nucleus and the volume of the whole cell so that the ratio between cytoplasm and nucleus can be counted. The volume of GC and nucleus were measured based on Pulley *et al*. (2013) and Andriyanto *et al*. (2015). In each observation field of microscope with microscope multiplication of 400x, the diameters of 50 cells and the diameters of their nuclei were also measured in the cutting of the largest cell. The GC and nucleus volumes were measured using the ball volume formula $V= 4/3 \times r^3 \times \pi$ ($\pi= 3.14$) with r as radius with the formula $r= (W/2 + H/2)/2$. W represents the largest diameter, while H is the smallest diameter in the same cell. The volume of the cytoplasm was calculated by measuring the difference between the total volume of GC and the volume of the nucleus. The percentage volume of cytoplasm to total volume of GC is the ratio of the total volume of cytoplasm (V cytoplasm) to the total volume of GC (V GC) in per cent that was calculated by using the formulas % V cytoplasm= V GC/100 x V cytoplasm. The percentage volume of the nucleus to the total volume of GC is a ratio of the total volume of the nucleus (V nucleus) to the total volume of GC (V GC) in % that was counted by using the formula % V nucleus = V GC/100 x V nucleus.

Histomorphology study of the uterus was conducted in the middle of the uterine horn with the parameters measured were the thickness of stratum functional of the endometrium (the outer layer of endometrium that contains uterine glands, μm), the number of uterine gland duct cross-sections (per lobe of stratum functional), the diameter of uterine gland duct (μm), inter-uterine gland distance (μm), the length of the uterine gland duct epithelial cell (μm), and the number of the uterine gland duct epithelial cell (cell/cut of uterine gland duct). All parameters were observed to study the quality of endometrium by measuring the quantitative characteristic of uterine glands.

The parameter of stratum functional thickness was measured from the basal membrane of stratum functional up to the end of the outermost lobes that were directly connected with the uterus lumen. Stratum functional is a part of the endometrium having uterine glands ducts functioning to secrete the uterine histotrophic. The parameter of the number of the uterine-gland duct was measured by counting the number of uterine gland ducts both cross diagonally and sagittally in each lobe of the stratum functional of the endometrium. The density of glands was counted by measuring the distance between glands (inter glands) at four sides of cut glands. Furthermore, the length and the number of the uterine gland duct epithelial cell were observed on the duct of each uterine gland. The observations were conducted in five (5) observational areas (with the magnification of 400x) randomly in each lobe of the stratum functional.

Data Analysis

The collected data were analyzed statistically using one-way ANOVA (SPSS 16, IBM, USA) with a completely randomized design with 3 treatments and 3 replications. The analysis was continued with the Duncan test when the difference was detected. Further, the cor-

relation analyses were conducted between the dose of PMSG and the thickness of the layer and the density of GC, between the thickness of the layer and the density of GC with the plasma concentration of E2, and between plasma E2 concentrations and the morphometric parameters of the uterus.

RESULTS

Quantitative Measures of GC

Micromorphological sections of GCL (Figure 2) in the pre-ovulatory follicle showed that heifers receiving PMSG at doses of 0.5 IU/kg BW and 1.0 IU/kg BW had higher stimulation of GC growth and development. The higher stimulations of GC growth and developments in heifers injected with PMSG with non-superovulation doses were indicated by the thicker GCL with a higher diameter and density of GC indicating the higher number of GC. The higher numbers and sizes of GC in heifers stimulated with PMSG at non-superovulation doses were also associated with the larger sizes (diameters and volumes) of the nucleus and cytoplasm that had associations with the higher capacity to synthesize and secrete E2 to simulate uterine growth and development.

The quantitative measurements of GC are presented in Table 1. Most of the GC histomorphology characteristics showed significant increases ($p<0.05$) along with the increasing doses of PMSG injection. The thickness of GCL, individual size of GC, and the ratio between the cytoplasm and nuclear size represent the strong GC growth and activities, even though the PMSG administrations were conducted at lower doses. These results were also confirmed by the significant increase of plasma E2 concentrations in heifers injected with PMSG at doses of 0.5 (IU/kg BW) and 1.0 (IU/kg BW), compared to control heifers without PMSG injection ($p<0.05$).

Quantitative Micromorphological Profiles of Endometrium

Histomorphology observations showed that heifers injected with non-superovulation doses of PMSG had a higher number of uterine glands with larger sizes or diameters (Figure 3). The higher number and size of stratum functional of endometrium in heifers injected with PMSG at non-superovulation doses is also associated with the thicker stratum functional of the endometrium, the higher number of the uterine gland cross-section per lobe of the stratum functional of the endometrium, the larger diameter of the uterine glands, the longer inter-uterine gap distance of the uterine glands, the higher height of glandular epithelial cell of uterine glands, and the higher number of cells per gland duct cross-section of the uterine glands (Table 2).

The uterine gland and epithelial cell parameters, as functional units in secreting uterine histotrophic, show a significant difference ($p<0.05$) compared to control

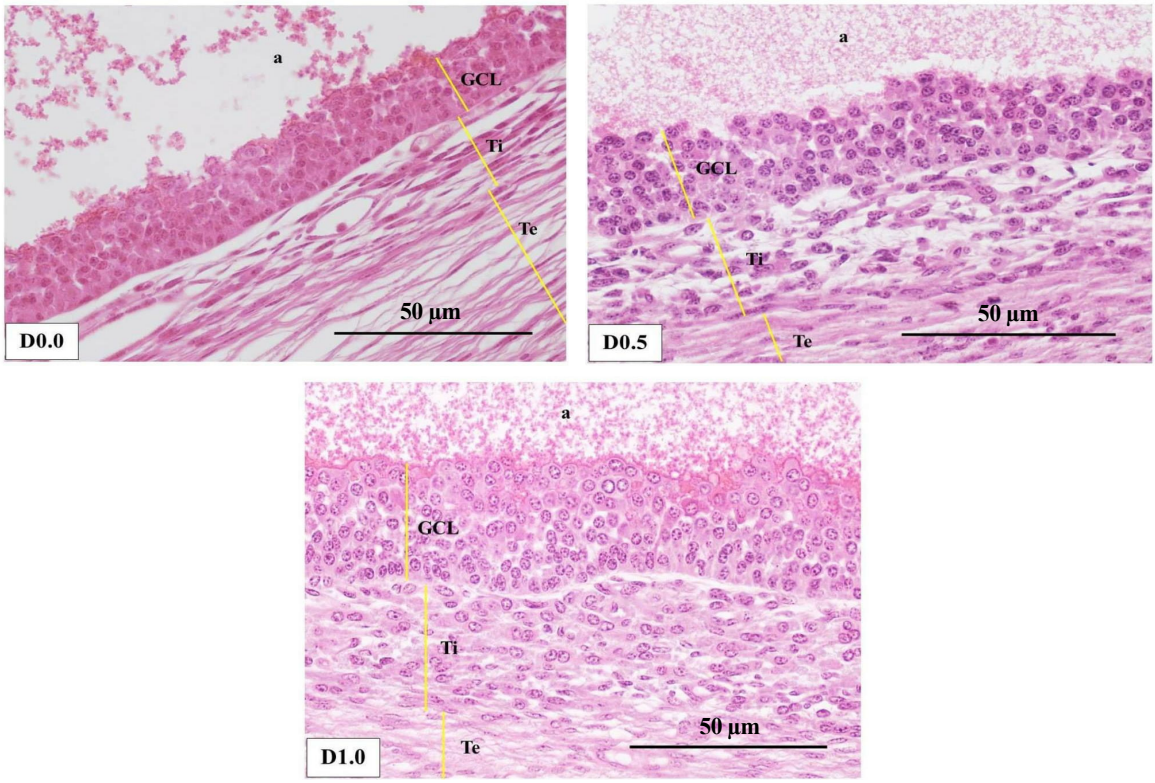

Figure 2. The histomorphological appearance of GCL of the pre-ovulatory follicle with Hematoxylin-Eosin (HE) stains. D0.0 was control heifer injected with PMSG at a dose of 0.0 IU/kg BW using blank solvent of PMSG. D0.5 was heifer injected with PMSG at a dose of 0.5 IU/kg BW. D1.0 was heifer injected with PMSG at a dose of 1.0 IU/kg BW. (a) follicle antrum, (GCL) granulosa cell layer, (Ti) internal theca cell layer, (Te) external theca cell layer. Note that the thickness of GCL in the control heifer (D0.0) was thinner compared to D0.5 and D1.0 heifers.

heifers. The significant increasing results ($p<0.05$) of the uterine gland duct number (per lobe), uterine gland diameter, along with significantly decreasing distance of inter-uterine gland ($p<0.05$) showed that the lower dose of PMSG injection could improve the growth and development of uterine gland duct and branch during estrus phase. Furthermore, the epithelial cell parameters also significantly increased in length and number ($p<0.05$) compared to control heifers as evidence of increasing activities in secreting uterine histotrophic. All of these measured quantitative parameters of uterine glands contribute to the capacity of the uterine glands to synthesize or transport and secrete all substances and materials required for prenatal growth of the embryo and fetus until parturition.

Correlation Analyses of PMSG Dose and Plasma E2 Concentrations with Quantitative Measures of GC of Pre-ovulatory Follicles

Regression analysis of the doses of PMSG injection on the quantitative measures of GC and E2 synthesis and secretions by GC showed a significant correlation ($p<0.05$) except for the density of GC (Table 3). Even

Table 1. Quantitative variables (mean ± SE) of granulosa cell (GC) and plasma estradiol (E2) concentrations during estrus phases in heifers administered with PMSG at doses of 0 IU/kg BW, 0.5 IU/kg BW, and 1.0 IU/kg BW

Variables	Doses of PMSG injection (IU/kg BW)		
	0	0.5	1
Granulosa cell layer thickness (μm)	20.94±0.87^{b}	28.57±3.71^{ab}	33.59±6.05^{a}
Granulosa cell density (cell/1000 μm^2)	65.53±6.24^{b}	83.80±2.09^{a}	83.20±0.60^{a}
Cell diameter (μm)	8.77±0.67^{b}	10.44±0.38^{a}	10.46±0.32^{a}
Nucleus diameter (μm)	4.98±0.42^{c}	6.63±0.19^{b}	6.91±0.14^{a}
Cell volume (μm^3)	359.45±84.86^{b}	597.17±64.81^{a}	600.61±55.05^{a}
Cytoplasm volume (μm^3)	293.33±79.39^{b}	444.06±66.9^{a}	427.67±51.91^{a}
Nucleus volume (μm^3)	66.11±16.28^{c}	153.11±12.88^{b}	172.93±10.63^{a}
Cytoplasm volume (%)	81.12±5.15^{a}	74.04±3.61^{b}	71.03±2.51^{c}
Nucleus volume (%)	18.88±5.15^{c}	25.96±3.61^{b}	28.97±2.51^{a}
Plasma E2 concentration (pg/mL)	130.17±13.91^{c}	181.55±22.05^{b}	278.00±10.63^{a}

Note: Means in the same row with different superscripts differ significantly ($p<0.05$).

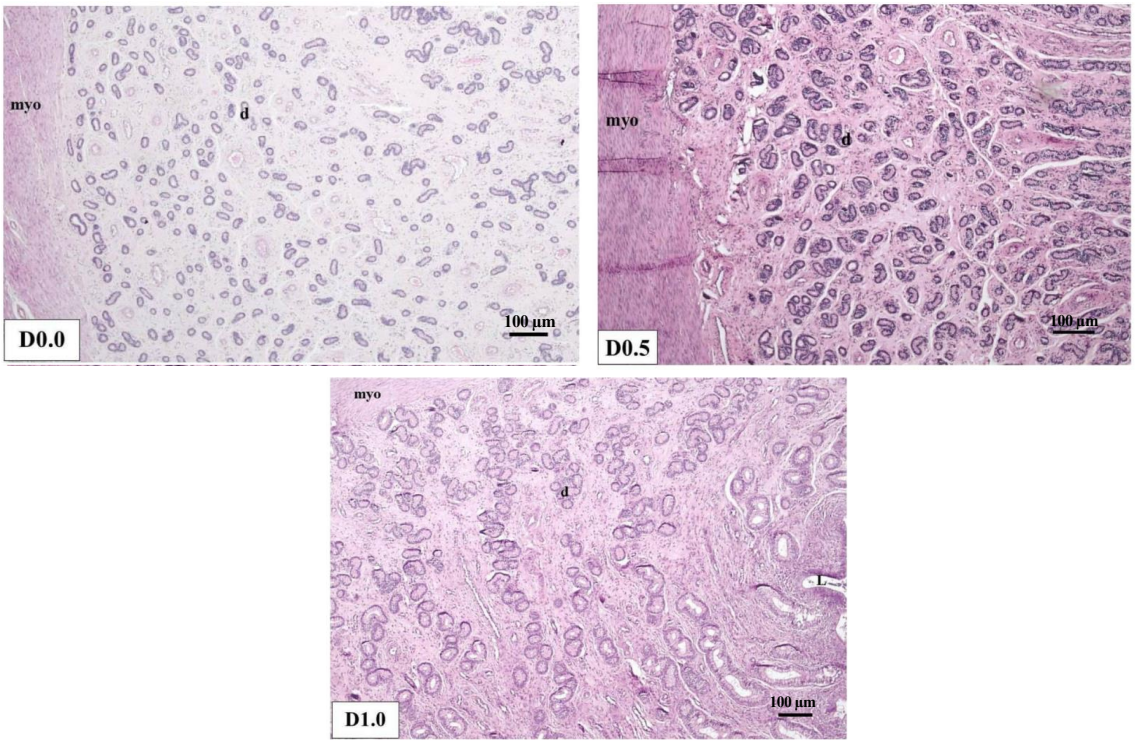

Figure 3. The histomorphological appearance of uterine glands with Hematoxylin-Eosin (HE) stains. D0.0 was control heifer administered with PMSG at a dose of 0.0 IU/kg BW using blank solvent of PMSG. D0.5 was heifer administered with PMSG at a dose of 0.5 IU/kg BW. D1.0 was heifer administered with PMSG at a dose of 1.0 IU/kg BW. (myo) myometrium, (L) uterine lumen, (d) uterine gland ducts. Note that the uterine duct gland diameter and its density were higher with increasing doses of PMSG from D0.0 to D0.5 and D1.0.

though the correlation was not significant, doses of PMSG injection and the density of GC had a positive correlation with a correlation coefficient of 0.80 ($p>0.05$). All parameters had positive correlations with the increased dose of PMSG injection, except the percentage volume of the cytoplasm. An increased dose of PMSG injection decreased the percentage volume of cytoplasm, implying that the increase in the volume and size of GC is contributed most by the higher increase in the nucleus volume than the volume of the cytoplasm. The nucleus volume had the highest correlation with the dose of PMSG injection. The increased growth and development of uterine glands with the increased dose of PMSG injection was reflected in the increased synthetic capacity of the GC to produce E2, as was reflected in the higher increase in plasma E2 concentrations. The dose of PMSG injection had the highest correlation with plasma E2 concentrations, with R value of 0.96.

Regression analysis of the effects of quantitative measures of GC and E2 synthesis and secretions showed that the thickness of GCL, the diameter of the nucleus, the diameter of GC, the volume of GC, and the density of GC did not have a significant correlation with the plasma E2 concentrations with correlation coefficients of 0.80, 0.78, 0.77, 0.77, and 0.68, respectively (Table 4). However, the volume of the nucleus, the percentage volume of the nucleus, the percentage volume of the cytoplasm, and the volume of the cytoplasm of GC had a significant correlation with the plasma E2 concentrations as an indication of the increased synthesis and secretion of E2 by the GC ($p<0.05$) with coefficient correlations of 0.86, 0.86, 0.86, and 0.69, respectively (Table 4).

Therefore, the use of a low dose of PMSG in heifers at the second follicle wave significantly stimulates the growth of GC, eventually improving the capacity of GC to synthesize E2.

Correlation Analyses of PMSG Dose and Plasma E2 Concentrations with Quantitative Measures of the Uterine Glands

Regression analysis showed that the dose of PMSG injection significantly had a positive correlation with the length of the epithelial cell of the uterine gland duct, the number of epithelial cells of the uterine gland duct, the thickness of stratum functional, and the number of duct cuts of the uterine gland with coefficient correlations of 0.94, 0.93, 0.84, and 0.72, respectively ($p<0.05$) (Table 5). The distance between ducts of the uterine glands had a significant negative correlation with the dose of PMSG injection with r= 0.86 ($p<0.05$). The diameter of the uterine gland duct did not significantly correlate with the dose of PMSG injection even though with a high correlation, i.e., 0.79.

The correlation analysis of plasma E2 concentrations with the quantitative measures of uterine glands showed that all quantitative measures of uterine glands

Table 2. Characteristics of endometrium morphology (mean ± SE) during estrus cycle in heifers administered with different doses of PMSG

Variables	Doses of PMSG (IU/kg BW)		
	0	0.5	1.0
The thickness of stratum functional* of the endometrium (μm)	2001.74±422.54^{b}	2603.72±129.66^{ab}	3091.06±429.97^{a}
The number of uterine gland duct cross-sections (per lobe)	222.943±63.42^{b}	396.61±104.96^{a}	400.67±14.44^{a}
Uterine-gland duct** diameter (μm)	26.46±6.76^{b}	36.40±0.67^{a}	38.66±2.29^{a}
Inter-uterine gland distance*** (μm)	33.65±3.54^{a}	14.38±1.21^{b}	13.48±3.49^{b}
The length of the epithelial cell of uterine glands duct (μm)	20.52±0.41^{c}	22.89±0.57^{b}	32.9±0.63^{a}
The number of the epithelial cell of uterine glands (cell/cut of uterine glands duct)	24.60±2.09^{c}	33.13±2.55^{b}	35.33±1.89^{a}

Note: Means in the same row with different superscripts differ significantly ($p<0.05$). *The functional layer of the endometrium that contains uterine gland. **Uterine gland duct cross sections inside the stratum functional. ***Distance between uterine gland ducts in four different directions, i.e., top, bottom, left, and right.

Table 3. Regression analysis of doses of PMSG administered (X variable) and the thickness of granulosa cell layer (GCL), density of granulosa cell (GC), plasma estradiol (E2) concentration, diameter of GC, diameter of GC nucleus, volume of GC, volume of GC nucleus, volume of GC cytoplasm, percentage volume of GC nucleus, and percentage volume of GC cytoplasm

Y variable	Regression equation	R	R^2	P
Thickness of GCL	Y = 12.66x + 21.37	0.83	0.70	0.005
Density of GC	Y = 17.67x + 68.68	0.80	0.64	0.100
Plasma E2 concentration	Y = 148.51x + 122.54	0.96	0.93	0.000
Diameter of GC	Y = 1.69x + 9.05	0.86	0.74	0.003
Diameter of GC nucleus	Y = 3.08x + 5.02	0.79	0.63	0.011
Volume of GC	Y = 241.1x + 398.5	0.86	0.74	0.003
Volume of GC nucleus	Y = 106.8x + 77.30	0.93	0.87	0.000
Volume of GC cytoplasm	Y = 134.3x + 321.1	0.79	0.62	0.012
Percentage volume of GC nucleus	Y = 10.09x + 19.55	0.91	0.83	0.001
Percentage volume of GC cytoplasm	Y = -10.09x + 80.44	0.91	0.83	0.001

Note: X variable= doses of PMSG injection, R= coefficient of correlation, R^2= coefficient of determination, $p<0.05$ considered significant.

were significantly positively stimulated by the increased plasma E2 concentrations, except the distance between ducts with a negative correlation with the plasma E2 concentration ($p<0.05$). Quantitative measures of uterine glands that have the highest correlations with the stimulation of plasma E2 concentrations were 1) the length of the epithelial cell of the uterine gland duct, 2) the number of the epithelial cells of the uterine gland duct, 3) the thickness of stratum functional, 4) the diameter of ducts of uterine glands, and 5) the number of duct cuts of uterine glands with correlations of 0.96, 0.84, 0.84, 0.70, and 0.69, respectively ($p<0.05$) (Table 6). The distance between ducts negatively correlated with the plasma E2 concentration ($p<0.05$) with $r = 0.76$. These observations confirmed that plasma E2 concentrations significantly stimulate the growth and development of uterine glands.

DISCUSSION

The observation conducted in the present study was limited to the measurement of growth and development of GC during the pre-ovulatory phase under the stimulation of a lower dose of PMSG and the degree of increase in E2 synthesis and secretion during the pre-ovulatory phase continued with the observations of growth and development of the uterine glands. In summary, the non-superovulation dose of PMSG injection, i.e., 0.5 IU/kg BW and 1.0 IU/kg BW, successfully stimulates the growth and development of GC in the pre-ovulatory follicles. However, the 1.0 IU/kg BW dose still had a propensity to endure the growth of the follicle into multiple dominant pre-ovulatory follicles, as shown in Figure 4. In this experiment, the significant increase in E2 secretion by the GC successfully stimulated the thickness of stratum functional of the endometrium, the

Table 4. Regression analysis of plasma E2 concentration (Y variable) and the thickness of granulosa cell layer (GCL), density of granulosa cell (GC), diameter of GC, diameter of GC nucleus, volume of GC, volume of GC nucleus, volume of GC cytoplasm, percentage volume of GC nucleus, and percentage volume of GC cytoplasm

X variable	Regression equation	R	R^2	P
Thickness of GCL	Y = 8.14x – 28.62	0.80	0.64	0.10
Density of GC	Y = 4.75x – 171.3	0.68	0.46	0.44
Diameter of GC	Y = 60.55x - 402.0	0.77	0.59	0.15
Diameter of GC nucleus	Y = 30.73x - 4.745	0.78	0.60	0.14
Volume of GC	Y = 0.424x - 23.29	0.77	0.60	0.15
Volume of GC nucleus	Y = 1.151x + 46.29	0.86	0.73	0.003
Volume of GC cytoplasm	Y = 0.626x - 46.43	0.69	0.48	0.039
Percentage volume of GC nucleus	Y = 12.02x - 99.15	0.86	0.74	0.003
Percentage volume of GC cytoplasm	Y = -12.02x + 1103	0.86	0.74	0.003

Note: Y variable= Plasma E2 concentration, R= coefficient of correlation, R^2= coefficient of determination, $p<0.05$ considered significant.

Table 5. Regression analysis of doses of PMSG (X variable) administered and the thickness of stratum functional, the number of the uterine gland cross sections, the diameter of ducts of uterine glands, inter-uterine gland duct distance, the length of the epithelial cell of uterine gland duct, and the number of epithelial cells of uterine gland duct

Y variable	Regression equation	R	R^2	p
Thickness of the stratum functional	Y = 1089x + 2020	0.84	0.70	0.005
Number of the uterine gland cross section	Y = 177.6x + 251.2	0.72	0.51	0.030
Diameters of the uterine gland duct	Y = 12.19x + 27.73	0.79	0.63	0.110
Inter-uterine gland duct distance	Y = -20.17x + 30.59	0.86	0.73	0.003
Length of epithelial cell of uterine gland duct	Y = 12.4x + 19.23	0.94	0.89	0.000
Number of epithelial cells of uterine gland duct	Y = 10.73x + 25.65	0.93	0.86	0.000

Note: X variable= doses of PMSG, R= coefficient of correlation, R^2= coefficient of determination, $p<0.05$ considered significant.

Table 6. Regression analysis of plasma E2 concentrations (X variable) and the thickness of stratum functional of endometrium, the number of the uterine gland duct cross sections, the diameter of the uterine gland ducts, inter-uterine gland duct distances, the length of epithelial cells of uterine gland duct, and the number of epithelial cells of uterine gland duct

Y variable	Regression equation	R	R^2	P
Thickness of stratum functional of endometrium	Y = 7.051x + 1177	0.84	0.70	0.005
Number of the uterine gland cross sections	Y = 1.101x + 123.4	0.69	0.50	0.042
Diameters of the uterine gland ducts	Y = 0.069x + 20.16	0.70	0.50	0.037
Inter-uterine gland duct distances	Y = -0.115x + 43.21	0.76	0.57	0.018
Length of epithelial cell of uterine gland duct	Y = 0.081x + 9.384	0.96	0.91	0.000
Number of epithelial cells of uterine gland duct	Y = 0.062x + 18.67	0.84	0.70	0.005

Note: X variable= plasma E2 concentrations, R= coefficient of correlation, R^2= coefficient of determination, $p<0.05$ considered significant.

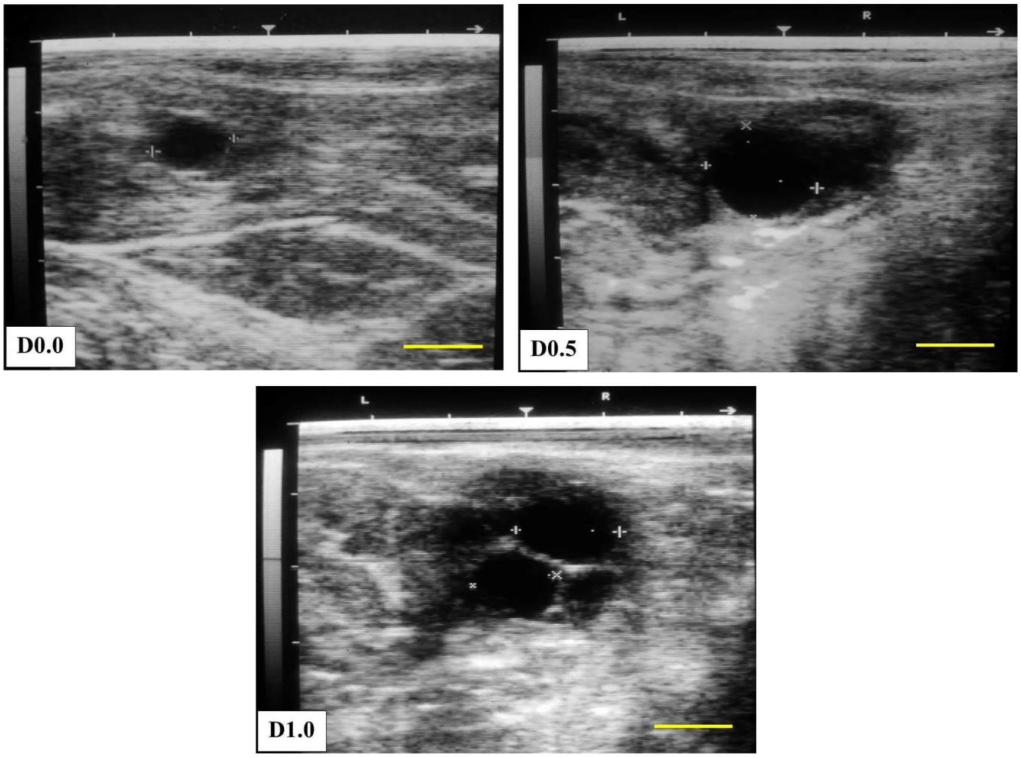

Figure 4. Ultrasonography images of the largest follicle during estrus phase (pre-ovulatory follicle) among treatment groups. D0.0 was control heifer administered with PMSG at a dose of 0.0 IU/kg BW using blank solvent of PMSG. D0.5 was heifer administered with PMSG at a dose of 0.5 IU/kg BW. D1.0 was heifer administered with PMSG at a dose of 1.0 IU/kg BW. Note that the diameter and number of follicles formed were higher with increasing doses of PMSG from D0.0 to D0.5 and D1.0.

number and density of uterine gland ducts, and the size of uterine glands as indicators of increased growth and development of uterine gland in the endometrium.

In general, we observed a complex mechanism since the beginning of PMSG injection on the reproductive tract. The first response to the PMSG injection is the increase in the growth and development of GC in the pre-ovulatory follicles. This finding also follows the prior research, which showed the improved follicle growth and development indicated by the greater pre-ovulatory follicles size of White Lampun cow (Jitjumnong *et al*., 2019) and Ongole-crossed heifer (Putro *et al*., 2020) treated with PMSG. The increase in the diameter of the dominant follicle due to the PMSG injection was also reported by Andriyanto *et al*. (2015) in Kacang goat, and this parameter had effects on the thickness and density of GC that also present in our study. Furthermore, in bovines, equine chorionic gonadotropin (eCG) induction at the removal of intravaginal progesterone device induces higher plasma E2 concentrations prior to ovulation (Núñez-Olivera *et al*., 2020), as well as the combination between FSH and eCG (Santos *et al*., 2018). These phenomena have a close relationship with histomorphometry changes of GC as a functional element in producing E2.

The PMSG works like FSH, i.e., by stimulating follicles that eventually recruit the follicle in a greater number. The increase in follicle recruitment continued with the stimulation of follicle development that eventually increasing the diameter and the number of dominant follicles, followed by an increasing level of E2 and ovulation rate at the end of the cycle (proestrus-estrus) (Sakaguchi *et al*., 2019; de Lima *et al*., 2020). At the normal estrus without PMSG stimulation, the largest follicle at estrus (pre-ovulatory follicle) was reported to have a significantly higher layer thickness and density of GC than the dominant follicle at follicle waves 1 and 2 (Singh & Adams, 2000). Therefore, a low dose of PMSG injection in the present study could improve the growth and development of GC up to the estrus phase, indicated by the increasing thickness of GCL and GC density more than in ordinary conditions.

The second response is the increase in E2 synthesis and secretion due to the increased growth and development of GC and increased PMSG injection doses. Under normal circumstances, E2 synthesis is closely related to FSH secretion as the main hormone that stimulates the growth and development of the follicles. This gonadotropin hormone is known to increase the synthesis of E2 when the GC has important substrates to convert androgen into E2, i.e., aromatase (Chwalisz & Fűrbass, 2014; Guillemin *et al*., 2015), while the activities of aromatase in GC of the pre-ovulatory follicle itself is known to increase even up to 700 folds (Strauss *et al*., 2014) during estrus phase. It was reported that during the estrus phase, there was an increase in the cytoplasmic activity of GC marked by the increase in the volume of cytoplasmic mitochondrial and the increase in the agranular cytoplasmic membrane (Sangha *et al*., 2012). Regan *et al*. (2018) also explained that in the mature GC (espe-

cially in the pre-ovulatory phase), the proliferation of organelle is occurred to accommodate the steroidogenic capacity meaning that there is an increase in the volume of the cytoplasm. However, in the present experiment, the nucleus of GC also had a high contribution to the increased synthetic capacity of the GC to synthesize and secrete E2 as it had a greater size compared to the control group. Based on all of these activities that occurred in the normal pre-ovulatory follicle (without induction with PMSG in the luteal phase), it is strongly assumed that injection of PMSG, even at a lower dose, can increase the higher synthetic activity of E2 through the increase in the volume and number of cytoplasmic and nucleus of GC without increasing in the number of ovulating follicles.

The third response is the increased E2 synthesis and secretion that directly affect the growth and development of uterine glands. This finding is also following several reports which explained that increasing levels of E2 at the estrus phase could affect the histomorphological changes and functions of the uterine glands (Gonella-Diaza *et al.*, 2017; Núñez-Olivera *et al.*, 2020). During the follicular phase (proestrus-estrus), it was reported an increase in the number of estradiol receptors α (ERα) in three parts of the uterus (Zeng *et al.*, 2017; Perry *et al.*, 2021). The increase in plasma E2 concentrations in the circulation and the increasing existence of Erα will cause the lengthening of uterine glands, followed by the production of estrus mucus by ciliated epithelial cells of the endometrium and secretory cells (Bunma *et al.*, 2021). The increase in the thickness of the stratum functional and the activity of the other uterine glands during the estrus phase is a direct effect of the high increase in E2 synthesis in the GC. This increases the synthesis and secretions of E2 and also have effects on improving the proliferation of uterine glands at the time of the luteal phase or at the beginning of pregnancy by increasing the secretions of uterine histotrophic (Benbia *et al.*, 2017).

Increased E2 synthesis and secretion without increasing the number of recruited follicles will improve the growth and development of uterine glands without increasing the number of implanted embryos. This steroid hormone is an early signal that begins the process of reproduction comprehensively from the stimulation of uterine glands growth and development for preparation of ovum and sperm for fertilization, fertilization, preparation of the uterus for implantation, implantation, and further growth and development of placenta, embryo, and fetus (Sugiura *et al.*, 2018). These processes eventually determine the nutrients and materials availabilities required by the growing and developing embryo and fetus (Sferuzzi-Perri *et al.*, 2013) and gene expression in the embryo and fetus (Fowden *et al.*, 2008; Fowden & Forhead, 2009). The availability of nutrients and material and genetic expression of embryo and fetus further determine the prenatal growth, birth weight, and the preweaning growth rate until adult age and finishing.

In addition to the roles of pregnant hormones (estradiol and progesterone), the other hormones such as glucocorticoid (Braun *et al.*, 2013; Fowden & Forhead, 2015; Fowden *et al.*, 2016) and thyroid hormones (Forhead & Fowden, 2014) also have roles in the prepartum maturation of body organs such as pulmonary gas exchange, thermogenesis, hepatic gluconeogenesis, and cardiac adaptations in preparations of the offspring to the environment after birth. The programming of intrauterine development of embryos and fetuses and the maturation of body organs will produce good offspring that will grow better with superior phenotype performances during postnatal and adult life. The improved uterine growth and development during early pregnancy will improve the availability of materials to optimize the growth and development of conceptus and further improve gene expression in the offspring eventually will improve the life quality during postnatal life until adult life through the process of intrauterine programming (Fowden & Forhead, 2004; Sferuzzi-Perri *et al.*, 2013).

The PMSG hormone is still widely used in animal reproductive technology, such as for superovulation purposes, treating anestrus animals, and estrus synchronization, followed by timed artificial insemination (TAI) (Murphy, 2012). Even though this glycoprotein class hormone has adverse effects due to its long half-life (around 45.6 hours in cows, according to Alvarez *et al.*, 2016), several studies showed that PMSG still has advantages in a particular area of research. Our previous observations in sheep showed that injection of sheep prior to mating with PMSG improved the productivity of smallholder sheep farms (Andriyanto & Manalu, 2012) as a positive impact on the production aspect. In other studies, injection of does with PMSG prior to mating showed the growth and development of dominant follicles that eventually improved estrus response, thus improving the mating process (Andriyanto *et al.*, 2015). The non-superovulation dose of the PMSG concept was also implemented in Kacang goats and showed a significant increase in endogenous secretion of estradiol and progesterone without superovulation response (Andriyanto *et al.*, 2017). Another study showed that injection of maternal goats prior to mating with a non-superovulation dose of PMSG significantly increased fetus length at 7 weeks of pregnancy (Arif *et al.*, 2018a). Furthermore, this method proved to improve estradiol and progesterone synthesis and secretion, which gave significant implications for improved lamb birth weight and produced lambs with higher immunity and resilience to *Haemonchus contortus* (Arif *et al.*, 2018b).

Observations in polytocous and oligotocous animals showed the use of gonadotropin injection prior to mating could improve the stimulation of the growth and development of granulose and thecal cells of the ovary eventually increase synthesizing capacity and secretions of the estradiol and progesterone at the beginning of reproduction cycle. These hormones are involved in the programming of the uterus and placental growth and development to produce materials and nutrients as well as the environment to support the optimum growth and development of embryos and fetuses during pregnancy. However, the non-superovulation doses of gonadotropin used to

improve the growth and development of granulose and thecal cells in the ovary are recommended to avoid the increased litter size above normal. The increase in litter size that is higher than the normal litter size will disturb the intrauterine programming that eventually interferes the prenatal growth and development. The use of gonadotropin at the dose below the superovulation doses will increase the growth and development of granulose and thecal cells in the recruited follicles, thus will increase the synthesis and secretions of estradiol and progesterone as well as other materials and factors that will program the growth and development of uterus and placenta. The optimum growth and development of the uterus and placenta under the programming of optimum concentrations of estradiol and progesterone will produce material and environment to support the optimum growth and development and genetic expressions of embryos and fetuses during prenatal growth. The optimum growth and development and genetic expression of the embryos and fetuses during prenatal growth and development will optimize the qualities of offspring produced during the postnatal and mature and adult lives.

In addition to the injection of gonadotropin at the non-superovulation doses to improve the growth and development of granulose and theca cells, another technology is available to improve the synthesis and secretions of estradiol and progesterone to start the uterine programming of embryos and fetal growth and development. The flushing technique is well known to improve the reproduction system in mammalian animals, where supplementation with 2.8% ALA from flaxseed oil gave the best results in terms of stimulating the highest number of large-sized pre-ovulatory follicles and the highest plasma estradiol concentration without sufficient improvement of follicle growth and development to reach the ovulation stage was found in does that were supplemented with lauric acid, an SFA from coconut oil (Nugroho *et al.,* 2021). This flushing technology can be combined with a non-superovulation dose of gonadotropin prior to mating to improve the growth and development of granulosa and thecal cells to increase the synthesis and secretion capacity.

All of these findings showed that an improved uterine environment during the estrus cycle and pregnancy will improve the growth and development of blastocyst to embryo until fetus and parturition to produce good offspring that will grow better with superior phenotype performances during postnatal and adult lives. It was proposed that improved uterine growth and development during early pregnancy will improve the growth and development of conceptus further improved gene expression in the offspring will improve the life quality during postnatal life until adult life through the process of intrauterine programming (Fowden & Forhead, 2004; Sferuzzi-Perri *et al.,* 2013). Therefore, the intrauterine programming of prenatal growth by injecting the maternal livestock with a lower dose of gonadotropin had a great potency in producing superior offspring in local animal breeds to increase animal production to meet the increasing demand for livestock products.

CONCLUSION

Based on these findings, a lower dose of PMSG injection in Ongole-grade heifers strongly affects the histomorphometrical changes of pre-ovulatory follicles that increase E2 synthesis and secretion that further stimulate the growth and development of uterine glands in the endometrium. It means that the low dose of PMSG can increase the reproductive performance at the time of estrus without increasing the ovulation rate. This low dose of PMSG injection can reduce the risk of multiple calving so that it can be used as an alternative method to develop intrauterine programming in large ruminant animals or other monotocous animals.

CONFLICT OF INTEREST

Wasmen Manalu and Arief Boediono serve as editors of the Tropical Animal Science Journal, but have no role in the decision to publish this article. All authors also certify that there is no conflict of interest with any financial, personal, or other people or organization related to the material discussed in the manuscript.

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