Conference Paper

Insulin-Like Growth Factor-I (IGF-I) from Crossbred Pregnant MareSerum to Increase Follicle Number of Mice (Mus musculus)

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Abstract

The purpose of the research was to know the effect of Insulin-Like Growth Factor-I (IGF-I) derived from pregnant crossbred mare serum (PMS) in mice (Mus musculus) folliculogenesis. The subjects of this research were 20 female mice. The research was arranged by Completely Randomized Design (CRD) with four treatments and five replications. The treatments were Co = 10 ng/ml of physiological NaCl, P1 = 10 ng/ml of IGF-I PMS, P2 = 20 ng/ml of IGF-I PMS, and P3 = 40 ng/ml of IGF-I PMS. Observed variables are the number of primary, secondary, tertiary and de Graff follicles. During the treatment, the estrous cycle was also observed. The data of follicles number were analyzed by Analysis of Variance (ANOVA) and followed by HSD (Honestly Significant Difference) test. The result showed that the addition of IGF-I PMS significantly affects ($p<0.05$) on increasing the primary and secondary follicles number. The addition of IGF-I PMS 20 ng/ml and 40 ng/ml can increase the primary and secondary follicles significantly ($p<0.05$).

Keywords: IGF-I crossbreed mare serum pregnant; follicle; Mus musculus.

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1. Introduction

The human need of animal protein is significantly increasing nowadays. It can be shown that Indonesian people consume 6.65% of meat during 2013-2014 [1] and is estimated that the number of consumption will keep rising up. On the contrary, the number of ranch industry is decreasing every year [2], especially in 2011 until 2014. At that time, the cattle ranch number in the slaughterhouse was significantly declining. In addition, the supplies of beef in some markets are decreasing as well. The government has attempted to solve this problem, like importing beef from Australia, but this has not

completely solved the problem. The people might not continually import the beef as the imported beef cost is much more expensive than the local one. Therefore, there was a thought to improve the productivity of livestock.

According to the regulations of the minister of agriculture no. 48 in 2016 about increasing the population of cows and buffaloes pregnancy rapidly. Article 5 verse 1 and 2 stated that the effort of increasing the population of cows and buffaloes pregnancy will be done by reproduction management system [3]. This system includes the examination for reproduction status and reproduction disorder, artificial insemination (AI) service and natural mating, fulfilling frozen semen and liquid N_2, controlling productive female individuals, and fulfilling forage and concentrate.Many problems, like reproduction, occur more often to stock farmers when they attempt to increase livestock population and productivity. The reproduction problems that usually occur are the cows are pregnant but there is not lust indication; reproduction hormone is abnormal (high progesterone), this causes false pregnancy and luteal cysts; the emergence of yellowish or greenery mucus with bad smell as there is inflammation inside the reproductive tract; unobserved lust; lack of feed which leads to ovarian hypofunction; repeated mating; and reproduction hormonal disorder (high estrogen) due to follicle cysts [4].

Reproduction or breeding is a process of forming a new individual whether it passes through natural mating or artificial mating [5]. Reproduction is a physiological process that is experienced by all living things in order to retain offspring and life sustainability [6]. This process requires reproduction hormone which has a role in reproduction system directly and indirectly. On the other hand, ovarium has also a significant role. It has two based functions: producing fertilizable oocyte that has faultless growth and secreting steroid hormone to set up the reproductive tract in a fertilization and implantation process [7] Thus when the ovary is in interference, it will disrupt reproduction system. A lot of previous research used bioactive materials which aimed to improve reproduction ability.

One of the bioactive materials that have a significant role in female animals reproduction system is Insulin-Like Growth Factor-I (IGF-I). IGF-I supplementation in maturation medium and oocyte culture can stimulate and increase the number of the mature oocytes. In addition, it also increases in vitro fertilization (IVF) [8] and the number of the embryo that reaches the stage of blastocyst stage for some animals, like pigs, cows [9], and buffaloes [10]. The result of this research [11] states that adding IGF-I during preantral cultures of pigs can intensify follicle diameter. Besides, it can increase mRNA Insulin-Like Growth Factor Receptor-I (IGFR-I) as well, by supplementing Follicle

Stimulating Hormone (FSH) during culture, and the oocyte will be processing meiosis stage after maturating. In addition [12], IGF can increase the proliferation of granulosa cells, steroidogenesis, and oocyte growth in mammals mainly. This research [13] also proves that identical events which happened on cows are related to IGF-I concentration enhancement toward serum and follicle itself. IGF-I from the ovary and/or from systemic circulation is an important thing in folliculogenesis regulation and is a mediator of the genetic component of ovulation on cows.

2. Materials and methods

This research applied laboratory experimental research with Completely Randomized Design (CRD) as the methods of the study. The subjects of this research were 20 Balb/C female mice in the age of 28-32 days which were got from Pusat Veterinaria Pharma (Pusvetma), Surabaya and were avowed by Research Ethics Commission of Faculty of Veterinary Medicine, Universitas Airlangga as ethically proper subjects. From 20 female mice, they were divided into four treatments groups. The mice in control group (C0) were injected by physiology NaCl 0.1 ml, treatment P1 were injected by IGF-I PMS0.1 ml (10 ng/ml), treatment P2 were injected by IGF-I PMS 0.1 ml (20 ng/ml), and treatment P3 were injected by IGF-I PMS 0.1 ml (40 ng/ml). All injections were done subcutaneously and the IGF-I PMS injections were done 5 times in 5 days. Before injecting, there would be vaginal swab toward the mice in order to know the status of the reproduction.

2.1. Experimental Animals Surgery and Preparing Mice Ovary Histopathology

In the end of the research, the experimental animals would be anesthetized by ether to become stagnate and did surgery to get the left ovary. Afterwards, the ovary was separated from its fat and soaked in 10% formalin solution for 1-2 days to fix the tissue. Subsequently, the ovary was ready to go on the next stage for making mice ovary histopathology preparation. This process was using routine dyeing techniquesHaematoxylin-Eosin in Veterinary Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga.

2.2. Identify and Calculate Mice Follicles

Ovary preparation was observed by using a microscope with ocular lens magnification of 10x and objective lens magnification of 4x-40x. From this observation, the follicle was classified based on the oocyte size inside the follicles, the size of follicles which is based on the number of cells that cover them, and follicles morphology [14]. Primary follicles consist of a layer of flat granulosa cells and cubical granulosa cells, or it is only the cubical shape, while for secondary follicles, they consist of two layers or more of cubical granulosa cells [15]. Next, small antral follicles (tertiary follicles) have segmented cavity with 2 antrum or more, while big antral follicles (follicle de Graff) have one cavity, called antrum, which continuously enlarged [16]. These kinds of the follicle (both preantral and antral) in ovary was calculated by examining all viewing fields, then the data was conducted and analyzed.

3. Results

The result of counting the number of mice primary follicles after given Insulin-Like Growth Factor-I (IGF-I)derived from crossbreed pregnant mare serum (PMS) showed that there was escalation number of the primary follicles. Giving this serum showed significantly affect ($p<0.05$) the treatment T1, T2, T3. The higher of the dose, the more increasing of the follicles. It can be seen that IGF-I PMS with a dose of 20 ng/ml (T2) and 40 ng/ml (P3) can increase the number of mice follicles into ($p<0.05$).

Similar to the primary one, the result of counting the number of mice secondary follicles after injected IGF-I derived from crossbreed pregnant mare serum (PMS) also showed significantly affect ($P<0.05$) the treatment T1, T2, and T3.IGF-I PMS with a dose of 20 ng/ml (P2) and 40 ng/ml (T3)can increase the number of mice secondary follicles, comparing to control group (Co) ($p<0.05$). Besides, IGF-I PMS with a dose of 20 ng/ml and 40 ng/ml (T2 and T3) have significantly affected ($p<0.05$) the treatment T1 in increasing mice secondary follicles number.

On the other hand, the result of mice tertiary follicles and de Graff follicles number after injected IGF-I derived from crossbreed pregnant mare serum (PMS) did not show any changes. Giving this treatment did not show significantly affect ($p>0.05$) as well toward mice tertiary follicles and de Graff follicles for treatment T1, T2, and T3.

To understand more about the follicles, each follicle is presented in Figure 1, while the graphic for mice primary, secondary, tertiary, and de Graff follicles number is presented

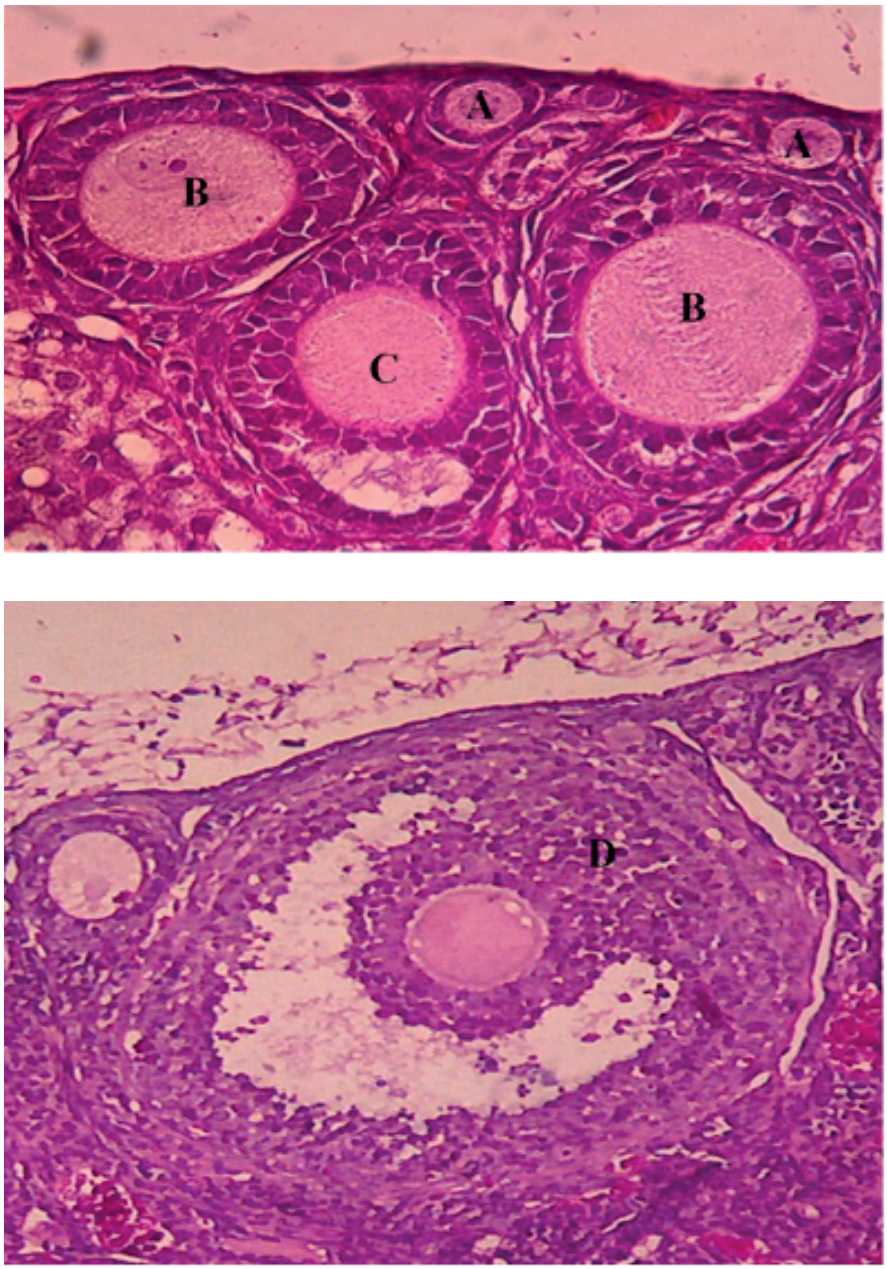

Figure 1: The result of the ovarian follicles identification in mice (*Mus musculus*) (Haematoxylin-eosin, 400x). In the figure above, (A) is a primary follicles composed of a flat layer of granulosa cells, (B) is a secondary follicle consisting of two or more layers of both cubic and cylindrical granulosa cells, and (C) is a tertiary follicles which is a follicle with some cavities called call-exner bodies and (D) is the de Graff with antral follicles containing liquor folliculi (Haematoxylin-Eosin, 100x).

in Figure 2. The results of counting primary, secondary, tertiary, and de Graff follicles number after injected IGF-I PMS are provided in Table 1.

The function of the ovary is to supply germinal cells which are needed for living things sustainability. Besides, ovary produces hormones that are useful for female

Table 1: The average and standard deviation of the number of primaries, secondary, tertiary, and de Graff follicles in mice ovary (Mus musculus) after given IGF-I PMS.

Treatment	Follicle number (x ± SD)			
	Primary Follicle	Secondary Follicle	Tertiary Follicle	de Graff Follicle
CO	$9.40^{a} \pm 6.58$	$6.20^{a} \pm 3.11$	$4.60^{a} \pm 2.88$	$3.40^{a} \pm 2.79$
T1	$14.00^{ab} \pm 2.82$	$8.40^{a} \pm 2.88$	$8.40^{a} \pm 2.88$	$3.00^{a} \pm 1.22$
T2	$23.80^{b} \pm 7.19$	$21.60^{b} \pm 6.34$	$8.60^{a} \pm 4.27$	$3.60^{a} \pm 1.14$
T3	$20.40^{b} \pm 4.87$	$20.00^{b} \pm 10.12$	$11.00^{a} \pm 4.41$	$5.40^{a} \pm 1.81$
Note: The different superscript in one column shows significant differences ($p<0.05$)				
T1 = injected with 10 ng/ml IGF-I PMS				
T2 = injected with 20 ng/ml IGF-I PMS				
T3 = injected with 40 ng/ml IGF-I PMS				

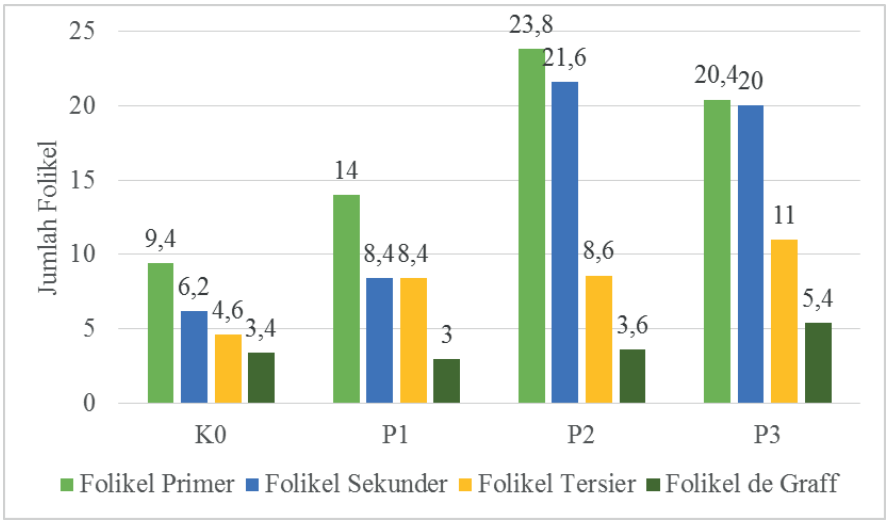

Figure 2: The average and standard deviation of the number of primaries, secondary, tertiary, and de Graff follicles in mice ovary (Mus musculus)after given IGF-I PMS.

individuals growth [18-20]. Female individuals were born with limited primordial follicles in which when it runs out, it cannot be added again. This thing leads to folliculogenesis that can be called as irreversible process [21-23]. Ovary folliculogenesis comprises all follicle growth, differentiation, and ovulation. More than 99% of follicles will degenerate or will have atresia. In mammals, the follicles are controlled by endocrine factors, like FSH and LH, and paracrine factor. Among those factors, there is an element called Insulin-Like Growth Factor (IGF) which has an important role in modulating gonadotropin in proliferation and differentiation of follicular cells [24].

Based on the result of this research, giving Insulin-Like Growth Factor (IGF)derived from crossbreed pregnant mare serum can increase the number of primary and secondary follicles significantly ($p<0.05$) comparing to the control group. Moreover, the

doses of 20 ng/ml and 40 ng/ml in IGF also affects the escalation of primary and secondary follicles significantly ($p<0.05$). On the other hand, the number of mice tertiary and de Graff follicles, injected with IGF, did not show significantly affect ($p>0.05$) in control group (Co). This proves that IGF-I can stimulate receptor cells type 1 in granulosa cells and theca cells [25] and the reaction from the receptor in granulosa cells will be raised by estrogen and gonadotropin. Subsequently, IGF-I receptor reaction will increase in small antral follicles [26].

According to this research, Insulin-Like Growth Factor-I (IGF-I) derived from cross-breed pregnant mare serum can advance the number of primary and secondary follicles because the mice have not been in puberty. This is corresponding with the follicular development theory proposed by [27] in which the dominant mice ovary that belongs to pre-puberty mice like this research's subjects is primary and secondary follicular development.

There is also another theory that states ovary follicular development is divided into two stages: preantral and antral. Preantral encompasses primordial follicle, primer follicle, and secondary follicle. This phase, can be called as gonadotropin-independent phase, is marked by oocyte growth and differentiation which is affected by some growth factors through the autocrine/paracrine system, like Transforming Growth Factor β (TGF-β) superfamily and insulin-like Growth Factor I (IGF-I). Another phase, antral or gonadotropin-independent, is marked by a significant escalation of the follicle size. This phase encompasses de Graff follicle. In addition, antral is controlled by Follicle Stimulating Hormone (FSH) and luteinizing Hormone (LH) and another growth factors. These another factor will stimulate proliferation cell and affect gonadotropin activity [28-30]. Therefore, there were only primary and secondary follicles which increase significantly in this research, while tertiary and de Graff follicles were not increasing.

The working mechanism of protein complex IGF-I and IGFBP-3 toward immature and mature follicles was explained in [31]. It stated that immature follicle, LH, assisted with IGF-I and activin in granulosa cell, will stimulate theca cell to form androgen. IGF-I and IGFBP-3 were binded and disparted by TIMP (Tissue InhibitoringMetalo Proteinase) with high concentration. However, the ability of disparting was not significant, so that the generated IGF-I would not be plentiful. Androgen was brought to granulosa cell, then it was changed into estradiol through aromatization process and was stimulated by IGF-I and FSH.

In mature follicle, LH, assisted with IGF-I and Inhibin in granulosa cell, would stimulate theca cell to form androgen. While IGFBP-3 would be disparted by TMP to produce IGF-I in large quantities. The androgen was brought to granulosa cell and changed

into estradiol through aromatization process. Afterwards, by controlled by aromatase inhibitor enzyme that was produced by granulosa cell, the androgen was stimulated by IGF-I and FSH to increase the secretion [31]. Based on this, the mice which have not been in estrous phase, they would get into the phase faster and it would be maintained during IGF-I injection.

Protein complex IGF-I and IGFBP-3 have an effect on forming FSH. This hormone, produced by the pituitary, will stimulate follicle growth in the ovary and form LH receptor in ovary follicles cells as well [32]. According to [33], injecting IGF-I and IGFBP-3 in diestrous phase can stimulate the anterior pituitary to produce LH and FSH to secrete estrogen in the blood. Estradiol secreting escalation by dominant follicle cells when ovulation happened will be causing lust, stimulate LH, and ovum excretion.

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