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The Effect of Leptin in Increasing the Quality of Goat Oocyte Maturation in Vitro

Pieter Kakisina

Faculty of Mathematics and Natural Sciences Pattimura University-Ambon, Indonesia

ABSTRACT

The aim of the research was to determine the effects of leptin in accelerating oocytes maturation. In this research, the addition of leptin in oocyte maturation medium to tissue culture medium-199 (TCM-199) + 10% fetal bovine serum (FBS) at concentrations of 0, 50, 100, 250, 500, 750 and 1000 ng/ml in incubation time 18, 22, 26 and 30 hours significantly increased oocytes percentage up to metaphase II (M II) along with increasing incubation time. The concentration of750 ng/ml with an incubation time of 30 hours is the most optimal in endorsing oocytes to reach M II.
KEYWORDS: leptin, oocyte maturation.

INTRODUCTION

Oocytes growth in the ovaries of all mammals do not occur at the same time because only oocytes that are able to respond to the release of gonadothrophin hormones that can continue the process of maturation through meiosis, development from prophase or the phase of germinal vesicle (GV) to metaphase two (M II) in the follicle, which will further be ovulated. If the growth can be well managed, immature oocytes in the ovaries can be used as sources of mature oocytes for the purpose of in-vitro fertilization program and as a source of recipient cytoplasm in somatic cell nuclear transfer to produce stem cell[1]. In the latest development, in-vitro culture system growth (IVG) on an experimental scale has been developed as a new technology using incompetent oocytes as a source of mature oocytes through in-vitro maturation[2,3,4].

Current provision of in-vitro embryos for the purposes of embryo transfer is still not able to meet the standards of living embryos with high viability. It is based on the pregnancy rate when in vitro-produced embryos transferred to a recipient produces only low pregnancy rates[5]. This low pregnancy rate requires an initial study on the molecular basis of reproductive maturation of oocytes. This is because the process of oocyte maturation is very complex and involves the role of growth factors. Until recently the synthesis and function of proteins and the molecular pathway mechanisms in the oocyte maturation process is still not widely known.

We require better studies of in-vitro embryo culture system so that it can be developed to increase the success of the production of embryos that have better quality and viability. This is because in some mammalian species, the results of oocyte in-vitro maturation (IVM), in-vitro fertilization (IVF) and embryo parthenogenetic are still quite low[6,7,8].

Leptin supplementation in IVM medium is an alternative to enhance the success of in-vitro embryo production because it is known that leptin, the 16-kDa hormone protein, plays an important role in reproduction, especially in the process of oocyte maturation. Research by Ryan, et alshowed that leptin has no direct effect on the spontaneous maturation due to inability to release the inhibition of oocyte maturation by phosphodiesterase3B (PDE3B) inhibitor, inhibitor3-isobutyl-1-methylxanthine(IBMX) and milrinone(MR), both the denuded oocytes(DO) and cumulus oocyte complex (COC)[9]. However, research shows that when oocytes are included in oocyte maturation medium, leptin significantly increased the proportion of oocytes in reaching M II and an increase occurs in the protein content of cyclin-B1 at M II stage oocytes. This proves that leptin plays a role in the maturation of nucleus and cytoplasm[10].

Due to contradictions of the two results of this study, it is necessary to carry out wider re-examination to other mammals than those studied by the two previous researchers. Leptin supplementation in IVM medium can improve the quality of oocyte maturation, so that it can cope with the decreased embryo quality resulting from in vitro fertilization and parthenogenetic embryo today.

METHOD

Oocyte in vitro maturation (IVM)

Oocyte aspiration of goat ovary was performed on follicles 2-6 mm in diameter[11]. The result of oocytes selection was transferred to 50 mm petri dishes containing washing medium three times (TCM-199 + 10%

***Corresponding Author:** Pieter Kakisina, Faculty of Mathematics and Natural Sciences Pattimura University-Ambon, Indonesia. E-mail: paet_kakisina@yahoo.com

FBS). Furthermore, good quality oocytes (quality A and B) were transferred into a 100 dropμL in culture dish. Each drop contains approximately 10 oocytes. Thereafter, it was incubated in a CO_2 incubator with 5% CO_2, in a temperature of 38 degrees C with maximum humidity. Successful IVM was assessed by observing the expansion of cumulus cells and the presence of MII oocytes of ways to shave the cumulus cells mechanically by repeated pipetting.

Analysis Data

Data were analyzed using two-way analysis of variance (ANOVA), and followed by post-hoc Tukey test with $\alpha = 0.05$, followed with Pearson regression test.

RESULTS

Oocytes assessment for IVM in this study was based on the morphology of oocytes according to Tanaka[12], namely: Quality A: round oocytes was evident, surrounded by whole cumulus oophorus more than two layers. Quality B: round oocytes was evident, surrounded by cumulus oophorus not full or in two layers. Oocytes used in this study can be seen in Fig 1. A, after IVM oocytes development showing little cumulus cell expansion was the quality one (K1) (Fig 1. B), while many cumulus cells that have expanded belonged to quality two (K2) (Fig 1. C).

To know that these oocytes had progressed to metaphase stage two (MII), the oocytes were shaved by repeated pipetting until the cumulus cells attached to oocytes became really loose and the oocytes became visibly denuded. Oocytes that had entered metaphase stage two are usually followed by the formation of the first polar body. Polar body is small cells part of karyokinetic process generated by the egg in oocyte maturation process, containing little or even no cytoplasm at all, but it has one set of chromosomes derived from meiotic process. Oocyte development after being cultured showed different expansion of cumulus cells according to the length of the incubation time, as shown in Fig 1.

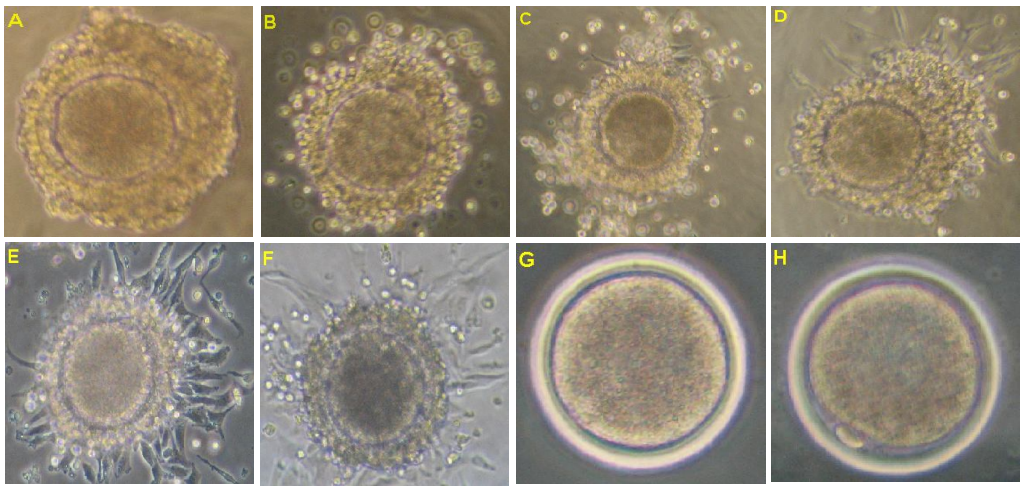

Fig.1. After oocyte maturation after being cultured.

Fig 1 is observations with olympus inverted microscope with different magnification. A. Oocytes before culture/0-hour, B. Cumulus cell expansion, C. Oocytes cultured after 18 hours, D. Oocytes cultured after 22 hours, E. Oocytes cultured after 26 hours, F. Oocytes cultured after 30 hours, G. Oocytes without M II , H. Oocytes with M II (A, B, C, D, E and F magnification 100x; G and H magnification 200x).

In-vitro maturation (IVM) success was marked by the expansion of cumulus cells surrounding the oocyte (Fig 1.B, C, D, E, F). There appeared to be differences between pre-cultured oocytes with cultured oocytes. Before culture, oocytes were surrounded by a compact and plated cumulus cells (quality A, B), whereas those had been cultured for 18 hours (Fig 1. C), 22 hours (Fig 1.D), 26 hours (Fig 1.E) and 30 hours (Fig 1. F) showed different cumulus cell expansion. Fig 1.G is already shaved oocytes and polar bodies appear there first, while Fig.1.H is oocytes with the first polar body.

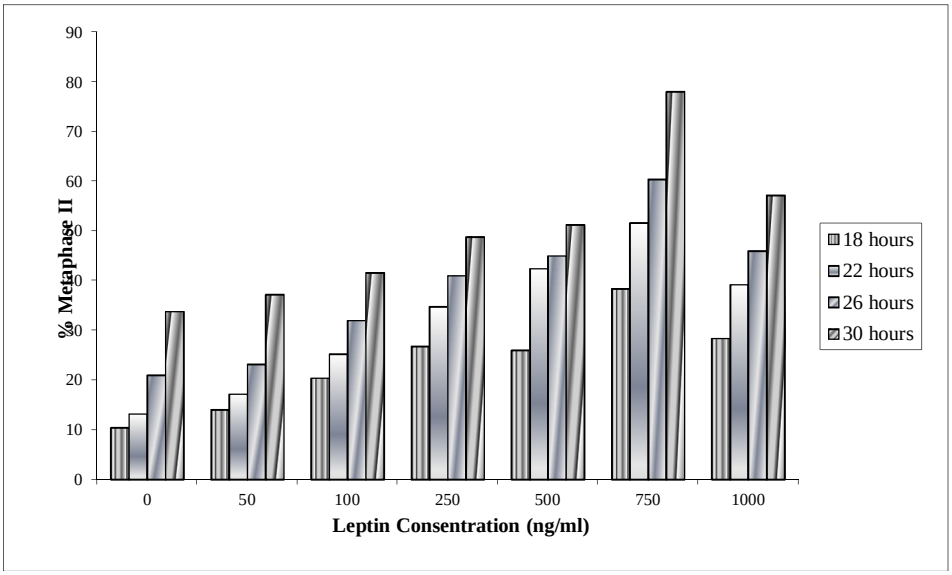

Fig. 2.Percentage of oocytes reaching metaphase II (M II).

Fig 2 is incubation at 18, 22, 26 and 30 hours with leptin concentrations of 0, 50, 100, 250, 500, 750 ng/ml showed an increase in the percentage of M II, whereas leptin concentration 1000 ng/ml at all incubation times showed a decrease in M II percentage.

The percentage of oocytes that reached MII in Fig 2 increased with leptin 50, 100, 250, 500 and 750 ng/ml, while higher leptin concentrations (1000 ng/ml) failed to stimulate oocyte to become mature due to the decline in the percentage of M II at all incubation times. The presence of leptin in the medium IVM causes oocytes to undergo progression to M II, with higher percentage than the mature ones without leptin and showed significant differences. In-vitro maturation (IVM) resulted oocytes with leptin supplementation at a concentration of 750 ng/ml with an incubation time of 30 hours showed the highest rate. Further test results using Tukey analysis showed that there were significant differences in the mean percentage of M II, except at a concentration of 500 ng/ml and 1000 ng/ml, no significant difference was found.

Based on data on M II percentage from four incubation times and seven concentrations, it was found that the highest mean percentage was the oocytes matured with leptin concentration of 750 ng/ml at 30 h incubation time, with a M II success rate was 78 ± 3.16 (Fig. 3). Then, 1000 ng/ml was 57.2 ± 1.79; 500 ng/ml was 51.2 ± 1.79; 250 ng/ml was 48.8 ± 1.79; 100 ng/ml of 41.6 ± 1,67; 50 ng/ml was 37.2 ± 1.1 and control 33.8 ± 1.1 .Fig 3 is concentration of 750 ng/ml showed the highest rate, followed by 1000, 500, 250.100, 50 and 0 ng/ml.

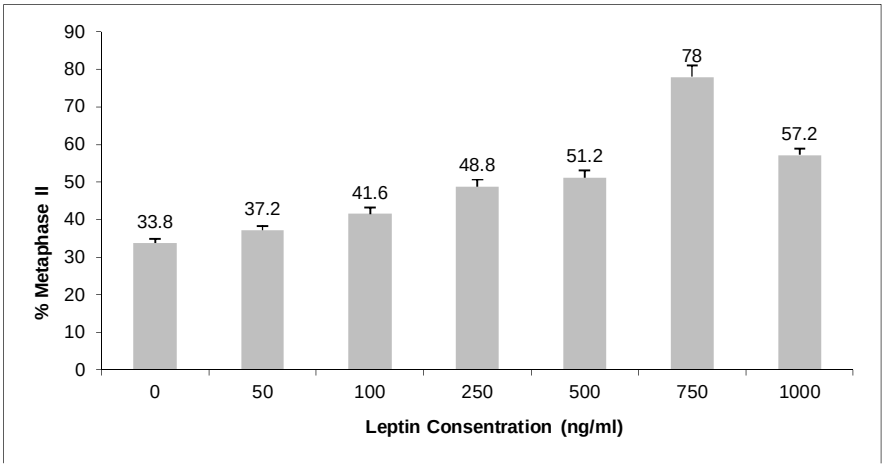

Fig 3. Effect of leptin concentration and incubation time to increase oocyte maturation resulting from in-vitro culture.

Results of two-way analysis of variance (ANOVA) showed that leptin administration with a concentration of 50, 100, 250, 500, 750 and 1000 ng/ml in TCM-199 medium had a significant influence on the increase of M II percentage in oocytes resulting from in-vitro culture with $p < 0.05$. Culture time also showed significant difference with $p < 0.05$. Furthermore, interaction between leptin concentration and culture time also showed a significant difference with $p < 0.05$ from the percentage of M II oocytes resulting from in-vitro culture.

Tukey test results to assess the effect of leptin concentration and incubation time on the percentage of M II in oocytes resulting from in-vitro culture, proved that the increase and decrease in M II percentage depended on the concentration given in TCM-199 medium.Tukey test results showed that leptin concentrations of 50, 100, 250, 500, 750 and 1000 ng/ml and controls showed a significant difference in the percentage of M II in oocytes resulted from in-vitro cultured with $\alpha = 0.05$. However, leptin concentration from 500 ng/mL to 1000 ng/ml showed no significant difference in $\alpha = 0.05$. To assess the relationship between leptin concentrations given with M II percentage we carried out pearson correlation analysis followed by regression analysis.

Pearson's correlation analysis showed that between X1 (concentration) and Y (percentage of M II) there was a very strong correlation between leptin concentrations and M II percentage. Then, between X2 (incubation time) and Y (percentage of M II) there was a strong correlation between culture time and the M II percentage. The overall significance test was based on modelsummary table, revealing R values of 0.880 with a probability value (sig. F Change) = 0,000 because sig. F Change value was $\alpha < 0.05$, leptin concentrations and incubation time simultaneously and significantly related to the percentage of the first polar body.

DISCUSSION

In general, oocytes cultured with the addition of leptin in TCM-199 medium + 10% FBS started showing cumulus cell expansion at the 18th hour, while those without leptin at 22nd hour. This means that leptin spur faster progress towards oocyte maturation. The observation of oocyte development in research was done visually through inverted microscope to observe the cumulus cell expansion. This is consistent with researchin goats [11,8], in cattle[7,6]. They found the development of in vitro maturation of oocytes with cumulus cell expansion observed as one of the indicators.

In this study, cumulus cell expansion was used as an indicator of the development of oocyte maturation because pre-ovulatory oocytes were surrounded by several cell layers, known as cumulus granulosa cells, which are closely related to oocytes through intercellular membrane process and gap junction that facilitates the exchange of glucose metabolites, ions and small signaling molecules[12]. Oocytes depends on cumulus cells because it serves to supply pyruvate and glucose metabolism in generating energy [13]. Cumulus cells are most directly exposed to mitogenic factors secreted by the oocytes [14].

In this study, cumulus cell expansion in goat oocyte was observed at 18th after culture. It is similar to bovine oocytes. Bovine oocyte cumulus cell expands after being incubated for 12 hours and up to 18 hours [15]. Cumulus cells affect oocyte maturation and nourish the oocytes because cumullus cells oophorus complex (COC) is able to communicate with the oocyte through microvilli that penetrate through the zonapellucida. Oocytes cultured without cumulus cells will undergo maturation failure or impaired maturation rate[16]. Based on this, this study used only oocytes with cumulus cells surrounding the oocytes more than one layer (Fig 1A).

The success of oocyte maturation also depends on the length of time of oocytes incubation. Time variation was very specific to each species. The length of incubation varies between 20 to 30 hours[17]. The best bovine oocyte maturation time is 24 to 30 hours[15] .Bovine oocyte maturation incubation time is maximally 31 hours, while the minimum incubation time is 20 hours[17]. Goat oocytes incubated in TCM-199 IVM medium supplemented with estrous cow serum (ECS) has the optimum maturation time of 30 hours[18]. Incubation time in this study 18, 22, 26 and 30 hours, which was still within the range of the time.

The results showed that the incubation time of 30 hours was the optimum time of goat oocyte maturation with rate of success in achieving M II as characterized by the formation of the first polar body of 78%. Some oocytes did not reach metaphase II perfectly up-to 30 hours due to varied oocytes condition during collection from each aspirated follicle and diverse oocytes size for IVM. The diversity was because oocytes were surrounded by layered cumulus cells, making it difficult to select the oocytes of the same size. One of the parameters of success for oocytes to reach metaphase II stage which can be observed directly is the presence of first polar body (PB I) because PB I appearance is a sign of achievement of oocytes maturation. Polar body is a product of meiosis during maturation. Oocytes that have reached the metaphase II stage is ready to be fertilized.

The addition of leptin in TCM-199 medium with concentrations of 50, 100, 250, 500 and 750 ng/ml at all observation time were able to induce oocytes to reach metaphase II as marked by the emergence of M II, resulting in an increase in the percentage of M II (Fig 5.2). These results are consistent with the research of Matsuoka[19] in mice, that leptin may affect oocyte maturation because it can activate signal transduction

activator of transcription 3 (STAT3). In cultured follicles, leptin significantly increases oocyte maturation. This suggests that leptin works indirectly through the theca cells to improve oocyte maturation [9].

High leptin concentrations 1000 ng/ml in IVM medium (TCM-199) appeared to reduce the percentage of oocytes reaching M II. This is because in high levels of insulin-like growth factor-I (IGF-I), the effect of FSH sensitization enhances progesterone and estradiol production from granulosa cells, but high levels of leptin can block the stimulatory effect of IGF-I in granulosa cells[20,21] suggested that the increased secretion of estradiol receptors induces luteinizing hormone (LH) which is the signal for the LH hormone secretion. LH secretion can inactivate gap junction proteins (Connexin 37 and 43) so that cAMP transfer into oocytes is inhibited. This causes a decrease in oocyte cAMP levels. The event will induce oocyte meiotic maturation[22].

Provision of leptin in the culture medium TCM-199 can improve oocyte maturation because leptin has a transmembrane receptor in oocytes that can activate mitogen-activated protein kinase (MAPK) pathway. Mitogen-activated protein kinase is a signal transduction pathway believed to play an important role during vertebrate oocyte meiotic maturation, because microinjection of antisense c-mos RNA, upstream MAPK kinase and suppression of MPF activity in swine oocytes showed the role of MAPK in increasing MPF activity [23,24]. Oocyte maturation involves activation of various signal transduction pathways that activate maturation-promoting factor (MPF) consisting of cyclin-B and Cdc2 kinase[25].

CONCLUSION

The addition of leptin in the medium TCM-199 + 10% FBS improves goat oocyte maturation in reaching M II. The optimum concentration of leptin able to increase the percentage of goat oocytes to reach M II is 750 ng/ml. Whereas, high leptin concentrations of 1000 ng/ml inhibits the oocytes to reach M II. In addition to leptin concentrations, incubation period also affects and is strongly correlated to M II percentage in goats oocytes resulting from in-vitro culture. Leptin concentration and incubation time clearly increases oocyte maturation. Further study is needed to explain leptin mechanism of action in improving oocyte maturation.

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