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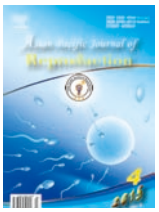

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BMP-15 m-RNA expression of mouse oocytes *in vitro* maturation in different droplet medium volume

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ABSTRACT

Objective: To investigate droplet medium volume effect on the BMP-15 mRNA expression. **Methods:** Oocytes are collected from mice ovaries by puncturing with a sterile 26-G needle. The droplet medium volumes are using 50 μL, 100 μL and 200 μL. The BMP-15 mRNA expression is determined in each group. **Results:** The results indicated that BMP-15 mRNA expression did not significantly differ when oocyte were cultured in 50 and 100 μL/droplet medium volume, but significant difference ($P < 0.05$) was found when oocytes were cultured in 200 μL/droplet medium volume. **Conclusions:** The highest BMP-15 m-RNA expression occur when oocytes are cultured in 200 μL/droplet medium volume.

1. Introduction

Follicle development (folliculogenesis) is a complex process that associated to the development of a follicles group at various stages of maturation. These processes depend on the interaction between hormone signals and ovarian growth factors. Granulosa cells differentiation and proliferation are the character of folliculogenesis. In addition, enlargement of oocytes also occur during folliculogenesis[1-3]. Oocytes secrete growth factors that have an important role in the folliculogenesis regulation and promotion, the action via paracrine signaling[4]. There are two important oocyte-secreted growth factors, also namely as OSF, bone morphogenetic protein-15 (BMP-15) and growth differentiation factor-9 (GDF-9[5]. mRNAs and proteins BMP-15 was detected at all phase in goat ovarian follicles development[6].

BMP-15 or GDF-9B was a member of the transforming growth factor- β (TGF- β)superfamily, played an important role for normal female fertility in mammals[7]. It had been reported that BMP-15 was expressed in an oocyte [1, 7, 9] and acted through receptors located in granulosa cells[10]. These protein played some key roles in promoting follicle growth [11, 12],cumulus cells expansion [13, 14]; the expression of connexin43 (Cx43) for intercellular communication [15] and promote glycolisis in cumulus cells[16]. BMP-15 level in follicular fluid appeared to be a potential factor in oocyte quality, implantation and pregnancy in human [17, 18]. In hen, BMP-15 inhibited progesterone production of granulosa cells, decreased in steroidogenic acute regulatory protein (STAR) level[19] and altered decreasing of corpus lutea number and litter size in mice[20]. Mutation of BMP-15 gene caused premature ovarian failure (POF) and POI(Primary Ovarian Insufficiency) in human [21-23].

In vitro maturation (IVM) of oocytes is widely used in assisted

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reproduction technologies in animal, and is increasingly used to treat human infertility. In the *in vitro* condition, oocyte gene expression is influenced by media culture condition. Media with serum increased the IGF2 (Insulin-Like Growth Factor-2) [24]. Another publication showed that culture medium affected the IGF2, LIF gene expression and apoptosis index [25]. The present study aimed to investigate the effect of different volume media culture on the oocytes m-RNA BMP-15 expression.

2. Materials and methods

2.1. Oocyte collection and in vitro maturation

Twenty-one-day-old mice were injected with 5 IU of pregnant mare serum gonadotropin (PMSG, Serotropin; Asuka, Tokyo, Japan), and ovaries were collected 46 h afterward for oocytes collecting. All animals were housed under controlled humidity, with a 12-hr light and 12-hr dark phase, temperature, and fed ad libitum. All experiments were conducted in accordance with the guide for the care and use of laboratory animals.

Ovaries were washed with Leibovitz's L-15 medium (Invitrogen, Grand Island NY) contained 4 mM hypoxanthine. COCs (Cumulus-oocyte complexes) were isolated by puncturing follicles with 26-gauge needles and collected in L-15-Medium/BSA. Only COCs with a uniform covering of compacted cumulus cells were used in the study. Furthermore COCs were culture in Waymouth's medium + 4 mM hypoxanthine for 18 hr at 37 ℃, in humidified atmosphere of 5% CO_2 to achieve metaphase II (M II) oocytes. Twenty (20) COCs were cultured in 3 group droplet volume medium which were 50 μL/droplet, 100 μL/ droplet and 200 μL/ droplet respectively under paraffin oil.

2.2. mRNA extraction, cDNA synthesis and analysis of mRNA expression

Fifty (50) μL hyaluronidase (Sigma) was added into the IVM culture dish to remove the cumulus cell from oocytes, slowly pipetting until cumulus cells free from oocytes. Metaphase II oocytes (20 oocytes) were transfer to the bottom of a tube containing 50 μL of cell lysis buffer. Total RNA from the three oocyte groups was isolated in parallel and guided by the manufacturer's instructions. To avoid contamination of genomic DNA, total RNA preparation were treated with DNase. The RNA quantity and quality were evaluated by spectrophotometer at 260/280 nm. The samples were stored at -80 ℃. Single-strand cDNA was reverse transcribed from 1 μg of RNA template using 1st strand cDNA Synthesis Kit. BMP-15 mRNA expression was quantified by PCR. The G3PDH (glyseraidehyde-3-phoaphate dehydrogenase) was used as intrinsic control. PCR was performed using rTaq polymerase (Takara, Shiga, Japan) and using primers 5-AGCAACCAGGTGACAGGA-3, and antisense primer, 5-CCTCCTTTACCAGGTCA-3 for BMP-15. DNA Primer for G3PDH was 5- CCACTCTTCCACCTTCGATG-3 and 5-GAGGGAGATGCTCAGTGTTG-3. The amplifications conditions was an initial denaturation step of 94 ℃ (5 min); followed by 35 cycles of 94 ℃(20 s), 56 ℃(45 s) for BMP-15 and 58 ℃ (20 s) for G3PDH and extension 72 ℃(60 s) and final extension at 72 ℃ for 3 min. The expression of mRNA was analyzed by Image J 1.47 program.

2.3. Statistic analysis

The data were analyzed using test of variance (ANOVA) technique with $P<0.05$. Tukey HSD test was used to compare the means. Both tests were conducted using SPSS 16 for Windows.

3. Results

3.1. Oocyte in vitro maturation

To analyze whether different droplet volume medium affect mRNA BMP-15 expression, we performed IVM under three different medium volumes (50 μL, 100 μL and 200 μL/droplet). The maturity of the oocytes was evaluated by observing first polar body. Oocyte was evaluated 18 h after incubation. The results of the study demonstrated that all of the oocytes reached the MII oocytes (Figure 1).

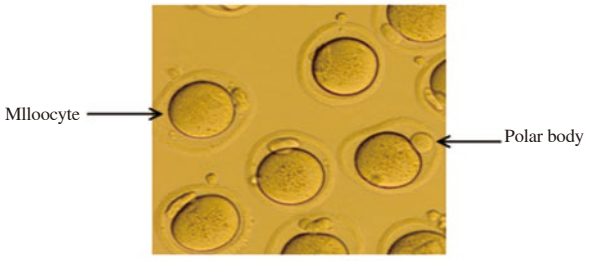

Figure 1. Moorphologial of MII Oocytes obtained from oocytes *in vitro* maturation.

3.2. BMP-15 m-RNA expression

We evaluated the effect of droplet volume medium on BMP-15 mRNA expression. Twenty (20) normal oocytes from each group were used for the analysis of the BMP-15 mRNA expression. As shown in the Figure 2, BMP-15 mRNA was expressed in the entire droplet volume medium. Relative abundance of BMP-15 mRNA expression did not significantly difference between 50 and 100 μL/ droplet medium, but displayed significantly higher ($P<0.05$) when oocytes were cultured in 200 μL/droplet medium. Thus, the highest BMP-15 m-RNA expression obtained when oocytes were cultured in 200 μL/droplet medium (Figure 3).

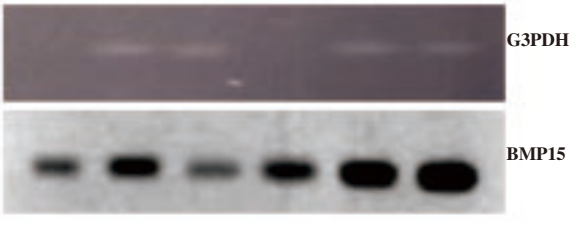

Figure 2. mRNA expression of BMP-15 in oocyte by PCR

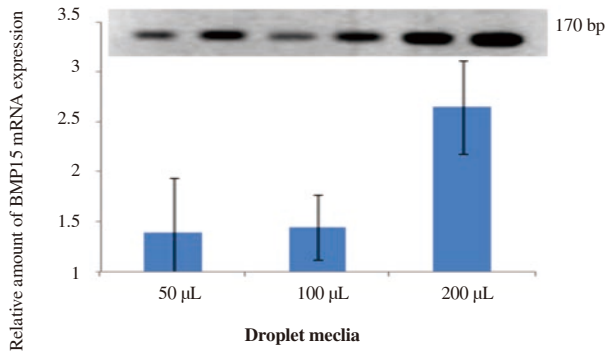

Figure 3. Relative mRNA levels of BMP-15 expression.

4. Discussion

This study demonstrated that different volume media droplet for oocytes *in vitro* maturation not affect to achieve MII oocytes because the Waymouth's medium contained components needed for oocyte maturation. The components media in the different volume media (50, 100 and 200 µL/droplet medium) would sufficient amount for oocytes maturation. Oocytes maturation rates was higher when oocytes was cultured in the 50, 100 and 200 µL/droplet medium than in the 20 µL/droplet medium[26]. In addition to droplet volume medium, oocytes collection method also had important role to achieve MII oocytes. In this study, we used puncturing method for COCs collection. Aspiration method was better to achieve MII oocyte than slicing method in black Iraqi goat[27] and buffalo[28]. Different studies had reported that there was no significant difference in the achievement of MII oocytes when oocytes were collected by slicing or aspiration method in goat[29, 30].

In mice, BMP-15 was secreted by oocyte and increase during follicular development and oocyte maturation [31, 32]. The functions of this protein were promoting biosynthesis of cholesterol in cumulus cells [33] and cumulus cell expansion [32]. There was no difference in the BMP-15 gene expression between in vivo and *in vitro* maturation of mice oocyte [34, 35]. We evaluated the effect of volume media on BMP-15 gene expression. Twenty (20) normal oocytes from each group were used for the analysis of BMP-15 mRNA expression. Figure 2 shows that the relative abundance of BMP-15 mRNA expression did not significantly differ between 50 and 100 µL/droplet media, but the expression displayed significantly higher ($P< 0.05$) when oocyte were cultured in 200 µL/droplet media. The highest BMP-15 m-RNA expression was obtained when oocytes were cultured in 200 µL/droplet medium (Figure 2). Components of media that used in this study consisted of amino acids, monosaccharide (galactosa), vitamins, inorganic salts and sodium pyruvat. The function of pyruvat was as an energy substrate and antioxidant [36]. Thus, in the 200 µL/droplet medium the higher volume media were more nutrient and oxygen to support oocyte activity. The culture atmosphere played a critical role during oocyte maturation *in vitro* [37]. Optimal volumes of Ham's F10 medium for early embryo development of mouse were 50 to 100 µl droplets [38]. Higher blastocyte cell number was achieved in 40 µL drops media than 5, 10, and 20 µL drops [39]. Component in the media had effect on gene expression. Different component media for IVM effected on the gene expression[25]. The serum addition in media for IVM increased Leukemia Inhibitory Factor (LIF) gene expression.

In conclusion, the result indicates that droplet medium volume give effect on BMP-15 mRNA expression. There is no difference expression when oocytes are cultured in 50 and 100 µL/droplet medium volumes. The highest BMP-15 mRNA expression is obtained when oocytes are cultured in 200 µL/droplet medium volume.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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