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Production and Characterization of Mouse Diploid Parthenogenetic Blastocyst Developed in Phosphate-Free Medium

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ABSTRACT

Parthenogenesis is an artificial oocytes activation process without paternal contribution. Blastocyst, derived from parthenogenesis, is one of potential source for pluripotent stem cell propagation. Unfortunately, previous studies reported that parthenogenetic embryo did not achieve exhilarating blastocyst rate. One of the component that predicted inhibit parthenogenetic embryo development is phosphate. Therefore, we try to modify culture medium in order to overcome that problem. The aim of this research was to produce and analyze the characteristics of parthenogenetic blastocyst developed in phosphate-free medium. Mouse oocytes obtained from adult female DDY by superovulation. The activator was strontium chloride 10 mM and diploidization with cytochalasin B 5 µg/ml. Medium for activation and culture medium were modified rat 1 cell embryo medium (MR1ECM) which is phosphate free. The results showed that parthenotes that were cultured in phosphate free medium reached higher blastocyst rate compared to the other groups. The increase of phosphate in culture medium lead to impaired parthenogenetic embryos development. Further experiment was made to analyze the differences between fertilized and parthenogenetic embryo in this medium. The experiment showed that diploid parthenogenetic could achieve high blastocyst rate (30.9±1.3%). The quality of diploid parthenogenetic blastocyst, based on cells number, viability, and ICM ratio, was lower than fertilized blastocyst.

1. Introduction

Embryonic stem cells (ESCs) comes from early embrional stage known as blastocyst. At this stage, embryo differentiates into two distinct lineages, trophectoderm and inner cell mass (Kidder 2014). The trophectoderm posseses epithelium characteristics and further develop and form extraembryonal tissue (Marikawa and Alarcon 2009). On the other hand, inner cell mass potentially capable to give rise to the three primary germ layer that later become all types of body cells. Therefore, stem cells derived from inner cell mass have pluripotency properties and can be classified as pluripotent stem cells.

Since the first invention of mouse ESCs in 1981 by Evans and Kaufman, then method for culturing human ESCs in 1998 by Thomson and Gearhardt, there are a lot of controversy related to this cells propagation (Robertson 2010). Most people against ESCs because they convice that embryos is a new life that has the same rights, moral, and legal status as an adult individual (Power and Rasko 2011). Hence, some countries prohibited ESCs research and restricted its funding.

An alternative source for pluripotent stem cells instead of fertilized embryo is parthenogenetic embryo (Baharvand 2009). Parthenogenesis is an artificial oocytes activation that lead to early embryonic development without fertilization by sperm (Paffoni *et al.* 2008). The discovery and the cellular mechanisms that occurs when sperms penetrate oocyte in the fertilization process become the basis knowledge of the development of parthenogenetic methods (Boediono *et al.* 1995). Unfortunately, parthenogenesis produces

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low number of good quality blastocyst, whereas blastocysts are the main material for stem cell propagation (Krivokharchenko *et al.* 2003).

One of the alleged element associated with blastocyst rate is phosphate. Haraguchi *et al.* (1996) reported that phosphate caused a negative effect on *in vitro* culture of AKR/N mice fertilized embryos, removal of phosphate elements was significantly improved the blastocyst rate up to 42.6%. The effects of phosphate also became an interesting finding in the study of Popova *et al.* (2011) that reported mouse fertilized embryos could well developed in modified medium rat 1 cell embryo medium (MR1ECM) which did not contain any phosphate. The effect of phosphate on *in vitro* culture of mouse parthenogenetic embryo has not been clear. Moreover, the characteristic of blastocyst derived from parthenogenesis in phosphate-free medium still not yet clear. Hence, the aim of this research was to produce and analyze the characteristics of diploid parthenogenetic blastocyst developed in phosphate free medium.

2. Materials and Methods

2.1. Animals

Adult female DDY mice were used in this study. Mice were housed under controlled temperature of 22-25°C and were kept on 12 h light conditions (06.00-18.00). Food and water supplied *ad libitum*. Experimental procedures of this research was approved by Animal Care and Ethics Committee of National Institute of Health Research and Development, Ministry of Health, Republic Indonesia (No:LB02.01/2/KE.150/2017).

2.2. Superovulation, Oocytes and Fertilized Embryos Collection

Female mice were induced to superovulation by intraperitoneal injection of 5 IU PMSG (Folligon®, Intervet, Netherland) followed by 5 IU hCG (Chorulon®, Intervet, Netherland) 48 h later. Mice were sacrificed 16 hours after hCG administration to collect cumulus oocytes complex. Hyaluronidase 0.1% (w/v) was used to remove cumulus cells. Metaphase-II oocytes were used for parthenogenesis. To obtain fertilized embryos for experiment 2, after hCG administration female mice were mated overnight with DDY males, then on the following morning the mice were examined for the presence of vaginal plugs. Handling medium was M2 medium (Sigma, St. Louis, USA).

2.3. Parthenogenesis

Oocytes were incubated for six hours exposure time in modified MR1ECM medium containing 10 mM strontium chloride ($SrCl_2$) (Sigma) and 5 µg/ml cytochalasin B at 37°C under 5% CO_2. The efficiency of activation was analyzed 6 h after treatment. Culture medium used in this study refers to the modified rat 1 cell embryo medium (MR1ECM) formulation reported by Popova *et al.* (2011).

2.4. Experiment 1: Effect of Phosphate on the Parthenogenetic Embryos Development

Six hours after activation, activated oocytes which had two pronuclei were cultured in phosphate-free medium (counted as 0 h). Parthenotes were washed three times then divided into three groups with different phosphate concentration: no phosphate additives; 1 and 2 mM phosphate. Embryos were cultured at 37°C and 5% CO_2 in drops of culture medium covered with mineral oil (Ovoil™, Vitrolife, Sweden). Embryos were examined at 48 and 96 h. One way ANOVA continued with Duncan test were used for data analysis.

2.5. Experiment 2: Comparison of Parthenogenetic and Fertilized Embryo Development in Phosphate-Free Medium

Female mice with positive vaginal plugs after mating were sacrified then the zygotes were collected 18 h after hCG administration. Zygotes collected in PBS + 10% FBS, then were washed three times in MR1ECM. Fertilized embryos and diploid parthenogenetic were separately cultured in drops of MR1ECM, phosphate-free culture medium, at 37°C and 5% CO_2. Embryos were examined at 48 and 96 h. *Student t-tests* were used to evaluate statistical differences of the parthenogenetic embryos development, cells number and viability of the blastocyst, and the data from ICM/blastocyst ratio measurement using Image-J software between the groups. A $p<0.05$ was considered significant.

2.5.1. Hoechst-Propidium Iodide (HPI) Staining

HPI staining was performed to determine the average number of cells and the rate of living or dead cells of the fertilized and parthenogenetic blastocyst. Blastocysts were incubated for 20 min at 37°C and 5% CO_2 in a 10 µg/ml Hoechst staining (Bisbenzimide H 33342, Sigma) and 10 µg/ml propidium iodide (Wako, Ozaka, Japan) then transferred to drop of PBS + 10% FBS and placed on an object glass gently covered with a cover glass. Stained blastocysts were

observed under fluorescent microscope at 330-365 nm for Hoechst 33342 dye and 540-608 nm for propidium iodide (Eclipse E 6000, Nikon, Japan). The NIH Image-J application used for counting the total cells and live cells (blue) or dead cells (red).

2.5.2. Image-J Measurement

Twenty five digital images of the fertilized and parthenogenetic blastocysts were taken in sequence of 3 images on different focal planes, magnification 200x. The ratio between ICM diameter and blastocyst diameter were calculated using Image-J open source software after calibration with microscope micrometer calibration ruler. The ICM diameter was measured between the apex of the ICM and its base on the innerborder of the blastocyst (Almagor *et al.* 2016). The blastocyst diameter defined as the distance between the outside borders of the TE.

3. Results

Chemical activation was used in this study to induce parthenogenesis. Strontium chloride was the activator while cytochalasin B was selected for diploidization. Activation can be done and able to produce diploid embryo with 6 hours exposure time as shown in Figure 1.

The result of the first experiment of this study showed that mice parthenogenetic embryos could develop into blastocyst stage and reached higher levels of development rate in phosphate-free culture medium. Inhibitory effect occured along with the addition of phosphate. There were no blastocysts in concentration of 2 mM phosphate in the culture medium as shown in Table 1.

Experiment 2 was conducted to compare the development and quality of parthenogenetic and fertilized blastocyst. The results showed that fertilized embryos developed better than parthenote. Blastocyst rate of fertilized embryo was 45.6%, higher than parthenogenetic embryo that reached 30.9% blastocyst rate. There were also a higher proportion of the parthenogenetic embryos which were arrested or grew slower at morula stage as shown in Table 2.

Hoecht-PI staining was performed to count the total cells number and to calculate the viability of the blastocyst. Parthenogenetic blastocyst has lower cell

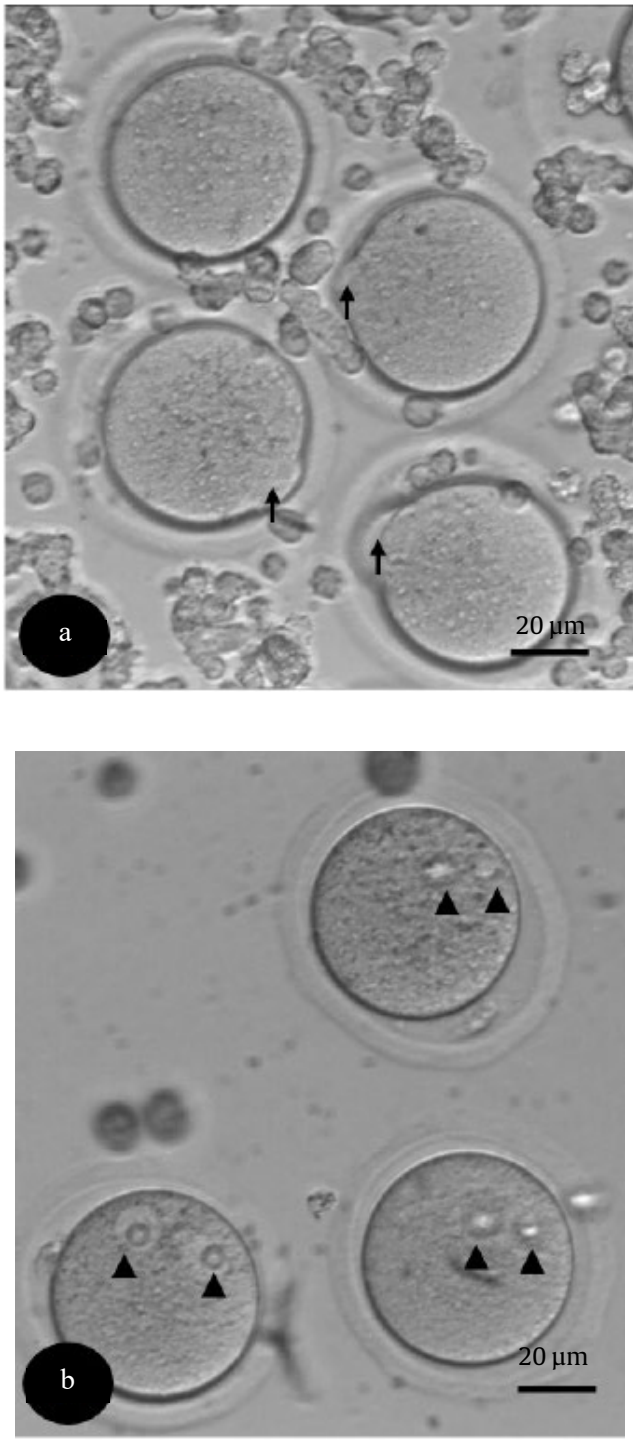


Figure 1. Diploidization of parthenogenetic mice oocytes (a) oocytes on metaphase II indicated by first polar body, prior to activation (black arrows: first polar body), (b) diploid parthenote with two pronuclei (black pointing triangle) after 6 hours exposure time in activation medium

Table 1. Effect of phosphate on the parthenogenetic embryos development

Phosphate concentration	Total No. of 2PN embryos	No. of embryos develop into (%)			
		2-cells	4-cells	Morula	Blastocyst
0	30	26 (86.6±1.3)	18 (59.8±3.8)[a]	13 (46.4±0.4)[a]	9 (29.9±1.9)[a]
1 mM	34	29 (85.6±2.9)	20 (53.1±2.9)[b]	6 (18.1±3.6)[b]	2 (9.4±2.4)[b]
2 mM	37	32 (86.8±1.8)	16 (43.4±0.9)[c]	3 (8.5±2.3)[c]	0 (0)[c]

Three replicated trials were carried out. Percentages of embryos are based on number of total 2PN embryos and are expressed as mean+SEM. [a, b, c]Values with different letters within a column indicates significant differences, $p<0.05$

Table 2. *In vitro* development of parthenogenetic embryo and fertilized embryo in modified rat medium (MR1ECM)

Group	Total No. of 2PN embryos	No. of embryos develop into (%)			
		2-cells	4-cells	Morula	Blastocyst
Parthenogenetic	71	63 (89.4±2.7)[a]	42 (59.6±1.8)[a]	34 (47.6±1.2)[a]	22 (30.9±1.3)[a]
Fertilized	70	64 (91.2±0.4)[b]	50 (72.1±1.1)[b]	38 (54.4±0.2)[b]	32 (45.6±0.2)[b]

Three replicated trials were carried out. Percentages of embryos are based on number of examined zygotes and are expressed as mean+SEM. [a, b]Values with different letters within a column indicates significant differences, $p<0.05$

numbers (51.3±2.9) compared to fertilized blastocyst (86.4±1.8). Besides, the percentage of live cells that indicated the viability of the parthenogenetic blastocyst was also lower than the fertilized as shown in Table 3. Stained blastocyst observed under fluorosence microscope can be seen in Figure 2.

Based on morphometrical analysis, diameter of fertilized blastocysts was 99.05±0.56 and not significantly different with parthenogenetic blastocyst with average of diameter 99.91±0.48. Nonetheless, the ratio of ICM-blastocyst of parthenogenetic blastocysts still lower than fertilized blastocyst as shown in Table 4. The measurement of blastocyst in NIH Image-J demonstrated in Figure 3.

4. Discussion

Parthenogenesis is a reproductive strategy for some species of the classes of insects, pisces and amphibians. In those reproductive strategy, embryos will develop without fertilization by sperm so that paternal contributions are not involved in the parthenogenesis process (Paffoni *et al.* 2008). Parthenogenesis does not occurs in the natural reproduction of mammals but some research found that oocyte can be artificially activated by mimicking fertilization mechanism.

Activation of the oocytes in the fertilization process is due to an increase of intracellular calcium. During fertilization, sperm penetrates the oocyte and induces intracellular calcium oscillation to activate the oocytes. The sperm factor phospholipase ζ (PLCζ) facilitates the hydrolysis of phosphatidylinositol 4.5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3). This event leads to calcium influx from reticulum endoplasmic as an intracellular calcium deposits (Saunders *et al.* 2002; Swann *et al.* 2004; Heytens *et al.* 2009). There will be inactivation of maturation promotion factor (MPF) and mitogen activated protein kinase (MAPk) following calcium influx, which promotes meiosis, DNA synthesis and pronucleus formation (Paffoni *et al.* 2008).

Artificial activation by chemical compounds can be done using some chemicals such as Ca^{2+} ionophores, ethanol 7%, strontium chloride, phorbol ester, ionomycin and thimerosal. Strontium chloride induces a recurrent increase by unbound the calcium of the oocyte and induce multiple oscillations from endoplasmic reticulum. Strontium is known as a very effective activator agent in mice oocytes (O'Neill *et al.* 1991) and could activated the mice oocytes after nuclear transfer (Wakayama *et al.* 1998). In order to produce diploid embryos from parthenogenesis mechanism, cytochalasin B is needed to prevent the second polar body extrusion.

Popova *et al.* (2011) published that development of early preimplantation embryos is altered by different *in vitro* culture conditions including medium composition. Experiment 1 showed that there was an inhibitory effect caused by phosphate addition in the culture medium started at 4 cell stage.

Table 3. Comparison of total cells number, percentage of live and dead cells from parthenogenetic blastocyst and fertilized blastocyst

Blastocyst	Cell numbers	Live cells (%)	Dead cells (%)
Parthenogenetic	51.3±2.9[a]	40.3±2.9 (78.7)[a]	10.9±2.1 (21.3)[a]
Fertilized	86.4±1.8[b]	78.8±1.5 (91.2)[b]	7.6±0.82.1 (8.8)[b]

Cell numbers are expressed as mean+SEM. [a, b]Values with different letters within a column indicates significant differences, p<0.05

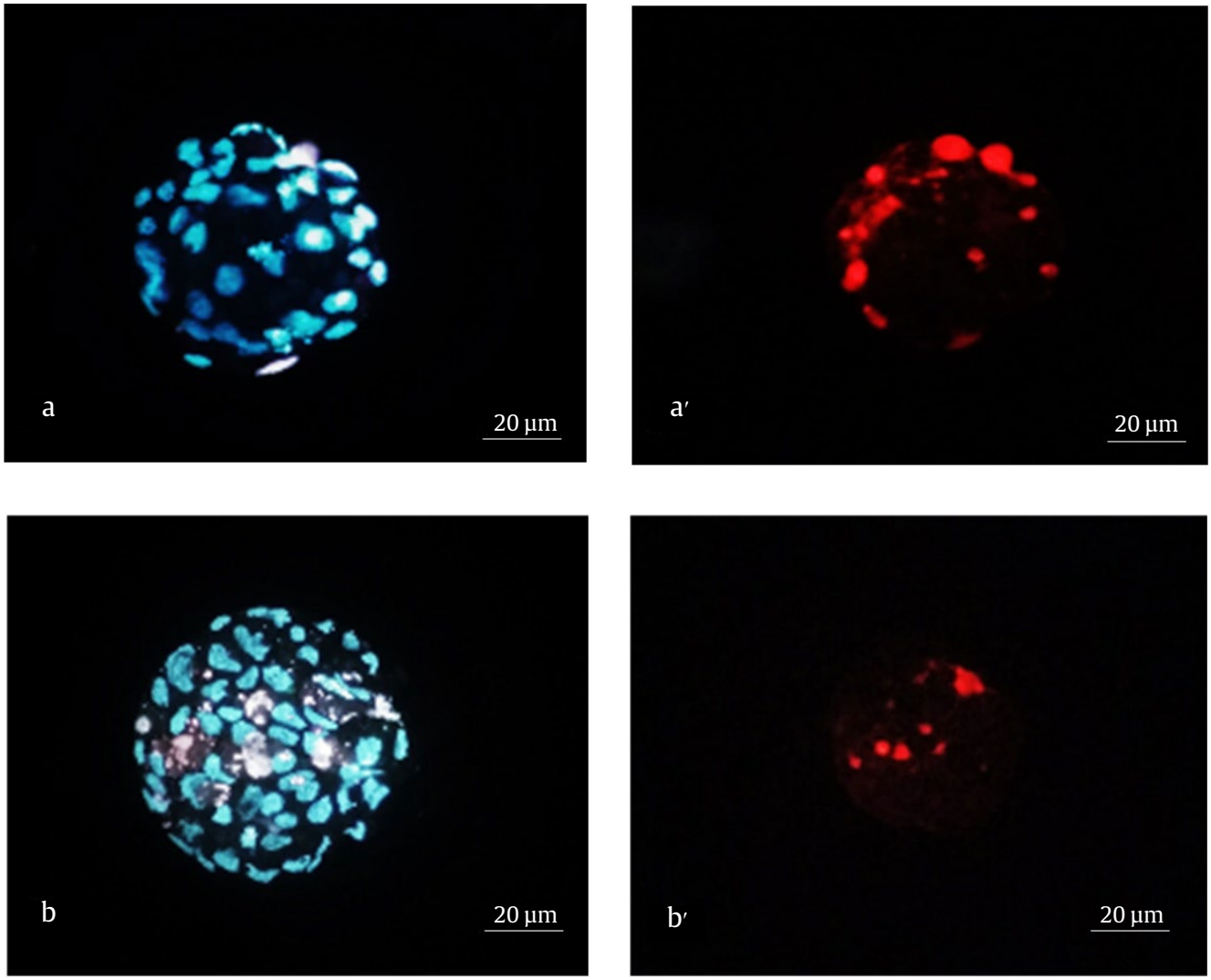
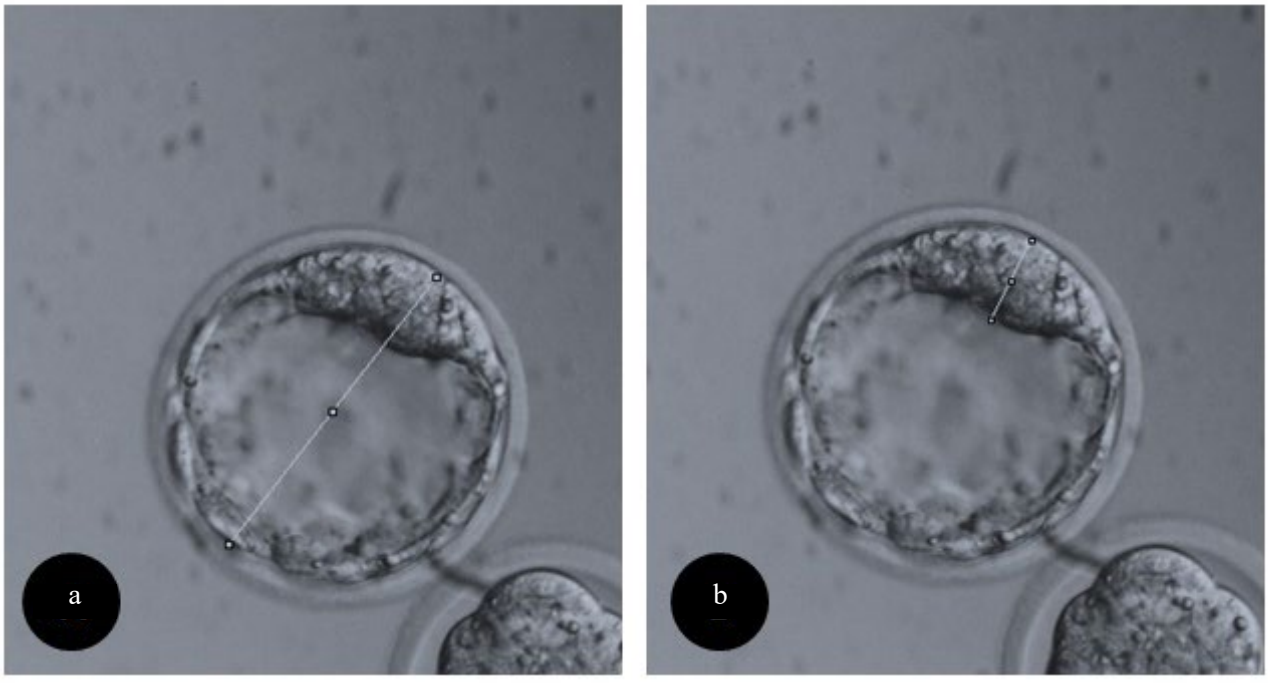


Figure 2. Hoechst-propidium iodide staining (a) live cells of parthenogenetic blastocyst, (a′) dead cells of parthenogenetic blastocyst, (b) live cells of fertlized blastocyst, (b′) dead cells of fertilized blastocyst

Table 4. Morphometrical characterictic of fertilized and parthenogenetic blastocyst developed in phosphate-free medium

	n	ø Blastocyst (µm)	ø ICM (µm)	Ratio ICM/Blastocyst
Parthenogenetic blastocyst	25	99.91±0.48	20.41±0.41[a]	0.21[a]
Fertilized blastocyst	25	99.05±0.56	31.94±0.58[b]	0.32[b]

Diameter (ø) are expressed as mean+SEM. [a, b]Values with different letters within a column indicates significant differences, p<0.05



Results

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	Area	Mean	StdDev	Min	Max	Angle	Length
1	16.778	135.087	20.023	72.061	175.619	56.070	99.931
2	4.461	124.173	33.936	75.852	200.938	62.949	26.519

Figure 3. Examples of simple method blastocyst morphometrical measurement in one focal planes using NIH Image-J (a) blastocyst diameter, (b) ICM diameter

Haraguchi *et al.* (1996) reported that there was significant decrease in the development of AKR/N mouse to 4-cell stage embryo with 0.001 mM phosphate and embryos did not reach morula stage in concentration of 1.0 mM phosphate. These phenomenon of sensitivity to phosphate were also reported found in rat embryos (Matsumoto and Sugawara 1998) and hamster embryo culture (Barnett and Bavister 1996).

The inhibitory effect of the presence of phosphate could be related to maturation promoting factor (MPF) activity. It has been reported that MPF plays an important role and control cell division activity (Yu *et al.* 2008). This kinase promotes the cell to enter the M phase. The presence of phosphate in culture medium may prevents second cleavage by inhibit the activation of MPF in the second cell cycle. The mechanism of MPF activity entails cyclin B synthesis and dephosphorylation of cdc2.

Experiment 2 was conducted to compare fertilized and parthenotes development in phosphate free medium. The results showed that fertilized embryo

developed better as indicated by higher development rate from cleavage stage and morula to blastocyst stage compared to parthenogenetic blastocyst. We predicted that it has correlation with different mechanism on embryonic genome activation.

Naturally, there will be a control transition from maternal genome to embryonic genome which is the first critical events in the life of the new organism (Latham and Schultz 2001). Previous study explained that genome activation in the mouse started at the 2-cell stage, with a minor activation event at the early 2-cell stage and a major activation event at the late 2-cell stage (Flach *et al.* 1982). The transitions of genomic control are needed to provide transcription factor that are required for DNA replication and cell cleavage (Jukam *et al.* 2017). Unlike fertilized embryo, parthenotes lack paternal factor so that there will be no transitions of genome control from maternal to zygotic genome activation. Because of that reason, parthenotes will not develop as good as fertilized embryos.

The blastocyst rate in modified phosphate free medium showed better results compared to previous studies. Hine *et al.* (2009) reported that the development rate of parthenogenetic embryos to blastocyst stage in KSOM medium was 13.30% while in CZB medium 6.11%. Murti *et al.* (2014) published 8.6% parthenogenetic development rate in CZB medium with 5.55 mM D-glucose. We analyzed and found that there was different phosphate concentration between KSOM and CZB medium. KSOM medium has a lower phosphate content compared to CZB medium. The KSOM medium contains 0.35 mM phosphate while the phosphate concentration in CZB medium is 1.18 mM (Nagy *et al.* 2003). This is strengthen the indication of the negative effects of phosphate on the development of parthenogenetic embryos.

One of the potential prospect of parthenogenetic blastocyst is to be used as an alternative sources for pluripotent stem cells (Daughtry and Mitalipov 2014). To reach this goal, we have to ensure that we did not only elevate the development rate of parthenogenetic blastocyst but also improve the quality of the blastocyst. Because of this reason, we counted mean cells number and the viability of the blastocyst by hoechst-pi staining. We found that parthenogenetic blastocyst had lower cell number compared to fertilized blastocyst. This is because the number of dead inner cell mass and trophoblast cells was significantly more in parthenogenetic embryos than in fertilized one (Niimura *et al.* 2002). Unfortunately, stained blastocyst can not be used to derivate parthenogenetic embryonic stem cells. Therefore, this study elaborate a noninvasive method to estimate the proportion of inner cell mass of the parthenogenetic blastocyst using digital images measurement approach. Almagor *et al.* (2016) reported that inner cell mass diameter and blastocyst diameter is correlated with successful pregnancy outcomes of single blastocyst transfers. The ICM-to-blastocyst diameter ratio is a one of the predictor of implantation and live birth in single-blastocyst transfers. In short, it can be used as one of blastocyst quality parameter.

The measurement of blastocyst morphometry showed that there was no difference between the diameter of parthenogenetic and fertilized blastocyst. There are some factors that affected blastocyst diameter including blastocoel volume and diameter of zona pelucida. The ratio of parthenogenetic blastocyst ICM was lower than a fertilized blastocyst. This is in line with the result of mean cell numbers calculation of parthenogenetic blastocyst cells that are also lower than the fertilized blastocyst.

In conclusion, mice parthenogenetic embryos develop better in phosphate-free medium. The quality of parthenogenetic blastocyst, based on cell number, viability, and ICM ratio parameters, was lower than for fertilized blastocyst. The characteristics of parthenogenetic diploid blastocyst in the phosphate-free medium are blastocyst rate up to 30%, have a mean cell number 51.3±2.9, viability of 78.7%, diameter of 99.91±0.48 µm and ICM/blastocyst ratio 0.21.

Conflicts of Interest

The authors declare that there is no conflict of interest on this research.

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