Oral Presentation (PCS-1)

Development of Mouse Parthenogenetic Embryos in Phosphate Free Medium

Vista Budiariati[1], Dwi Budiono[1], Mokhamad Fahrudin[1], Berry Juliandi[2], Ratih Rinendyaputri[3], Arief Boediono[1]*

[1]Department of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine, Bogor Agricultural University, West Java 16680, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, West Java 16680, Indonesia
[3]Center for Biomedical and Basic Technology of Health, Ministry of Health, Indonesia
*Corresponding author's email: ab@apps.ipb.ac.id

Keywords: development, embryos, fertilized, parthenogenetic, phosphate.

INTRODUCTION

Parthenogenesis is an artificial oocytes activation process without paternal contribution so that embryos will develop without fertilization [3]. The process of parthenogenesis as a reproductive strategy occurs in species of insect, pisces, or amphibian, which not require any implantation. Naturally, parthenogenesis is not common in mammals, but by understanding cellular mechanism during fertilization, it is possible to artificially activate mammalian oocytes.

Blastocyst, derived from parthenogenesis, can be used for developmental study, embryo reconstruction, and one of potential source for pluripotent stem cells. Unfortunately, previous studies reported that parthenogenetic embryo did not achieve exhilarating blastocyst rate.

One of the component that has been predicted to inhibit parthenogenetic embryo development 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% [1]. The effects of phosphate also became an interesting finding in the study that reported mouse fertilized embryos could well developed in modified medium rat 1 cell embryo medium (MR1ECM) which not contained any phosphate [2].

The effect of phosphate on in vitro culture of mouse parthenogenetic embryo has not been clear. The aim of this research was to analyze inhibitory effect caused by phosphate in the medium and compare the development pattern between parthenogenetic and fertilized embryos in order to reach optimal production of parthenogenetic blastocyst for further purposes.

MATERIALS AND METHODS

Superovulation and Oocyte Collection. Female DDY 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, after hCG administration female mice were mated overnight with DDY males, then on the following morning, mice were examined for the presence of vaginal plugs. Handling medium was M2 medium (Sigma, St. Louis, USA). Experimental procedures of this research was approved by Animal Care and Ethics Committee of Agency of Health Research and Development, Ministry of Health, Republic Indonesia (No: LB02.01/2/KE.150/2017).

Parthenogenesis. Oocytes were incubated for six hours exposure time in modified MR1ECM medium containing 10mM strontium chloride (SrCl2) (Sigma) and 5 µg/ml cytochalasin B at 37°C under 5% CO2. 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 [2].

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 mM; and 2 mM phosphate. Embryos were cultured at 37°C and 5% CO2 in drops of culture medium covered with mineral oil (OvoilTM, Vitrolife, Sweden). Embryos were examined at 48 h and 96 h. One way ANOVA continued with Duncan test were used for data analysis.

Experiment 2: Comparison of parthenogenetic and fertilized embryo in phosphate-free medium. Female mice with positive vaginal plugs 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% CO2 Embryos were examined at 48h and 96h. Student t-tests were used to evaluate statistical differences of the parthenogenetic embryos development.

RESULT AND DISCUSSION

The first experiment of this study showed that activation can be done and able to produce diploid embryo with 2 pronuclei in 6 hours exposure time (Figure 1).

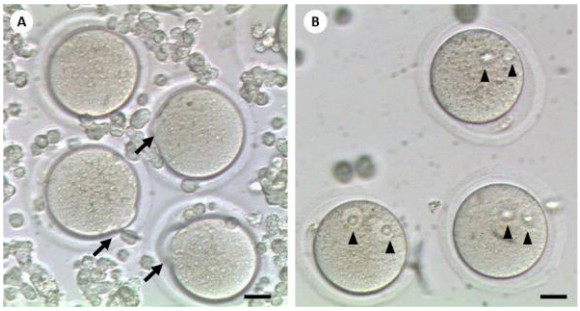

Figure 1. Diploidization of parthenogenetic mice oocytes (A) oocytes on metaphase II indicated by first polar body, prior to activation (black arrows: first polar body); (B) diploid parthenote with two pronuclei (black pointing triangle) after 6 hours exposure time in activation medium. Scale bars = 20 µm

Parthenotes that were cultured in free phosphate medium reached higher blastocyst rate compared to other groups (Table 1).

Table 1. Effect of phosphate on the parthenogenetic embryos development

P	∑	No. of embryos develop into (%)			
		2-cells	4-cells	Morula	Blasto cyst
I	30	26 (86.6±1.3)	18 (59.8±3.8)[a]	13 (46.4±0.4)[a]	9 (29.9±1.9)[a]
II	34	29 (85.6±2.9)	20 (53.1±2.9)[b]	6 (18.1±3.6)[b]	2 (9.4±2.4)[b]
III	37	32 (86.8±1.8)	16 (43.4±0.9)[c]	3 (8.5±2.3)[c]	0 (0)[c]

Phosphate concentration (P); 0 mM (I); 1 mM (II); 2mM (III); Total no. of 2PN embryos (∑). [a,b,c]Values with different letters within a column indicates significant differences, P < 0.05

The increase of phosphate concentration in culture medium lead to impairement of parthenogenetic embryos development. Decreased level of embryonic development was occurred in 4 cell stage to blastocyst. There were no blastocyst that can be obtained from culture medium with 2mM phosphate concentration.

Blastocyst rate in free phosphate medium was better than previous studies which used medium that contained phosphate. Hine (2009) reported that the development rate of parthenogenetic embryos to blastocyst in KSOM medium was 13.30% while in CZB medium 6.11% [3]. Murti *et al.* (2014) published 8.6% parthenogenetic rate in CZB medium + 5.55 mM D-glucose [4]. These comparation strengthen the indication of negative effect of phosphate on the development of parthenogenetic embryos.

Inhibitory effect caused by phosphate could be related to maturation/ metaphase-I promoting factor (MPF) activity. The presence of phosphate in culture medium may prevents second cleavage by inhibit MPF activation in second cell cycle. The mechanism of MPF activity entails cyclin B synthesis and dephosphorylation of inactive form of MPF (pre-MPF) by cdc25 phosphatase. Phosphate will disrupt that dephosphorylation process. Mouse parthenotes may have different expression of *cdc25* genes that lead to phosphate sensitivity.

Further experiment was made to analyze the differences between fertilized and parthenogenetic embryo in free phosphate medium. The result of the second experiment showed that fertilized embryo developed better as indicated by higher development rate from cleavage stage and morula to blastocyst stage compared to parthenogenones (Table 2).

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

	∑	No. of embryos develop into (%)			
		2-cells	4-cells	Morula	Blastocyst
P	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]
F	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]

Parthenogenetic embryos (P); fertilized embryos (F); Total no. of embryos (∑). [a,b,c]Values with different letters within a column indicates significant differences, P < 0.05

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. 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. The transitions of genomic control are needed to provide transcription factor that are required for DNA replication and cell cleavage.

Unlike fertilized embryo, parthenotes lack paternal factor so that there will be no transitions of genomic control from maternal to zygotic genome activation. Because of that reason, parthenotes will not develop as good as fertilized embryo.

CONCLUSION

In conclusion, mice parthenogenetic embryos develop better in phosphate-free medium. The development of parthenogenetic embryo was lower than for fertilized.

ACKNOWLEDGMENTS

This study was funded and supported by Directorate General of Higher Education (DGHE) Indonesia through the PMDSU Study Grant Program 136/SP2H/LT/DRPM/IV/2017. The authors also would like to thank the Center for Biomedical and Health Basic Technology of Health, Ministry of Health, Indonesia for the laboratory facilities.

REFERENCES

[1] Haraguchi S, Naito K, Azuma S, Sato E, Nagahama Y, Yamashita M, Toyoda Y. 1996. Effects of phosphate on in vitro 2-cell block of AKR/N mouse embryos based on changes in cdc2 kinase activity and phosphorylation states. *Biol Reprod.* 55(3): 598-603.

[2] Popova E, Bader M, Krivokharchenko A. 2011. Effect of culture conditions on viability of mouse and rat embryos developed in vitro. *Genes (Basel)*. 2: 332-344.

[3] Hine TM. 2009. Pengembangan Metode Kultur Embryonic Stem Cells dari Embrio Hasil Fertilisasi dan Produksinya dari Embrio Partenogenetik Mencit [disertasi]. Bogor (ID): Institut Pertanian Bogor.

[4] Murti H, Fahrudin M, Setiadi MA, Setiawan B, Boediono A. 2014. Perkembangan Praimplantasi Embrio Mencit dengan Materi Genetik yang Berasal dari Paternal, Maternal, dan Inti Sel Somatik. *J Vet.* 15(1): 1-10.