

Mice Oocytes Respond after Vitrification Followed by Artificial Activation Using a Various Concentration of Strontium Chloride and Cytochalasin B

(RESPON OOSIT MENCIT PASCA VITRIFIKASI TERHADAP AKTIVASI BUATAN DENGAN MENGGUNAKAN STRONSIUM KLOORIDA DAN *CYTOCHALASIN B* PADA BERBAGAI KONSENTRASI)

Muhammad Rosyd Ridlo¹, Rini Widyastuti^{2,3}, Alkaustariyah Lubis⁴,
Mokhamad Fahrudin⁵, Arief Boediono⁵

¹Veterinary Nursing Study Program,

Vocational College. Universitas Gadjah Mada.

^{2,2}Reproduction and Artificial Insemination Laboratory.

Department of Livestock Production.

Faculty of Animal Husbandry. Padjadjaran University.

³Veterinary Study Program. Faculty of Medicine. Padjadjaran University.

⁴Medical Study Program Student. Faculty of Medicine. Padjadjaran University.

⁵Division of Anatomy, Histology and Embryology.

Department of Anatomy, Physiology and Pharmacology.

Bogor Agricultural University.

Jl. Yacaranda. Sekip Unit II. Yogyakarta, indonesia 55281

Phone +628977022127; Email: rosyidridlodrh@gmail.com

ABSTRACT

Oocyte vitrification is the important part of gamete preservation for further purpose. The objective of this study was to evaluate the response or development of vitrified-mice oocyte following activation using various concentrations of Strontium Chloride (Sr Cl_2). Oocytes were collected from superovulation-induced female mice. Oocytes vitrification was then performed using a gradual equilibration of 2 M Ethylene Glycol in 0.25 M sucrose and 7 M Ethylene Glycol on 0.5 sucrose. Subsequently, the vitrified oocytes were thawed and activated using various Strontium Chloride concentration in each group. Control 1 is unvitrified oocyte and without Sr Cl_2 . Control 2 is unvitrified oocyte then activated by 20 mM Sr Cl_2 . Zero (0) mM Sr Cl_2 is vitrified oocyte without Sr Cl_2 . Group Ten (10) mM is vitrified oocyte then activated by 10 mM Sr Cl_2 . Group Twenty (20) mM is vitrified oocyte then activated by 20 mM Sr Cl_2 . The viability of vitrified-thawed oocytes was observed based on ooplasm integrity. Whereas the oocytes respond to artificial activation was observed based on pronucleus formation after 10 hours of activation. The result showed that 39% of oocyte degenerated following vitrification. The respond of vitrified-thawed oocytes following artificial activation using Strontium Chloride was significantly lower compared to fresh oocytes ($p < 0,05$). Interestingly the highest percentage of activated oocytes (36.36%) was present in a group achieved 20 mM Strontium Chloride. As conclusion is Strontium Chloride 20mM has a best result (36,36%) to activate vitrified oocyte than 0 mM and 10 mM of Strontium Chloride.

Keywords: mice oocyte; vitrification; activation; strotium chloride

ABSTRAK

Vitrifikasi oosit merupakan bagian penting dari pengawetan sel gamet untuk tujuan lebih lanjut. Penelitian ini bertujuan untuk mengetahui pengaruh vitrifikasi oosit mencit dengan metode *Open Pulled Straw* (OPS) terhadap viabilitas oosit serta respons oosit yang telah dibekukan terhadap aktivasi buatan dengan menggunakan berbagai konsentrasi Stronsium Klorida (Sr Cl_2). Oosit diperoleh dari mencit betina dewasa kelamin yang telah disuperovulasi. Pada seri pertama penelitian dilakukan vitrifikasi terhadap oosit dengan menggunakan equilibrasi bertingkat di dalam *Etilen Glikol* (EG) 2 M dalam 0,25 M

sukrosa dan 7,0 M EG dalam 0,5 M sukrosa. Grup kontrol 1 merupakan oosit segar yang tidak divitrifikasi dan tidak dilanjutkan aktivasi menggunakan Sr Cl₂. Grup kontrol 2 merupakan oosit segar yang tidak divitrifikasi, namun dilanjutkan dengan aktivasi menggunakan 20 mM Sr Cl₂. Grup 0 mM Sr Cl₂ merupakan oosit yang divitrifikasi kemudian tidak dilanjutkan dengan aktivasi menggunakan Sr Cl₂. Grup 10 mM Sr Cl₂ merupakan oosit yang divitrifikasi kemudian dilanjutkan dengan aktivasi 10 mM Sr Cl₂. Grup 20 mM Sr Cl₂ merupakan oosit yang divitrifikasi kemudian dilanjutkan dengan aktivasi 20 mM Sr Cl₂. Viabilitas oosit setelah vitrifikasi diamati berdasarkan pada keutuhan membran ooplasma, sedangkan respon oosit pascaaktivasi diamati berdasarkan pada pembentukan pronukleus. Hasil pengamatan menunjukkan bahwa 39% oosit mengalami degenerasi akibat vitrifikasi. Respons oosit pasca vitrifikasi yang diaktivasi dengan menggunakan stronsium klorida dengan berbagai konsentrasi secara signifikan tampak lebih rendah apabila dibandingkan dengan kelompok kontrol ($p < 0,05$). Pada kelompok vitrifikasi yang dilanjutkan dengan aktivasi oleh 20 mM strontium menunjukkan sebanyak 36,36% oosit teraktivasi pada tahap dua pronukleus (PN). Hasil tersebut lebih tinggi apabila dibandingkan dengan kelompok lain yang diaktivasi dengan menggunakan strontium. Sebagai kesimpulan stronsium klorida 20 mM memberikan hasil terbaik (36,36%) dalam mengaktivasi oosit yang telah divitrifikasi dibandingkan kelompok Stronsium Klorida 0 mM dan 10 mM.

Kata-kata kunci: oosit menci; vitrifikasi; aktivasi; strontium

INTRODUCTION

Embryonic stem cells are a pluripotency cell which allows proliferation and differentiation into a variety of cells type. The exceptional of embryonic stem cells has promising therapy benefit in neurodegenerative diseases such as Parkinson, Alzheimer, and leukemia (Chen *et al.*, 2014, Wu *et al.*, 2015). Embryos cell differentiation happen at day five after fertilization when trophoblast and embryoblast are separated. Embryoblast will differentiate become a body cell type. If the embryoblast is separated and cultured in favorable condition, embryoblast may continue to proliferate and preserve its pluripotency (Maherali, 2008).

Slow freezing and vitrification technique are still developing and currently, vitrification is often used for cryopreservation (Shaw *et al.*, 2003). Vitrification is one of freezing methods which eliminate ice crystal formation in oocyte cytoplasm to reduce cryo damage during the freezing process. Therefore, study of oocyte vitrification will give basic knowledge about an optimal condition for oocyte after vitrification. This process also highly affects the oocytes potency to develop up to an embryo phase.

The activation of frozen oocytes is a crucial step in the production of embryonic stem cells from frozen oocytes banking. The activated oocytes are characterized by increase of intracellular calcium level by oscillation as the result of rising inositol triphosphate (IP3). This oscillation will induce the oocytes to continue meiosis, pronucleus formation, and DNA synthesis. The activated oocyte then proceed several phases until embryo developed (Tosti *et al.*, 2016). Naturally, oocyte activation occurs

the following fertilization and this is related with reactivation of oocyte that had stopped on metaphase II of meiosis II (Yanagida *et al.*, 2008). Parthenogenetically oocyte activation can be created artificially using agents which able to mimic sperm induction to the oocyte. Some of the methods used are single phase electric current, protein synthesis inhibitor, ethanol, and strontium.

In the previous study, strontium has been used to activate mice oocytes (Idris *et al.*, 2013; Kishigami *et al.*, 2007; Saili *et al.*, 2012). However, the results were varied. Artificial activation using strontium chloride (SrCl₂, Sigma) able to assist oocyte activation after ICSI formed two pronuclei (2PN) (Saili *et al.*, 2012). Utilization of strontium as artificial activation post vitrification has not much been used so that further research is needed. The purpose of this study was to evaluate the viability of mice oocytes after vitrification and response of vitrified-warmed oocytes following artificial activation using various concentration of strontium

RESEARCH METHODS

Oocyte collection

Oocytes were collected from female mice (*Mus musculus albinus*) DDY strain age 6–12 week (1,5 to 3 months) that previously super ovulated using 5 IU pregnant mares serum gonadotropin (PMSG) (Intervet, Holland) followed by 5 IU (hCG) (Intervet, Holland) per mice followed by 5 IU of human chorionic gonadotropin (HCG) (Intervet, Holland) injection after 48 hours post PMSG treatment. Oocyte was harvested after 14 to 14,5 hours after HCG

injection (Okabe, 2013). Female mice then moved into vasectomized male mice cage with the proportion of 1:1 (Monogamous Pair Mating) (Hafez, 1993). Oocytes were collected by cutting fallopian tube then slashed using the tip of a 26G syringe. Cumulus oophorus was removed using 90 Unit/mg hyaluronidase (Catalogue number: H3757, Sigma, USA). Subsequently, oocytes were washed three times using phosphate buffer saline (PBS) which contain 10% serum.

Vitrification

Oocyte vitrification was done using graded equilibrium. Oocyte was put into 2.0 M ethylene glycol (EG) (Wako; BM 62,07) in 0.25 M sucrose (Sigma; BM 342,3) for 5 minutes and 7.0 M ethylene glycol (EG) in 0.5 M sucrose for 45 seconds. Oocyte immediately put into 0.25 mL Open pulled straw, then plunged into liquid nitrogen. After 30 minutes oocyte was separated and warmed in 37°C water for 30 seconds. Oocyte subsequently put into PBS containing 0.5 M sucrose and 10% serum and incubated for 30 minutes in 37°C Oocyte membrane was observed using dissection microscope (Olympus, Japan).

Activation

Oocyte activation was done by exposing oocyte into *Chatot- Ziomec -Bavister* (CZB) medium without calcium which contains Sr Cl₂ at 0, 10 and 20 mM for 30 minutes. Control 1 is unvitrified oocyte and without Sr Cl₂. Control 2 is unvitrified oocyte with 20 mM Sr Cl₂. Zero (0) mM Sr Cl₂ is vitrified oocyte without Sr Cl₂. Ten (10) mM is vitrified oocyte then activated with 10 mM Sr Cl₂. Twenty (20) mM is vitrified oocyte then activated with 20 mM Sr Cl₂. Then all treated oocyte were activated and washed three times using CZB + calcium. The oocytes were then incubated using CZB + calcium contained 5 µg/ml *cytochalasin B* (Sigma, USA) for six hours followed by four hours cultured in CZB. Activation and incubation were done on a sterile petri dish (Falcon 35-1008, USA) in 37°C, CO₂ 5%. The development of pronucleus was observed using 1% aceto-orcein staining.

Statistical Analysis

All data were analyzed using SPSS software version 16 (SPSS Inc, Chicago, IL, USA). Oocytes viability post vitrification was analyzed using Chi-Square method. Oocytes response post vitrification followed by activation using a various concentration of strontium analyzed

using one-way ANOVA followed by Tukey multiple comparison tests. P<0.05 was considered statistically significant.

RESULT AND DISCUSSION

Post-warming oocyte morphology can be determined by oocyte membrane intact post-vitrification. Non-viable oocytes marked with shrunken or lysis of cytoplasm. The viable oocytes marked with intact cytoplasm after warming and three hours culture. Table 1 showed a percentage of viable oocyte post-vitrification and warming. The result showed that the vitrification process significantly reduces the oocyte viability compared to non-vitrified oocytes (61,53% vs 100%, p<0.05).

The present result similar reported by Picton (2002) that oocyte viability post vitrification warming about 63%. Dinnyes (2000) reported a better result, that 79% oocyte still viable and about 86% still surrounded by cumulus oophorus following vitrification warming. The lower oocytes viability in our results affected by an internal and external factor such as exposed with a chemical substance, mechanic stressor and temperature changes which can disrupt cellular and subcellular mechanism leading to deranged cell function and cell death (Smith *et al.*, 2011). Oocytes are very sensitive because only contain one cell which may vulnerable to environmental changes. One of the external factors that affect oocyte viability is a cryoprotectant. High concentration cryoprotectant induces a high osmotic pressure and induces plasma membrane damage, nucleus, even organelles. Long exposure toward cryoprotectant generates toxic effect resulting in low viability (Smith *et al.*, 2011). Therefore, reducing cryoprotectant toxicity should be taken into consideration and applied in the appropriate time. Although cryoprotectant is toxic, combination using of intracellular and extracellular cryoprotectant could reduce toxicity effect and decrease high- pressure osmotic damage of cryoprotectants. Sucrose act as an extracellular cryoprotectant can counterbalance fluid rate and help membrane stability during rehydration process (Widyastuti *et al.*, 2017).

The observation of oocytes response post vitrification was using strontium in various concentration followed by exposing to *cytochalasin B* to induce oocytes activation. The activated oocytes was indicated by the presence of second polar body and formation of female

pronucleus (n). Exposing oocyte toward culture medium containing strontium followed by cytochalazine B is able to activate unfertilized oocyte. After 10 hours of activation, oocyte showed two pronuclei whereas inactivated oocyte only showed one pronucleus (Figure 1).

Table 2 presented the oocyte activation using strontium in various concentrations. Exposing oocyte to strontium has a significant effect on oocytes activation and 20 mM strontium could significantly activate oocytes more than another concentration (12 %, $p < 0,05$). Oocytes with more

Table 1. Percentage of viable vitrification and non vitrification.

Type of Treatments	Number of Oocyte	Number of Vitrified Oocyte	Intact Oocyte (%)	Degenerated Oocyte (%)
Control	36	0	36 (100) ^a	0 (0,00) ^b
Vitrification	143	143	88 (61,53) ^b	55 (38,47) ^a

Different superscribe value in the same column indicates significant differences between two groups $p < 0.05$

Table 2. Oocyte activation using strontium in various concentration

Type of Treatments	Activated oocytes	Pro Nucleus Development of Oocyte (%)			
		0 PN	1 PN	2 PN	>2 PN
Control I *	0	36(100,00) ^a	0 (0,00) ^a	0 (0,00) ^a	0(0,00)
Control II **	46	4 (8,69) ^b	10 (21,74) ^b	30(65,23) ^b	2(4,34)
0 mM Strontium	70	45 (64,28) ^c	24 (34,28) ^b	1 (1,43) ^c	0(0,00)
10 mM Strontium	25	16 (64,00) ^d	5(20,00) ^{bc}	0 (0,00) ^a	0(0,00)
20 mM Strontium	33	17 (51,51) ^d	4(12,12) ^{bc}	12(36,36) ^c	0(0,00)

Different superscribe value in the same column indicates significant differences between groups $p < 0.05$ PN: Pronucleus; * Negative Control of Oocyte Activation; ** Positive Control of Oocyte Activation.

Note: Control 1 is unvitrified oocyte and without Sr Cl₂. Control 2 is unvitrified oocyte with 20 mM Sr Cl₂. Zero (0) mM Sr Cl₂ is vitrified oocyte withouth Sr Cl₂. Ten (10) mM is vitrified oocyte then activated with 10 mM Sr Cl₂. Twenty (20) mM is vitrified oocyte then activated with 20 mM Sr Cl₂

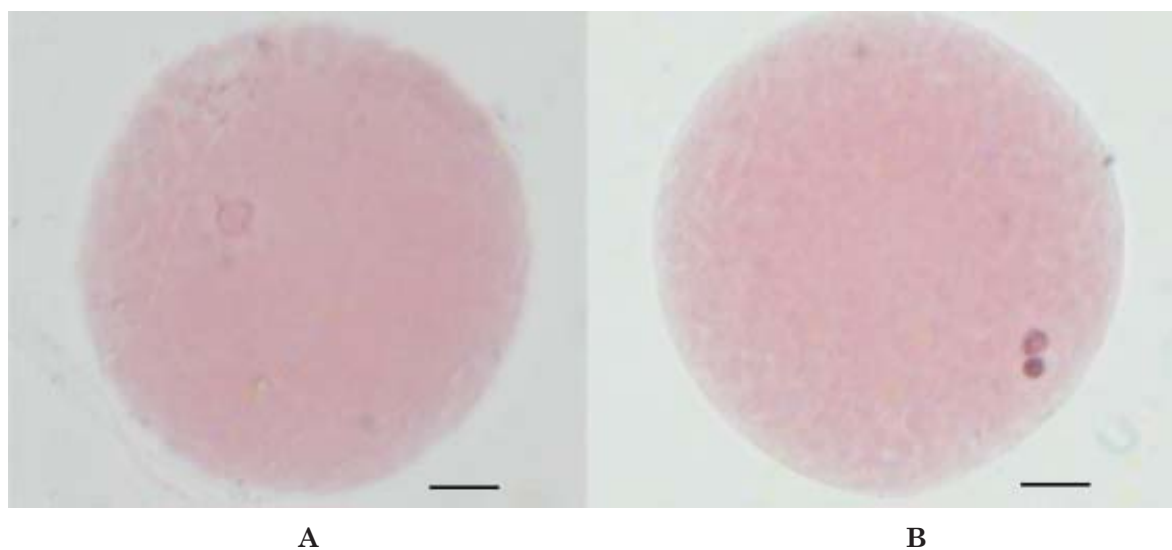


Figure 1. Morphology of oocyte post artificial activation using strontium. A. Unactivated oocyte. B. Activated oocyte

than 2 PN was observed about 4,34% in control II group but it had no significant different compared to other four groups ($P < 0,05$).

The formation of PN may be caused by karyokinetic without cytokinetic. Moreover, the ununiformed nucleus proliferation can cause polyploidy. When oocyte was activated by sperm, the sperm will condensate and formed male pronucleus (n) then both male and female pronucleus will fuse (syngamy). The newly formed cell is called zygote (2n). However, when oocyte was activated by the artificial agent, the male pronuclear will not presence, so the activated oocytes is still haploid (n). In order to produce diploid oocytes, the release of the second polar body must be resisted. This process can be aided by using cytochalasine B. Thereby the resulting oocyte will be diploid but second polar body is not released. The present study indicated the oocyte which had 2 PN was diploid (2n).

Most important step in meiosis-mitosis is protein activation in cytoplasm induced by maturation/ meiosis/ mitosis-promoting factor (MPF). MPF has two protein complex which consists of cyclin and p34^{cdc2}. The cessation of the oocyte in metaphase II is resulting from MPF high activity (Kuwayama, 2007), however in pronucleus stage MPF is reduced. The decrease is caused by activation stimuli which induce raise in calcium wave from the cytoplasm. This process was needed to activate cyclin so MPF will be inactivated. Cytostatic factor (CSF) and MPF inactivated by calcium are believed to be the key of oocyte activation to proceed its development (Idris *et al.*, 2013)

CONCLUSSION

Vitrification has an effect on decreasing the integrity of the oocyte membrane. The best strontium concentration to activate oocytes is 20 mM in the strontium activation group, 0 Mm., 10 mM and 20 mM.

SUGGESTION

Further research needs to be carried out related to vitrification technique. Further studies need to be carried out on higher strontium concentrations.

ACKNOWLEDGMENT

This study was supported by our friends, they are Toif Hidayatullah and Mu'tasim Billah. Thank you very much for the technical assistance.

REFERENCES

- Chen KG, Mallon BS, McKay RD, Robey PG. 2014. Human pluripotent stem cell culture: consideration for maintenance, expansion, and therapeutics. *Cell Stem Cell* 14(1): 13-26
- Dinnyes A, Wallace GA, Rail WF. 2000. Effect of Genotype on the Efficiency of Mouse Embryo Cryopreservation by Vitrification or Slow Freezing Methods. *Mol Reprod Dev* 40: 429.
- Idris SK, Abdullah RB, Embong WKW, Rahman MJ. 2013. Comparison between different combination of chemical treatment on parthogenetic activation of mouse oocytes and its subsequent embryonic development. *Anim Cells Syst* 17(3): 196-202
- Kishigami S, Wakayama T. 2007. Efficient Strontium-Induced Activation of Mouse Oocytes in Standard Culture Media by Chelating Calcium. *J Reprod Dev* 53(6): 1207-1215
- Kuwayama M. 2007. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the cryotop method. *Theriogenology* 67(1): 73-80
- Maherali N. 2008. Guideline and technique for the generation of induced pluripotent stem cells. *Cell Stem Cell* 3(6): 595-605
- Okabe M. 2013. The cell biology of mammalian fertilization. *Development* 140: 4471-1479
- Picton HM, Gosden RG, Leibo SP. 2002. Cryopreservation of Oocytes and Ovarian tissue. Gamete Source, Manipulation and Disposition. Pp: 142-150.
- Saili T, Djuwita I, Setiadi MA, Agungpriyono S, Boediono A. 2012. Oocyte Activation Using Strontium Chloride Following Injection of Freeze-Dried Ram Spermatozoa. *J Veteriner* 13(3): 220-226

- Shaw JM, Jones GM. 2003. Terminology Associated with Vitrification and other Cyopreservation Procedures for Oocytes and Embryos. *Hum Reprod Update* 9: 583-605.
- Smith GD, Motta EE, Serafini P. 2011. Theoretical and experimental basis of oocyte vitrification. *Reproductive BioMedicine Online* 23: 298–306
- Tosti E, Menezo Y. 2016. Gamete activation: basic knowledge and clinical application. *Human Reprod Update* 22(4): 420-439
- Widyastuti R, Khoirinaya C, Ridlo MR, Syamsunarno MRAA. 2017. Perbandingan Viabilitas Oosit pasca Vitrifikasi pada Konsentrasi Sukrosa yang berbeda. *Majalah Kedokteran Bandung*. 49(4): 252-258.
- Wu J, Belmonte JC. 2015. Dynamic pluripotent stem cells and their application. *Cell Stem Cell* 17(5): 509-525
- Yanagida K, Fujikura Y, Katayose H. 2008. The Present Status of Artificial Oocyte Activation in Assisted Reproductive Technology. *Reprod Med Biol* 7: 133-142.