Optimization of Activation Methods for Mouse Oocytes Using Calcium-free CZB Medium, $SrCl_2$, and Cytochalasin B in Vitro

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Abstract

Embryonic stem cells can be obtained by generating an embryo through fertilization; however, an embryo can also be generated asexually through parthenogenesis. This procedure will overcome the ethical issues regarding the use of embryos initially generated for reproductive purposes. The aim of this study was to obtain an optimized oocyte activation method through parthenogenesis by using mice oocytes as a model. Ten mM $SrCl_2$ and 5 µg/ml Cytochalasin B (CB) in calcium-free Chatot Ziomek Bavister (CZB) were used as a medium for an in vitro activation of mouse oocytes. Treatment combinations for the oocyte activation methods were (A) activation in CZB & $SrCl_2$ (prepared in stock) for two hours and in CZB & CB for four hours; (B) activation in CZB & $SrCl_2$ (fresh medium) for two hours and in CZB & CB for four hours; and (C) activation in CZB & CB & $SrCl_2$ (fresh medium) for six hours. The results show that the activation rate of mouse oocytes with method C has been the best among all the protocols. This optimized protocol clearly provides a new insight in the generation of embryos for further use, particularly for producing embryonic stem cells.

Keywords*: embryonic stem cell, activation, $SrCl_2$.*

Introduction

An activation method has commonly been used in manipulating embryos, especially for the production of parthenogenetic and cloned embryos.[1,2] Those embryos can be artificially activated to be developed into its blastocyst stage, from which the inner cell mass (ICM) can potentially be derived as a source of parthenogenetic embryonic stem cells (pESCs) and nuclear transfer embryonic stem cells (ntESCs).[3,4] ESCs can be differentiated into specific cells for repairing human degenerative or damaged tissue.[5] The application of mouse oocyte manipulation has potentially evolved as an experimental model because the use of human oocytes is ethically limited.[6]

Unfertilized mouse oocytes are ovulated from the ovarium at metaphase II (MII). This process is marked by an extrusion of the first polar body (PB I).[7] The cytostatic factor (CSF) affects the MII oocytes to stay stable until the fertilization.[8] It is believed that the arrest of the MII of the oocytes is caused by the high activity of Maturation Promoting Factor (MPF) and the CSF present in the cytoplasm.[9]

An artificial activation of the MII oocytes is a modification of a natural fertilization. During the fertilization, the binding of the sperm by the oocyte plasma membrane induces the

intracellular Ca^{2+} release into the oocyte.[10] There are several ways to activate oocytes for the parthenogenetic activation purpose. The artificial oocyte activation can be conducted through stimulating Ca^{2+} oscilation (increasing intracellular Ca^{2+} release) and inhibiting MPF & Mitogen Activating Factor kinase (MPFk) using cycloheximide & puromycin to prolong the periods of the incubation in the mouse oocytes. The use of Ca^{2+} stimulating substance is common in parthenogenesis and nuclear transfer studies.

An activation method of the MII mouse oocytes can be conducted by electrical and chemical techniques. Some of the commonly employed agents for mouse oocyte activation are ethanol, Ca^{2+} ionophore, and $SrCl_2$.[11] Strontium chloride ($SrCl_2$) has been successfully used to activate mouse oocytes after the nuclear transfer. Probably, strontium chloride induces multiple Ca^{2+} transients not only by displacing bound Ca^{2+} in the oocyte but also by inducing intracellular Ca^{2+} release. In order to prevent the second polar body (PB II) extrusion, Cytochalasin B should be added into the activation medium or culture medium after the activation treatment. In fact, Cytochalasin B has the capability to disrupt actin filaments and inhibit cytokinesis.[12]

The activation rate of mouse oocytes can be affected by the type of the mouse strain, the quality of the oocytes, the type and concentration of the chemical reagents, the time of activation treatment, and the combination of the activation medium.[11] This study investigated an optimal method of mouse oocyte activation by using a treatment combination of calcium-free CZB medium, $SrCl_2$, and Cytochalasin B.

Materials and Methods

Superovulation

A superovulation was stimulated by gonadotropin hormone as previously described.[11] Briefly, mature 8-10 weeks old Ddy female mice were induced to superovulate by an intraperitoneal (i.p.) injection of 5-7.5 IU *Pregnant Mare's Serum Gonadotrophin/* PMSG (Intervet International BV, Folligon, Boxmeer, Holland) and 46-48 h later by an i.p. injection of 5-7.5 IU *human Chorionic Gonadotrophin/* hCG (Intervet International BV, Chorulon, Boxmeer, Holland).

Oocytes Collection

Oocytes collection was conducted through an observation using a stereo microscope (Nikon, SMZ-2T, Japan) as previously described.[11] During the period of 14-16 h after the hCG injection, the mice were humanly sacrificed by cervical dislocation. The body wall (peritoneum) was cut to open the abdominal cavity. The oviduct was pulled from the uterus and the ovary and transferred into a droplet of M2 medium (Specialty medium, MR-015P-D, Phillipsburg, New Jersey, USA) at a 35mm petri dish (Nunc, 153066, Roskilde, Denmark). A 27 G×½" needle (Terumo, NN*2713R, Philippines) was used to slice a larger part of the oviduct transparently. Cumulus-oocytes complexes (COC) were turned out from the oviduct and transferred immediately into a droplet of M2 medium supplemented with 0.1% hyaluronidase (Sigma, H4272, St.Louis, MO, USA) to disperse the cumulus cells form the oocytes. Approximately ten minutes after the treatment using hyaluronidase, the cumulus–free oocytes were transferred into a droplet of glucose-free

CZB medium supplemented with 1 mg/ml Bovine Serum Albumin/ BSA (Sigma, A3311, St. Louis, MO, USA), covered with mineral oil (Sigma, M8410, St. Lois, MO, USA), and kept at 37°C under 5% CO_2 in an incubator (Sanyo, MCO-95, Japan). Approximately 15 minutes after the incubation, the oocyte morphology was observed to asses the quality of the oocytes. The parameters of good quality oocytes are ± 85 µm in diameter, homogeny cytoplasm (not fragmented cytoplasm), PB I extrusion, and normal distance between pellucid zone and cytoplasm.

Vital Staining

A vital staining was conducted to observe the viability of the oocytes. The oocytes were stained by using PBS (Gibco, 21600-010, Grand Island, NY, USA) medium supplemented with 10 µg/ml Hoechst 33342 (Invitrogen, H1399, Eugene, USA) and 10 µg/ml Propidium Iodide (Sigma, P4170, St. Lois, MO, USA) for ten minutes. The results of the vital staining were observed on Terazaki/minitrays plate (Nunc, 163118, Roskilde, Denmark) under fluorescent microscope (Nikon, E600, Japan) with 380 nm of wave length. In this vital staining, the living oocytes were stained by Hoechst 33342 (blue) and the dead oocytes were stained by Propidium Iodide (red).

Oocytes Activation

The oocytes activation was conducted in calcium-free CZB medium, 10 mM $SrCl_2$ (Sigma, 255521, St. Louis, MO, USA), 5 µg/ml Cytochalasin B (Sigma, C6762, St.Louis, MO, USA) as previously described but with modification.[11,13,14] Only good qualities of mouse embryos were used for further treatment. Treatment combination for oocytes activation methods were (A) activation in CZB & $SrCl_2$ (prepared in stock) for two hours and in CZB & CB for four hours; (B) activation in CZB & $SrCl_2$ (fresh medium) for two hours and in CZB & CB for four hours; (C) activation in CZB & CB & $SrCl_2$ (fresh medium) for six hours. The present study (treatment A and B) compared a different combination of calcium-free CZB medium and $SrCl_2$ to determine the capability of $SrCl_2$ prepared in a stock and in a fresh medium in the mouse oocytes activation in vitro. Treatment C is commonly used as an activation protocol in mouse oocytes after the nuclear transfer. Prolonged time exposure of CB is not affecting the activation process, but it is necessary to expose the activated oocytes to CB until the pro-nuclear formation is reached (six hours). This is the way to obtain diploid parthenogenetic embryos.

In Vitro Culture and Determination of Activation Efficacy

The in vitro culture of the activated oocytes was conducted as described previously (13). Briefly, following a serial washing in CZB medium, embryos were put in drops of glucose-free CZB medium supplemented with 1 mg/ml BSA. They were then kept at 37°C under atmosphere of 5% CO_2 in air for 24 hours. Six hours after activation, the activated oocytes were observed under inverted oocytes to determine the presence of pronucleus, and were then returned to the incubator to assess further the in vitro development of the oocytes following the activation.

Statistical Methods

Completely Randomized Design (CRD) was used as experimental design

in the present study as previously described.[15] The data were evaluated by one way analysis of variance (ANOVA), and the differences between the treatment groups were determined by a post hoc, Tukey-Honestly Significantly Different (HSD) test. The differences at a probability value (P) of 0.05 or less were considered to be significant. All of statistical analyses were conducted by using SPSS 15.0 for windows software.

Results and Discussion

In a natural ovulation, female mice could release 8 to 12 oocytes depending on the strain. A high number of oocytes were needed in the manipulation of the embryos, in which they were commonly produced through superovulation. The optimal PMSG and hCG dosages were varied in 2.5 to 10 IU depending on their strain.[11]

The results (table 1.) showed that the highest mouse oocytes production was observed on a superovulation with a 7.5 IU dosage of PMSG and hCG (17.29 oocyte/mouse) and it was significantly different (P≤0.05) from that with a 5 IU dosage of PMSG and hCG (12.79 oocyte/mouse). A superovulation with a 7.5 IU dosage of PMSG and hCG also increased the number of the bad quality oocytes (14.22%) and was significantly different (P≤0.05) from a 5 IU dosage of PMSG and hCG (9.22%). However, the results indicated that the average of good quality oocytes on superovulation with a 7.5 IU dosage of PMSG and hCG was the highest (14.83 oocytes/mouse) and it was significantly different from that with a 5 IU dosage of PMSG and hCG (11.61 oocytes/mouse).

Based on the results, it was evident that the significant improvement of ddy mouse oocyte production was caused by the superovulation with a 7.5 IU dosage of PMSG and hCG. However, this treatment also affected the quality of the oocytes to be worse than the ones treated by a 5 IU dosage of PMSG and hCG. It was assumed that the higher dosage would influence the hormonal balance system and the growth of follicles to become mature. They would also affect the metaphase I chromosome alligment and increase aneuploidy in mouse oocytes maturated in vitro.[16] The previous study showed that a superovulation method could decrease embryonic development capability in vitro and in vivo.[17] Moreover, the method could increase abnormal blastocsyts, slow fetus development, and aberrant DNA methylation of imprinted loci.[18,19]

Table 1. Production of Mouse Oocytes by Superovulation

Dosages of PMSG & HCG	No. of Mouse	Total Number of Oocytes	Average (oocytes/ mouse)	Quality of oocytes based on morphology observation (%)	
				Good (oocytes/mouse)	Bad (oocytes/mouse)
5 IU	28	358	12.79[a]	11.61 (90.78)[a]	1.18 (9.22)[a]
7,5 IU	59	1020	17.29[b]	14.83 (85.78)[b]	2.46 (14.22)[b]

Note: values within columns with different superscripts are significantly different (P≤0.05).

Table 2. Activation Rate of Mouse Oocytes at Twenty Hours Post Activation

Activation Methods	No. of Oocytes Assessed	Activation Rate (%)	
		Not Cleveage	Cleveage
A	250	169 (67.60)[a]	81 (32.40)[a]
B	146	36 (24.66)[b]	110 (75.34)[b]
C	215	6 (2.79)[c]	209 (97.21)[c]

Note: values within columns with different superscripts are significantly different (P≤0.05).
Activation Methods:
(A) Activation in CZB & $SrCl_2$ (prepared in stock) for two hours and in CZB & CB for four hours;
(B) Activation in CZB & $SrCl_2$ (fresh medium) for two hours and in CZB & CB for four hours;
(C) Activation in CZB & CB & $SrCl_2$ (fresh medium) for six hours.

In the present study (Tabel 2.), the activation rate of mouse oocytes with treatment C was the highest (97.21%) and significantly different (P≤0.05) from other treatments, i.e. treatment A (32.40%) and B (75.34%). Furthermore, the results indicated that treatment C with an activation in calcium-free CZB & CB & $SrCl_2$ (fresh medium) for six hours was the optimal activation method for mouse oocytes in vitro.

Calcium-free CZB medium was commonly used as the basic medium for oocyte activation.[14,20] In an artificial activation, the extracellular calcium could influence the capability of $SrCl_2$ to induce the oscillation of the intracellular ion calcium. The supplementation of $SrCl_2$ into the medium was one of the methods to increase the ion Ca^{2+} signal oscillation in oocytes so that it would be capable to induce the biochemical reaction.[8] It would trigger CSF degradation by ubiquitin or proteosome system. Ion Sr^{2+} could increase the ion Ca^{2+} oscillation of MII oocytes through InsP3 receptor.[21] In order to inactivate MPF (the concentration would decrease), the inactive CSF would trigger the activation of APC and destroy cyclin A and cyclin B.[22] The activated APC would induce the MII oocytes into anaphase.[9]

In treatment C, it was assumed that six hours' exposure of $SrCl_2$ would be optimal to induce the ion Ca^{2+} oscillation, whereas two hours' exposure of $SrCl_2$ (treatment A & B) showed lower results of activation rate than treatment C. The activation rate of the mouse oocytes was influenced by using a medium stock ($SrCl_2$ and calcium-free CZB prepared in a stock). It was assumed that the medium stock would decrease the capability of $SrCl_2$ to induce the ion Ca^{2+} oscillation of the MII oocytes. In treatment A, $SrCl_2$ kept as a frozen solution had a lower activation rate than that prepared as a fresh solution. In order to reduce the time consumed for the activation protocols, the frozen stock of the activator agents has widely been used in parthenogenetic activation (Ca^{2+}ionophore, Ionomycin, phorbol ester, and thimerosal) without significantly affecting the subsequent development in vitro of the activated oocytes. However, the factor(s) responsible of this discrepancy is unknown yet.[23]

The confirmation of the oocytes' viability showed that the good quality oocytes would markedly be in blue color, whereas the bad quality oocytes would markedly be in red color (figure 1.D). In vital staining, Propidium Iodide

only penetrates into cytoplasm in the dead oocytes. After the activation treatment, the extrusion of PB II was successfully inhibited by adding Cytochalasin B, which generated diploid parthenogenetic embryos.[12] This was evident in the formation of two pronucleus formation at six hours post activation (figure 1.E). At 20 hours post activation, the activated oocytes would develop into two cell stage oocytes (figure 1.F).

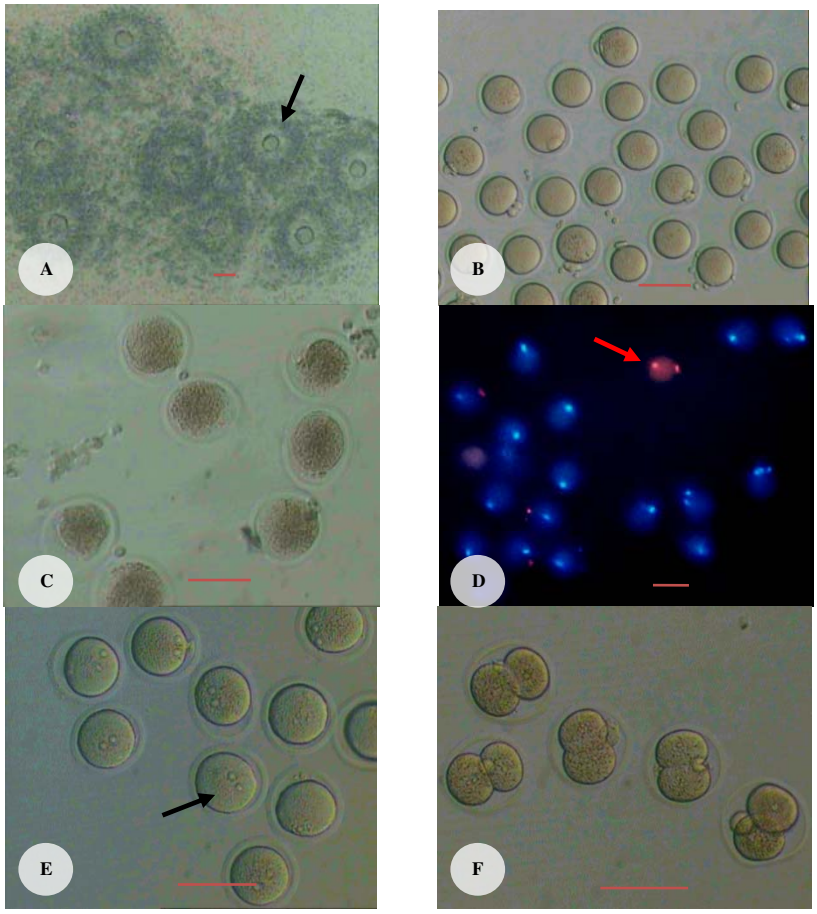

Figure 1. Morphology of Mouse Oocytes Prior to Superovulation, followed by Isolation and Activation. A. Cumulus-Oocytes Complexes (COC) after isolation from oviduct; B. Good quality oocytes based on a morphology observation ; C. Bad quality oocytes based on a morphology observation; D. Hoechst 33342-PI staining (blue = life, red = die); E. Formation of two pronucleus (2PN) at six hours post activation; F. Activated oocytes forming two-cell embryos at twenty hours post activation. Bar = 100 µm.

Conclusions and Suggestions

Optimum results of in vitro activation of mouse oocytes can be achieved by a treatment combination of the activation in calcium-free CZB & CB & $SrCl_2$ (fresh medium) for six hours. A superovulation method with a 7.5 IU dosage of both PMSG and hCG was optimal for ddy female mouse. Clearly, by using the optimized protocol, parthenogenetic embryos were able to be generated more efficiently. The use of embryos to generate embryonic stem cells will assist further research for finding more treatments of diseases including a more rapid drug screening process.

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