Original Article

In vitro Activation of Mouse Oocytes through Intracellular $Ca2^+$ Regulation

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Abstract

Background: Ca^{2+} signaling pathway is suggested to play an essential role in mediating oocyte maturation. **Aims:** The aim of this study was to evaluate intracellular Ca^{2+} of resistant immature oocytes that failed to resume meiosis following subsequent *in vitro* culture reach metaphase II after calcium ionophore A23187 activation. **Settings and Design:** This *in vitro* analytical experimental study was conducted at Animal Science Laboratory of Indonesian Medical Education and Research Institute (IMERI), Human Reproductive Infertility and Family Planning of IMERI, and Electrophysiology Imaging of Terpadu Laboratory, Faculty of Medicine, University of Indonesia. **Methods:** A total of 308 oocytes classed as resistant immature following *in vitro* culture were randomly allocated to control (n = 113) and treatment groups (n = 195). The oocyte activation group was exposed to A23187 solution for 15 min and then washed extensively. Maturation was evaluated by observing the first polar body extrusion 20–24 h after A23187 exposure. Ca^{2+} imaging was conducted using a confocal laser scanning microscope to identify the dynamic of Ca^{2+} response. **Statistical Analysis:** SPSS 20, Chi-square, and Mann–Whitney U-test were used in this study. **Results:** Activation of resistant immature oocytes with A23187 significantly increased the number of oocyte maturation compared with the control group ($P < 0.001$). Furthermore, fluorescent intensity measurements exhibited a significant increase in the germinal vesicle stage when activated ($P = 0.005$), as well as the metaphase I stage, even though differences were not significant ($P = 0.146$). **Conclusion:** Artificial activation of resistant immature oocyte using chemical A23187/calcimycin was adequate to initiate meiosis progress.

Keywords: *Artificial oocyte activation, $Ca2^+$ signaling, calcimycin, calcium ionophore A23187, meiotic arrest*

Introduction

A23187 is a chemical agent that is commonly used to increase the intracellular Ca^2 + levels, especially in parthenogenetic studies, leading to oocyte activation,[1-18] and it has been used to treat patients with a previous history of low fertilization and poor embryo development.[18-25] Successful maturation of immature oocytes after exposure to A23187 has been reported in both mammals and nonmammals.[26,27] However, the effect of A23187 on resistant immaturity has not yet been investigated. This study aimed to investigate whether the modification of intracellular Ca^{2+} regulation of resistant immature oocytes that failed to resume meiosis after overnight culture could reach metaphase II (MII) after A23187 activation.

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Methods

Oocytes collection

A total of 308 mouse oocytes classed as resistant immature following *in vitro* culture were randomly allocated to

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control (n = 113) and treatment groups (n = 195). This study conducted at Animal Science Laboratory of IMERI, Human Reproductive, Infertility, and Family Planning Research Center of IMERI, and Electrophysiology Imaging of Terpadu Laboratory, Faculty of Medicine, University of Indonesia. Immature oocyte after *in vitro* culture was included in this study, whereas exclusion criteria were mature and degenerated oocytes.

Immature mouse oocytes were obtained by priming 8–12-week-old DDY hybrid female mice with 7.5 IU of a pregnant mare's serum gonadotropin (Intervet, The Netherlands) (intraperitoneal). A total of 44–48-h later ovarian dissection was performed to collect immature oocytes using a 26.5G sterilized needle in G-MOPS medium (Vitrolife, Sweden). Subsequently, maturation cultures were divided into two groups based on cumulus status (intact or without cumulus cells) [Figure 1] in G-IVF medium (Vitrolife, Sweden) for 14–16 h at 37°C and 5% CO_2. Oocytes in both germinal vesicle (GV) and MI stages were randomly allocated to activation treatment and control groups by an embryologist [Figure 2].

Activation of resistant immature oocyte

Both resistant immature oocytes in either GV or MI stages were exposed to 10 μM/L A23187 (Sigma-Aldrich) in G-MOPS PLUS medium (30–40 μL activation drop) from stock solution dissolved in dimethyl sulfoxide (Invitrogen) (1:99) for 15 min. Exposure was accomplished in the darkroom at 37°C and maintained inside the incubator without CO_2 during treatment. The oocytes were then extensively washed in G-MOPS medium and recultured at 37°C, 5% CO_2. The effectiveness of A23187 activation was evaluated 14–16 h later based on the first polar body extrusion. Since the cumulus cells were denudated due to matured observation, the activation does not involve the cumulus cells. To observe whether preactivation cumulus status implied response of resistant immature oocytes during activation, the activation was performed separately according to cumulus cell status at the time of collection.

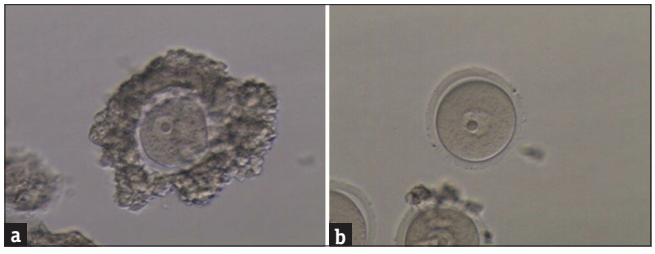

Figure 1: Immature oocytes collected from ovaries (Bar 50 μm), (a) oocyte with intact cumulus cells, (b) oocyte without cumulus cells

Measurement of intracellular Ca^{2+} intensity with confocal laser scanning microscopy

Oocytes that exhibited resistance to meiotic maturation were exposed to 0.2% pronase for 2 min to remove the zona pellucida and then washed extensively. The oocytes were loaded to 10 μM/L Fura Red AM ester (Invitrogen) and 0.2% Pluronic F-127 (Invitrogen) for 30 min in G-MOPS medium. After washing extensively, the oocytes were cultured for 30 min at 37°C and 5% CO_2 to complete the intracellular Fura Red de-esterification. The basal concentration of free Ca^{2+} intensity was measured in a glass-bottomed dish using a confocal laser scanning microscope 700 (Zeiss). A fluorescent measurement was made to observe calcium dynamic every 15 s (time series) with a specific filter that provided excitation at 405 nm and 488 nm wavelengths. Oocytes were then treated with a 10-μL droplet of A23187 solution to collect the free Ca^{2+} intensity during activation. Free intracellular Ca^{2+} concentration either in basal or activation was obtained from the fluorescence ratio between 405 and 488 wavelengths (expressed in relative fluorescent intensity).

Study approval

Ethical approval was issued by the Ethics Committee of Health Research of the Faculty of Medicine, University of Indonesia; Committee reference number: 0187/UN2. F1/ETHIC/2018, approved on March 5, 2018.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (Release 20.0, SPSS, Inc., Chicago, IL, USA). The categorical variable (maturation, degeneration, and spontaneous cleave) was compared using the nonparametric Chi-square test. The Mann–Whitney U-test was used to compare the continuous data (Ca^{2+} intensity) since it was not normally distributed. Confidence interval 95% was used in this study.

Results

Resistant immature oocyte response to A23187 exposure

The maturation rate of resistant immature oocytes post-A23187 activation was significantly higher compared to the control group (25.64% [50/195] vs. 1.77% [2/113], $P < 0.001$, respectively). There was a significant increase in oocyte degeneration compared to the control group (6.15% [12/195] vs. 0.88% [1/113], $P = 0.036$, respectively). A23187 exposure also led to a significant increase in spontaneous cleavage compared to the control group (20% [39/195] vs. 8.85% [10/113], $P = 0.010$, respectively) [Table 1].

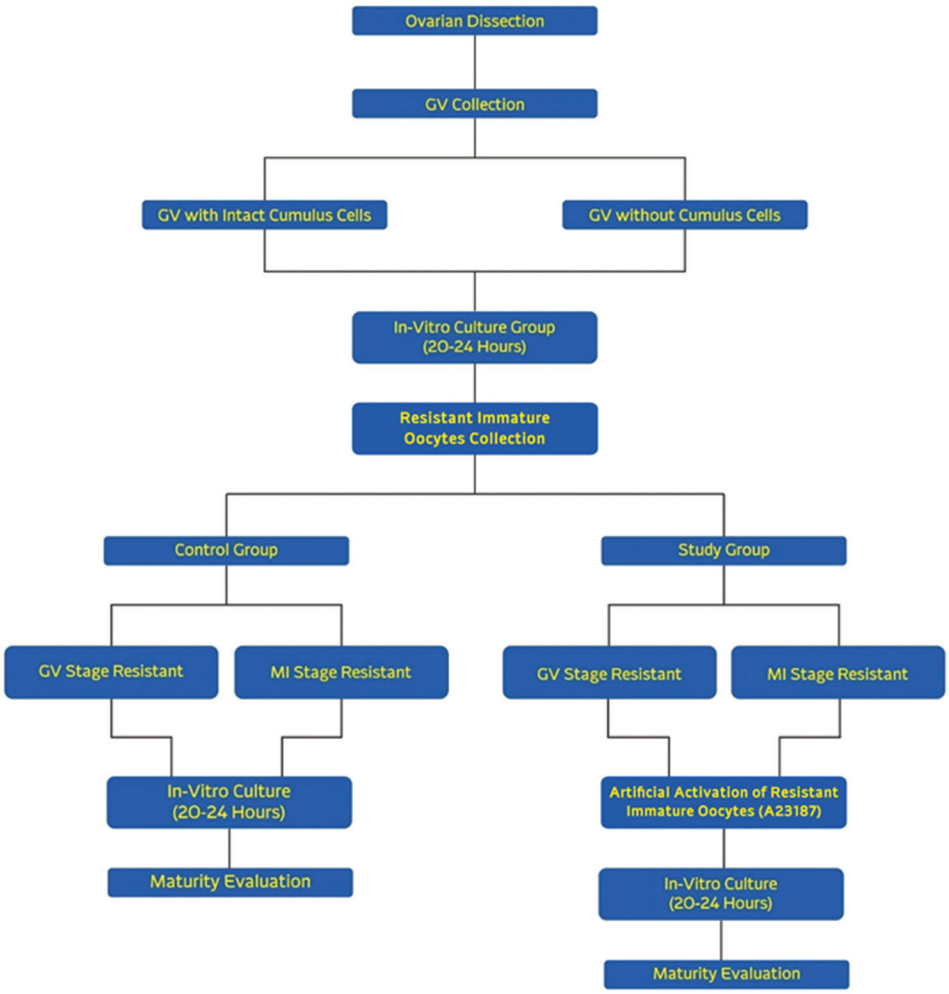

Figure 2: The schematic study of resistant immature oocyte activation

Table 1: Resistant immature oocyte response to calcium A23187 exposure

	Activation of calcium A23187 (*n*=195)	Control (*n*=113)	*P*
Number of post activation maturations (%)	50 (25.64)	2 (1.77)	<0.001**
Number of post-activation degeneration (%)	12 (6.15)	1 (0.88)	0.036*
Number of spontaneous cleavage (%)	39 (20)	10 (8.85)	0.010*

Data presented as number of oocytes and percentage (*n* (%)); **P*<0.05; ***P*<0.001

Resistant immature oocyte response to A23187 exposure based on preactivation cumulus status

Preactivation cumulus status of resistant immature oocytes either in intact or without cumulus did not influence the response of those oocytes to A23187 activation to resume the meiosis progress. As shown in Table 2, no significant difference was found in both oocytes with intact and without cumulus cells in the term of maturation (29.73% [22/74] vs. 23.72% [28/121], P = 0.307, respectively). Similarly, degeneration rate (4.08% [3/74] vs. 10.74% [13/121], P = 0.099, respectively) and spontaneous cleavage (20.27% [15/74] vs. 11.57% [14/121], P = 0.098, respectively) after activation were not significantly different between the groups.

Resistant immature oocyte response to A23187 exposure based on meiotic arrest stage

The stage of meiotic arrest (GV or MI) at the time of A23187 exposure did not affect the ability to resume meiosis progression after exposure (22.22% [18/81] vs. 28.07 [32/114], P = 0.357, respectively) [Table 3].

Measurement of intracellular calcium intensity during A23187 activation

To identify free intracellular Ca^{2+} response during activation, the activation and basal intensity levels (without activation) of each stage were compared. During activation, exposure of A23187 to oocytes at the GV stage exhibited a significant increase in intensity

Table 2: Response of postactivation oocytes with and without cumulus cells after 20-24-h culture

	Oocytes with intact cumulus cell (*n*=74)	Oocytes without cumulus cell (*n*=121)	*P*
Number of post-activation maturations (%)	22 (29.73)	28 (23.72)	0.307
Number of post-activation degeneration (%)	3 (4.08)	13 (10.74)	0.099
Number of spontaneous cleavage (%)	15 (20.27)	14 (11.57)	0.098

Data presented as number of oocytes and percentage (*n* (%)); **P*<0.05; ***P*<0.001

Table 3: Oocytes response based on meiotic arrest stage to A23187 exposure

	Metaphase II (%)	*P*
Total GV (prophase I) stage oocytes (*n*=81)	18/81 (22.22)	0.357
Total MI (metaphase I) stage oocytes (*n*=114)	32/114 (28.07)	

Data presented as number of oocytes and percentage (n (%)); **P*<0.05; ***P*<0.001. GV=Germinal vesicle, MI=Metaphase I

Table 4: Baseline calcium fluorescence intensity and A23187-induced level

Variable	*n*	Median±IQR	*P*
GV stage oocytes			
Basal calcium intensity	17	0.1500 ± 0.03	0.005*
Activation calcium intensity		0.2400 ± 0.22	
MI stage oocytes			
Basal calcium intensity	17	0.2200 ±0.05	0.146
Activation calcium intensity		0.2400 ± 0.15	

**P*-value <0.05; ***P*-value <0.001. GV=Germinal vesicle, MI= Metaphase I, IQR= Interquartile range

compared to those at the basal level (0.2400 ± 0.22 vs 0.1500 ± 0.03, P = 0.005, respectively). Oocytes at the MI stage also showed increasing intensity during activation but did not differ significantly compared to the basal level (0.2400 ± 0.15 vs 0.2200 ±0.05, P = 0.146, respectively) [Table 4].

Discussion

In this experimental study, the use of A23187 to activate oocytes that showed resistance to meiotic maturation after a 20–24-h *in vitro* culture was adequate to promote meiotic progression through the increase of intracellular Ca^{2+} levels. A23187 is a specific lipophilic molecule that mediates the exchange of protons and divalent cations through an electroneutral exchange transport mechanism (two compounds of A23187 bind to one calcium ion).[28,29] Ionophore exposure may have the ability to mimic similar cell responses when activated by hormones through the Ca^{2+} mobilization, which is not only derived from extracellular influx but also by efflux from intracellular storage of endoplasmic reticulum.[30]

A23187 may exert its effects on oocyte maturation through triggering the activity of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) enzyme.

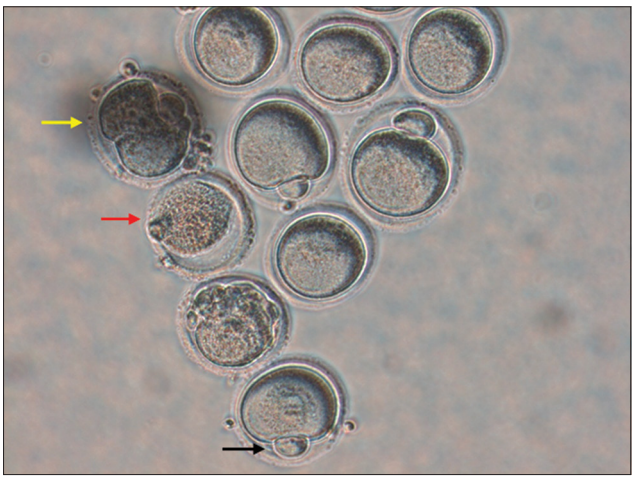

Figure 3: Resistant immature oocytes responses to A23187 activation (Bar 50 µm); metaphase II (black arrow), degeneration (red arrow), spontaneous cleaves (yellow arrow)

During activation, A23187 may activate the CaMKII autophosphorylation and maintain enzyme activity, even without the presence of calcium at a later stage.[31,32] CaMKII is an enzyme that is postulated to mediate the transition of chromosomes into anaphase. Once the CaMKII multifunctional enzyme is activated, the downstream activity of CaMKII altogether with ATP mediates meiotic progress, as shown by the colocalization of calmodulin interacting with CaMKII in the meiotic spindle immediately after A23187 activation.[32]

In this study, oocyte degeneration and spontaneous cleavage after A23187 activation also significantly increased [Table 1]. Calcium downstream signaling seems to activate several proteins to induce cell cycle progression or may prompt programmed cell death. Regardless of the CaMKII enzyme activity, calcineurin (Ca^{2+}/CaM-dependent phosphatase) is also believed to be activated by calcium signals. Downstream effects of CaMKII or calcineurin enzymes lead to the activation of numerous transcription factors that regulate gene transcription factors such as nuclear factor of activated T-cells (NFAT) and Nuclear Factor Kappa B (NFkB).[33] Afterward, NFAT and NFkB activate the expression of numerous targeted genes to determine whether the cells will enter into the cell cycle or the death process. In this research, it was not fully

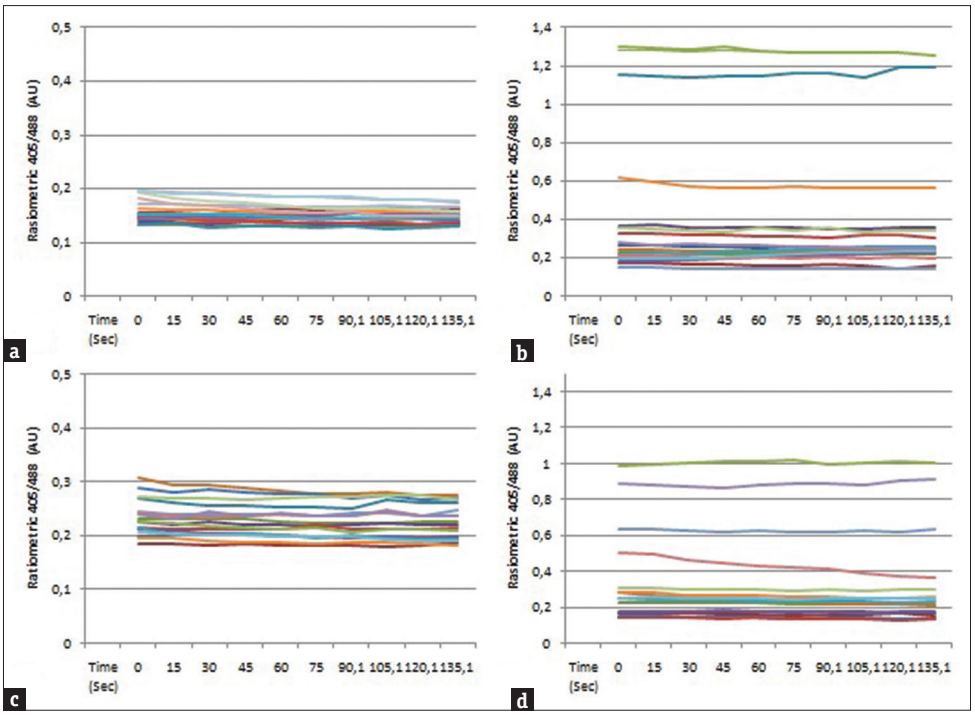

Figure 4: Ratiometric fluorescent intensity of resistant immature oocytes, (a) basal intensity of resistant immature germinal vesicle stage, (b) fluorescent intensity of resistant immature germinal vesicle during activation to A23187, (c) basal intensity of resistant immature metaphase I stage, (d) fluorescent intensity of resistant immature metaphase I during activation to A23187 (Intensity was expressed in relative fluorescent intensity (RFI), each line color represents the value of the samples)

elucidated how postactivation of resistant immature oocytes has shown such different responses in the same concentration and duration of exposure.

Resistant immature oocytes cultured with intact or without cumulus cells did not show significant differences during A23187 activation in terms of maturation [Table 2]. The quality of resistant immature oocytes in both the groups was assumed to be defective of the same condition in the nucleus and/or cytoplasmic factors. Notably, denudation was conducted after *in vitro* maturation culture to assess the meiotic progress; as a consequence, the activation process was performed without involving the cumulus cells.

Our study also indicated that exposing resistant immature oocytes to A23187 results in increased stages of the meiotic progress of either prophase I or MI to MII [Figure 3]. It is accepted that A23187 induces a single-wave pattern of Ca^{2+} that remains high during exposure.[34-36] Although the pattern of calcium release from the endoplasmic reticulum and through membrane transports does not show oscillation compared to physiological processes, it is assumed that oocytes can tolerate the change of calcium concentration at a certain level.[37] Furthermore, some studies indicate that oocytes induced by A23187 can develop to the blastocyst stage.[37-39]

The intensity of intracellular Ca^{2+}-level measurements of this study showed that resistant immature oocytes in both GV and MI stages responded to the activation individually [Figure 4]. The reason for this is unknown and requires further investigation. Nonetheless, this response may represent the amount of intracellular calcium in the ER as well as the channel activity which is responsible for regulating calcium entry into the cells.[40]

Limitations of this study were applied. First, we do not perform molecular investigation about the ploidy status and normality of MII spindle formation of matured oocytes derived from activation, since this study only focused on the fluorescent intensity level of resistant immature during activation. Second, the RFI of matured oocyte after activation followed by fertilization was not investigated.

Conclusion

Artificial activation using A23187 could promote meiosis progression of resistant immature mouse oocytes either in GV or MI stages. The use of mouse oocytes in investigating the effect of A23187 activation is benefited as the experimental proxy to show the possible option for triggering maturation. Afterward, further study is needed to evaluate the developmental potential of the matured oocyte generate from activation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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