Regulation of $[Ca^{2+}]_i$ Oscillations and Mitochondrial Activity by Various Calcium Transporters in Mouse Oocytes

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Abstract
Oocyte activation inefficiency is one of the reasons for female infertility and Ca²⁺ functions play a critical role in the regulation of oocyte activation. We used various inhibitors of Ca²⁺ channels and pumps located on the plasma membrane, the endoplasmic reticulum or both, including sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPases (SERCAs, the main Ca²⁺ pumps which decrease the intracellular Ca²⁺ level by reaccumulating Ca²⁺ into the sarcoplasmic reticulum), transient receptor potential (TRP) ion channel subfamily member 7 (TRPM7, a Ca²⁺/Mg²⁺-permeable non-selective cation channel), T-type Ca²⁺ channels and calcium channel Orai1, to investigate their roles in Ca²⁺ oscillation patterns and mitochondrial membrane potential during oocyte activation by real-time recording. Our results show that SERCAs, TRPM7 and T-type Ca²⁺ channels are important for initiation and maintenance of Ca²⁺ oscillations, which is required for mitochondrial membrane potential changes during oocyte activation, as well as for subsequent pronuclear formation and transition to embryo development, while the function of calcium channel Orai1 is not confirmed. Increasing the knowledge of these transporters may provide a theoretical basis for improving oocyte activation in human assisted reproduction clinics.

Introduction
According to reports by the World Health Organization in 2016, at least one of ten couples in developed countries cannot have children within five years of marriage, half of which are due to female infertility [1]. Oocyte activation inefficiency is a major problem causing female infertility [2]. Oocytes are arrested at metaphase of the second meiosis until fertilization takes place. Directly following sperm penetration, oocyte activation begins with a series of crucial steps triggered by periodical repetitive increases and decreases of intracellular Ca²⁺ ([Ca²⁺]ᵢ) concentrations known as [Ca²⁺]ᵢ oscillations [3, 4]. Although [Ca²⁺]ᵢ oscillations are required for oocyte activation [5], very little is known about which Ca²⁺ transporter participates in producing [Ca²⁺]ᵢ oscillations during oocyte activation.

The importance of Ca²⁺ functions in the regulation of oocyte activation is increasingly being
recognized [6]. There are many important channel proteins involved in Ca\(^{2+}\) transportation in oocytes. Ca\(^{2+}\) transporters on the plasma membrane can be controlled by voltage, ligand, second messengers, Ca\(^{2+}\) concentration, or other interactions [7]. Ca\(^{2+}\) influx into the cytoplasm is mediated by a diverse population of Ca\(^{2+}\) transporters exhibiting significant diversities in their gating and activation mechanisms. Once the oocyte is activated, the [Ca\(^{2+}\)]\(_i\) oscillations are produced by simultaneous intracellular Ca\(^{2+}\) storage release and the extracellular Ca\(^{2+}\) influx [8]. Hence, it is important to elucidate the molecular mechanisms of [Ca\(^{2+}\)]\(_i\) oscillations at activation. According to our previous research, it can be seen that [Ca\(^{2+}\)]\(_i\) oscillations are regulated by mitochondria during activation [9]. Mitochondria are the main organelles for energy supplement in the cell. We believe that [Ca\(^{2+}\)]\(_i\) oscillations in the cytoplasm will have similar effects as Ca\(^{2+}\) regulates mitochondria in cardiomyocytes [10]. At present, the transporters involved in the regulation of [Ca\(^{2+}\)]\(_i\) oscillations and mitochondria activity have not been completely determined.

In this study, specific inhibitors for different Ca\(^{2+}\) transporters were introduced to detect the [Ca\(^{2+}\)]\(_i\) oscillation patterns and mitochondria membrane potential dynamic changes, in order to evaluate the role of Ca\(^{2+}\) transporters in oocyte activation. Studying the function and regulatory mechanisms of these transporters have great importance for both understanding oocyte activation mechanisms and for improving clinical assisted reproductive technologies (ART).

Materials And Methods

Ethics statement

Female 8 weeks ICR mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were handled in accordance with the institutional animal care policies of the Institute of Zoology, Chinese Academy of Sciences. Mice were maintained under a 12-h light and 12-h darkness cycle in a specific pathogen-free stage at the Central Animal Laboratory of the Institute of Zoology. The Laboratory Animal Care and Use Committee of the Institute of Zoology approved this study.
Oocytes Collection And Parthenogenetic Activation
Female mice were injected with 10 IU pregnant mare serum gonadotropin (PMSG, Ningbo Hormone Product Co. Ltd., Cixi, China) followed 44 to 48 hours later by injection of 10 IU human chorionic gonadotropin (HCG, Ningbo Hormone Product Co. Ltd., Cixi, China). After 15 h of HCG, ovulated cumulus-oocyte complexes (COCs) were collected and denuded in 1 mg/ml hyaluronidase. \([\text{Ca}^{2+}]_i\) oscillations induced by 10 mM strontium chloride (SrCl\(_2\), 10025-70-4, Sangon Biotech, Shanghai, China) in Ca\(^{2+}\)-free CZB incubating medium containing various Ca\(^{2+}\) channel modulators four hours. After parthenogenetic activation, embryos were moved into KSOM (MR-106, Merck Millipore, USA) for further culture.

Real-time Recording Of \([\text{Ca}^{2+}]_i\) Changes
Oocyte \([\text{Ca}^{2+}]_i\) oscillations were detected using 2 µg/mL Fluo-4 AM (488 nm excitation, 525 nm emission) in the partheno-activation solution. Real-time images after parthenogenetic activation of oocytes which were co-incubated with various Ca\(^{2+}\) channel modulators were obtained using a time-lapse confocal laser microscope (UltraVIEW-VoX; PerkinElmer, MA, USA) and recorded at 2 frames per minute. \([\text{Ca}^{2+}]_i\) intensity was detected using an argon laser. A software Volocity was used to analyze fluorescence intensity.

Real-time Recording Of Oocyte Mitochondrial Potential
In our study, we set out to confirm the \([\text{Ca}^{2+}]_i\) oscillations regulatory function in mitochondrial activity during oocyte activation. Oocytes were denuded, and pre-incubated in M2 culture medium (M7167, Sigma-Aldrich, USA) with 2 µg/mL JC-1 (C2005, Beyotime, Beijing, China), a cell permeable voltage-sensitive fluorescent mitochondrial dye, for 15 minutes. In order to reduce the toxicity of JC-1 for long-term observation under laser confocal microscopy, we reduced the concentration of JC-1 to 0.5 µg/ml in the activating solution. Briefly, the lower potential mitochondria were imaged as JC-1 monomer emitting in green (488 nm excitation, 561 nm emission), and energized highly potential mitochondria were imaged in aggregated JC-1 that emits in red (488 nm excitation, 561 nm emission) as fluorescence transporters. Oocyte mitochondrial potential dynamic changes after treatment with
various Ca2+ channel modulators were recorded after partenogenetic activation using a time-lapse confocal laser microscope (UltraVIEW-VoX; PerkinElmer, MA, USA) at identical magnification and gain settings throughout the experiments. A software Volocity was used to analyze fluorescence intensity.

Statistical Analysis
All experiments were conducted at least three times. No less than 40 oocytes were collected and examined in each group each time. We presented information of samples with Means and Standard Deviations (SD). Results were analyzed by SPSS 19.0. The significance of differences among groups was analyzed by the Chi-square test, and p values less than 0.05 were considered statistically significant.

Results
Inhibitors of Ca2+ channels affect the efficiency of oocyte activation
We firstly examined the dose-dependent effects of inhibitors of SERCAs, TRPM7, T-type Ca2+ channels and Orai1 on the efficiency of SrCl2-induced oocyte parthenogenetic activation and found a suitable concentration. The percentages of survival oocytes showing clear cell membrane and high cytoplasmic refraction after activation, pronuclear formation and 2-cell cleaved embryos were shown in Table 1. We applied Thapsigargin (10522, Cayman, Michigan, USA), a SERCAs inhibitor, with gradient concentrations of 0.5, 1, and 10 µM. When its concentration rose to 10 µM, a suggested working concentration by manufacturer, [Ca2+]i would rise slowly, and oocytes died during the activation process. NS-8593 (N-195, Alomone, Jerusalem BioPark, Jerusalem, Israel), a TRPM7 inhibitor, was applied with gradient concentrations of 0.1, 1, and 5 µM. The oocyte survival rate was 37.5% when treating with 1 µM NS-8593. When treating with 5 µM NS-8593, half of suggested concentration by manufacturer, all oocytes died in a short time. Mibefradil (Mib, HY-15553, MCE, NJ 08852, USA), a T-type Ca2+ channels blocker, was added with gradient concentrations of 0.5, 5, and 10 µM. Under suggested concentration of 10 µM, activation of most oocytes was blocked. When applying GSK-7975A (HY12507, MCE, NJ 08852, USA), an Orai1blocker, with gradient concentrations of 10, 100 µM, and 1 mM. Oocytes were still activated at concentrations of 10 µM and 100 µM GSK-7975A. Pronuclear formation was suppressed significantly when GSK-7975A reached 1 mM, but this
concentration had severe cytotoxicity. We found that SERCAs, TRPM7 and T-type Ca$^{2+}$ channels played an important role in activation, while inhibition of Orai1 does not seem to affect oocyte activation. In our subsequent experiments, we selected a moderate concentration of inhibitors to observe the effect of [Ca$^{2+}$]$_i$ oscillations on mitochondrial dynamic potential.

### Table 1
Effect of calcium channel inhibitors on oocyte parthenogenetic activation

| Inhibitor | Concentration | No. oocytes | Repeats | PA No. | PN No. | 2-Cell |
|-----------|---------------|-------------|---------|--------|--------|--------|
| Ctrl      |               | 240         | 5       | 98.33 ± 1.24% | 93.25 ± 5.32% | 82.90 ± 7.53% |
| Thapsigargin (Tha) | 0.5 µM | 240 | 5 | 84.2 ± 5.72% | 65.7 ± 9.56%* | 67.6 ± 7.59% |
|           | 1 µM         | 240         | 5       | 66.2 ± 9.24% | 42.1 ± 8.56%* | 26.1 ± 12.45%* |
|           | 10 µM        | 240         | 5       | 0       | X      | X |
| NS-8593   | 0.1 µM       | 240         | 5       | 83.3 ± 6.28% | 75.2 ± 9.57% | 86.7 ± 7.50% |
|           | 1 µM         | 240         | 5       | 37.5 ± 12.53%* | 52.9 ± 7.84%* | 57.7 ± 11.56%* |
|           | 5 µM         | 240         | 5       | 0       | X      | X |
| Mibefradil (Mib) | 0.5 µM | 240 | 5 | 77.3 ± 5.74% | 55.6 ± 10.60% | 67.5 ± 8.82% |
|           | 5 µM         | 240         | 5       | 36.7 ± 5.68%* | 42.3 ± 5.93%* | 66.4 ± 10.34% |
|           | 10 µM        | 240         | 5       | 3.6 ± 2.40%* | 0      | X |
| GSK-7975  | 10 µM        | 240         | 5       | 95.4 ± 3.21% | 84.5 ± 6.34% | 82.7 ± 7.26% |
|           | 100 µM       | 240         | 5       | 83.8 ± 5.21% | 82.6 ± 5.83% | 58.4 ± 11.57% |
|           | 1 mM         | 240         | 5       | 63.5 ± 7.95%* | 0      | X |

Note: Tha indicates SERCAs inhibitor Thapsigargin. NS-8593 is a TRPM7 specific inhibitor. Mib indicates T-type Ca$^{2+}$ channels inhibitor Mibebradil. GSK-7975 is an Orai1 specific inhibitor. PA indicates survival embryos of all activated oocytes after activation. PN indicates pronuclear embryos from surviving oocytes. 2-Cell indicates cleaved embryos in pronuclear fertilized eggs. "X" indicates none available data. The significance of differences between Inhibitors and Ctrl group were analyzed by the Chi-square test and p<0.05 (*) was considered statistically significant.

[Ca$^{2+}$]$_i$ And Mitochondrial Potential Dynamic Changes During Activation

Cytoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) oscillations and mitochondrial dynamic potential changes during the activation of oocytes were observed by time-lapse confocal laser microscopy (UltraVIEW-VoX, PerkinElmer, Massachusetts, USA). Normal [Ca$^{2+}$]$_i$ oscillations were induced by SrCl$_2$ stimulation (Fig. 1A). We found that mitochondria were activated from the cortex to the internal area of the oocyte and finally aggregated around the chromosomes (Fig. 1B). In order to analyze changes in oocyte mitochondrial membrane potential, a relative fluorescence intensity analysis and a high membrane potential/low membrane potential ratio were introduced. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). The overall membrane potential showed two elevated peaks and then returned to a basal level (Fig. 1C, D).

Effect Of SERCAs Inhibitor Thapsigargin On Oocyte Activation

Endoplasmic reticulum (ER) is a major Ca$^{2+}$ storage component in the cytoplasm. SERCAs are the
main transporters for cytoplasmic Ca\textsuperscript{2+} refilling to the ER. We wanted to observe changes in [Ca\textsuperscript{2+}]\textsubscript{i} oscillations and mitochondrial activity during oocyte activation after the Ca\textsuperscript{2+} refill was blocked by SERCAs inhibitor Thapsigargin (Tha) (Fig. 2). The survival rate was 66.2\% when treating with 1 µM Tha (Table 1), by which concentration [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (Fig. 2A) and pronuclear formation were sufficiently suppressed. Mitochondrial membrane potential continued to decrease under Ca\textsuperscript{2+} refill inhibition by 1 µM Tha (Fig. 2B). Under suggested working concentration by manufacturer, 10 µM Tha will completely inhibit Ca\textsuperscript{2+} refill into ER, and oocyte will die during activation (Fig. 2A). Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). Thus, Ca\textsuperscript{2+} refilling to ER through SERCAs plays an important role in [Ca\textsuperscript{2+}]\textsubscript{i} oscillations and mitochondrial activation during oocyte activation.

**Effect Of TRPM7 Inhibitor NS-8593 On Oocyte Activation**

TRPM7 is highly expressed in the GV and MII oocytes, mainly distributed on the plasma membrane. We inhibited TRPM7 activity by its specific inhibitor NS-8593 to study its role in oocyte activation (Fig. 3). The [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (Fig. 3A) and pronuclear formation were sufficiently suppressed when 1 µM NS-8593 was used to inhibit the transport of Ca\textsuperscript{2+} through TRPM7. The dynamic mitochondrial membrane potential of oocyte was observed after treatment with 1 µM NS-8593 (Fig. 3B). At this concentration, mitochondrial membrane potential continually decreased. Depolarization of mitochondria was found in the case of long-term inhibition (Fig. 3B). When treating with 5 µM NS-8593, half of suggested concentration given by manufacturer, [Ca\textsuperscript{2+}]\textsubscript{i} slowly rose and finally induced oocyte death. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). Thus, [Ca\textsuperscript{2+}]\textsubscript{i} oscillations regulated through TRPM7 are important for mitochondrial activity during oocyte activation.

**Effect of T-type Ca\textsuperscript{2+} channels inhibitor Mibefradil on oocyte activation**

T-type Ca\textsuperscript{2+} channels include Cav3.1, Cav3.2, and Cav3.3 subtypes are distributed on the plasma membrane. Mibefradil can suppress all three T-type Ca\textsuperscript{2+} channels. Mibefradil was introduced to
study the role of T-type Ca\textsuperscript{2+} channels in [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (Fig. 4). The mitochondrial membrane potential was observed in the 5 µM group, half of suggested concentration by manufacturer. We found that the mitochondrial membrane potential continued to decrease after inhibiting the transport of Ca\textsuperscript{2+} through T-type Ca\textsuperscript{2+} channels. When the concentration was increased to 10 µM, Ca\textsuperscript{2+} slowly increased, which induced rapid death of most oocytes. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). Based on these results it can be concluded that the transport of Ca\textsuperscript{2+} through T-type Ca\textsuperscript{2+} channels is important for [Ca\textsuperscript{2+}]\textsubscript{i} oscillations and mitochondrial activity in oocyte activation and survival.

Effect Of Orai1 Inhibitor GSK-7975a On Oocyte Activation

Orai1 is distributed on the plasma membrane. We studied the role of Orai1 in oocyte activation by treating oocytes with the specific inhibitor GSK-7975A. The mitochondrial membrane potential was observed at 1 mM, which showed irregular changes compared to the Ctrl group (Fig. 5). Most oocytes survived until 4 hours of activation, but none of them formed a pronucleus due to cytotoxicity. Ordinate is marked as Relative Fluorescence Intensity (relative to the fluorescence intensity of the start point). It was not confirmed whether this [Ca\textsuperscript{2+}]\textsubscript{i} oscillation pattern and mitochondria membrane potential changes were caused by complete inhibition of Orai1 or by cytotoxicity induced by the high concentration of 1 mM GSK-7975A. The role of Orai1 in the activation of oocytes requires additional evidence to confirm.

Effects Of Inhibitors On Long-lasting [Ca\textsuperscript{2+}]\textsubscript{i} Oscillations

The mechanisms of initiation and maintenance of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations are believed to be different. However, it is unclear which transporters participate in the initiation or maintenance. The effects of several transporters on the maintenance of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations were investigated in our study (Fig. 6). By using time-lapse confocal laser microscopy we observed the effect of various inhibitors on the maintenance of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations after some initial oscillations (Fig. 6A). The same concentration was applied as in the mitochondrial membrane potential observation study mentioned above.
Ruthenium Red (MACKLIN, Beijing, China) completely inhibited oocyte activation as a non-specific cation channels inhibitor. It was found that the addition of Ruthenium Red, Thapsigargin and GSK-7975A inhibited Ca^{2+} transport, and the [Ca^{2+}]_{i} oscillations were blocked from the time of inhibitor addition. After addition of Mibefradil and NS-8593, the intracellular Ca^{2+} increased continuously, and the cell death rate was higher than in the ctrl and other groups. Based on these results it can be concluded that the three Ca^{2+} transporters, SERCAs, TRPM7 and T-type Ca^{2+} channels, are not only involved in [Ca^{2+}]_{i} oscillation initiation but also in [Ca^{2+}]_{i} oscillations maintenance. Interestingly, the effect of Ruthenium Red and other inhibitor additions into culture of oocytes which had already initiated [Ca^{2+}]_{i} oscillations in oocytes did not significantly influence oocyte activation (Fig. 6B), suggesting that oocyte activation and pronuclear formation require only a small amount of [Ca^{2+}]_{i} oscillations. The function of long-lasting [Ca^{2+}]_{i} oscillations in embryo development needs further study.

Discussion
Obese, diabetic and aging women typically suffer from abnormal body metabolism such as hypertension, hyperglycemia and hyperlipidemia [11–16], causing long-term stress in oocytes [17], which severely damages the quality of oocytes, thereby leading to lower pregnancy rate [18]. Insufficient oocyte activation and mitochondrial damage were considered to be major causes for embryo developmental disorders[19, 20].

Ca^{2+} is one of the major signal molecules that regulate various cell functions including cell cycle progression, arrest and apoptosis. Oocyte activation induces a continuous series of oocyte intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) increases and decreases known as [Ca^{2+}]_{i} oscillations, which encode oocyte activation events, including liberation from the MII arrest, pronucleus formation and the transition to embryo development [21]. Release of Ca^{2+} from internal stores and Ca^{2+} influx from the extracellular matrix induce moderate increases in [Ca^{2+}]_{i} levels. The increase of [Ca^{2+}]_{i}
generally lasts about 2 minutes. As Ca\textsuperscript{2+} refills back to the ER or efflux from the cytoplasm to prepare for the next peak of oscillation, the elevated [Ca\textsuperscript{2+}]\textsubscript{i} will return to baseline levels, resulting in an average of a ten to twenty minutes resting interval. [Ca\textsuperscript{2+}]\textsubscript{i} oscillations will last 5–6 hours until pronuclear formation [9]. The repeated elevation and recovery of Ca\textsuperscript{2+} signaling is tightly regulated, and the strictly ordered Ca\textsuperscript{2+} signal will coordinate the interaction of various organelles in the oocyte for its activation. Ca\textsuperscript{2+} transporters and regulators could become potential therapy targets for infertile women especially for in vitro fertilization failure.

During activation, Ca\textsuperscript{2+} shuttles through the cell, and the transporter is likely to be located near IP3R and downstream organelles [22]; the cell membrane, lysosomes, the nucleus, vesicles and mitochondria may be targets of Ca\textsuperscript{2+} release. Endoplasmic reticulum (ER) is a main intracellular Ca\textsuperscript{2+} store, where the Ca\textsuperscript{2+} concentration increases to 300 or even 1,000 µM [23]. The pathway for Ca\textsuperscript{2+} efflux from the ER into the cytoplasm has not yet been well identified. SOCE or Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channels (CRAC), were first described in immune cells where they have been shown to be critical for their function. Accordingly, defects in SOCE in humans are associated with severe immune-deficiencies [24]. In oocytes, the predominant [Ca\textsuperscript{2+}]\textsubscript{i} increase pathway appears to be achieved through store-operated Ca\textsuperscript{2+} entry (SOCE) processes. Ca\textsuperscript{2+} enters the cytosol from the endoplasmic reticulum (ER), which in turn opens one of ER channel, sarcoplasmic reticulum/ER Ca-ATPase (SERCAs), to transport Ca\textsuperscript{2+} back to ER [25]. Total cellular Ca\textsuperscript{2+} was estimated by the addition of 10 µM Thapsigargin (Tha), a SERCAs inhibitor, which induced complete release of Ca\textsuperscript{2+} from ER [26]. When ER Ca\textsuperscript{2+} stores had been significantly depleted by Tha, sperm no longer triggered [Ca\textsuperscript{2+}]\textsubscript{i} oscillations [27]. In our study, we used three gradient concentrations of 0.5, 1 and 10 µM of Tha. Ten µM Tha kept oocytes at a higher [Ca\textsuperscript{2+}]\textsubscript{i} and all oocytes died before the end of the activation process (Fig. 2). However, when oocyte [Ca\textsuperscript{2+}]\textsubscript{i} oscillations were suppressed by 1 µM Tha, more than half of
the oocytes survived more than 4 hours. Under such moderate concentration, the effects of SERCAs on mitochondrial activity can be observed for a long term. Oocyte mitochondria membrane potential continued to decrease under Ca\textsuperscript{2+} refilling inhibition with 1 µM Tha incubation. Not only [Ca\textsuperscript{2+}]\textsubscript{i} oscillations but also mitochondria activity were suppressed by Tha that induced blocking of ER Ca\textsuperscript{2+} refilling. Finally, oocytes cannot be activated under SERCAs inhibition.

Recently, a member of the TRP channels family, TRPM7, was found to be expressed in mouse GV, MII oocytes and 2-cell embryos [28]. TRPM7 belongs to the subfamily of melastatin and exhibits a ubiquitous tissue distribution. Trpm7 knock-out caused E14.5 embryonic lethality [29]. Using inhibitor NS-8593 suppression of the transporter hours after activation reduced progression to the blastocyst stage [28]. Oocytes treated with 10 µM NS-8593 and fertilized in vitro display impaired Ca\textsuperscript{2+} oscillations [30]. We applied NS-8593 with gradient concentrations of 0.1, 1, and 5 µM (Table 1 and Fig. 3A). After treating with 5 µM NS-8593, [Ca\textsuperscript{2+}]\textsubscript{i} slowly rose, which finally induced oocyte death.

Treatment with 1 µM NS-8593 kept the survival rate at 37.5% (Fig. 3), while the [Ca\textsuperscript{2+}]\textsubscript{i} oscillations and pronuclear formation were sufficiently suppressed. Under such moderate concentration of 1 µM NS-8593, the effects of TRPM7 on mitochondrial activity can be observed for a long term.

Mitochondrial activity did not exhibit the same activation state as the Ctrl in the case of TRPM7 inhibition with NS-8593. Mitochondrial membrane potential continually decreased when inhibiting the transport of Ca\textsuperscript{2+} through TRPM7 with 1 µM NS-8593 (Fig. 3). The TRPM7 on the cell membrane of oocytes has a significant effect on the [Ca\textsuperscript{2+}]\textsubscript{i} oscillation patterns in oocytes, and [Ca\textsuperscript{2+}]\textsubscript{i} oscillations achieved through TRPM7 is important for mitochondrial activity and oocyte activation.

Mibefradil is a T-type channel inhibitor. Mibefradil was developed as a cardiovascular hypertension and angina remedy [31]. Mibefradil has been repurposed as an anti-cancer drug [32]. However, its underlying mechanisms are still unclear. The mechanism of the anti-cancer therapy is thought to be via the blockage of Ca\textsuperscript{2+} influx through T-type channels. Mibefradil blocks all three subtypes of T-type channels, including Cav3.1, Cav3.2, and Cav3.3, with an IC50 (Semi-lethal concentration) of 5.8-
7.2 µM. In our study, inhibition of Ca\(^{2+}\) release by high concentrations of Mibefradil impaired intracellular Ca\(^{2+}\) dynamics and thus affected cell viability. We applied Mibefradil at three gradient concentrations of 0.5, 5, and 10 µM (Table 1 and Fig. 4). In order to observe for a long term, a moderate concentration of 5 µM NS-8593 was selected to study the effects of T-type channels on mitochondrial activity. The mitochondrial membrane potential continued to decrease after inhibiting the transport of Ca\(^{2+}\) through T-type Ca\(^{2+}\) channels with 5 µM Mibefradil. When the concentration reached 10 µM, Ca\(^{2+}\) rapidly increased, which induced rapid death in most oocytes. Thus, transport of Ca\(^{2+}\) through T-type Ca\(^{2+}\) channels is important for mitochondrial activity and oocyte activation.

Orais are 4 transmembrane proteins that form highly Ca\(^{2+}\)-selective channels [33]. Orais has three family members, ORAI1, 2 and 3 [34]. Loss-of-function mutation of ORAI1 caused immune deficiency [35] and dysfunction of thrombus formation [36]. SOCE is also mediated through the ORAI channels at the outer membrane. 10 µM GSK-7975A has been reported to induce maximal inhibition of Ca\(^{2+}\) influx in Jurkat T-cells [37]. We applied GSK-7975A at gradient concentrations of 10, 100 µM, and 1 mM. We found that oocytes could not be activated when GSK-7975A below 1 mM was used to effectively inhibit Ca\(^{2+}\) influx. Mitochondrial dynamic membrane potential showed irregular changes compared to the Ctrl group (Fig. 5). Most of the oocytes survived up to 4 hours post activation even at a concentration as high as 1 mM, but none of them formed a pronucleus. Female mice were fertile after knocking out ORAI1 [30]. It is not yet clear whether this \([\text{Ca}^{2+}]_i\) oscillation pattern and membrane potential changes were caused by complete inhibition of Orai1 or by cytotoxicity induced by the high concentration of 1 mM. The role of Orai1 in the activation of oocytes requires additional evidence to confirm.

The mechanisms of initiation and maintenance of \([\text{Ca}^{2+}]_i\) oscillations are different [38]. However, it is unclear which transporters participate in the initiation or maintenance of \([\text{Ca}^{2+}]_i\) oscillations. The effects of several transporters on the maintenance of \([\text{Ca}^{2+}]_i\) oscillations were investigated in our
study (Fig. 6). We found that the addition of Ruthenium Red, Thapsigargin and GSK-7975A all inhibited the maintenance of \([Ca^{2+}]_i\) oscillations. After addition of Mibefradil and NS-8593, the intracellular \(Ca^{2+}\) increased continuously, and the cell death rate was higher than in the ctrl and other groups. All these results suggest that three \(Ca^{2+}\) transporters, SERCAs, TRPM7 and T-type \(Ca^{2+}\) channels are involved in both the initiation and maintenance of \([Ca^{2+}]_i\) oscillations. Interestingly, the addition of Ruthenium Red and other inhibitors into culture of oocytes which had initiated \([Ca^{2+}]_i\) oscillations did not significantly influence oocyte activation (Fig. 6B), suggesting that oocyte activation required only a small amount of \([Ca^{2+}]_i\) oscillations.

In summary, we applied ER-associated \(Ca^{2+}\) transporter SERCAs inhibitor Thapsigargin, TRPM7 inhibitor NS-8593, T-type \(Ca^{2+}\) channels inhibitor Mibefradil, and Orai1 inhibitor GSK-7975A to understand the regulation of \([Ca^{2+}]_i\) oscillations and mitochondrial activity during oocyte activation, and showed inhibition of SERCAs, TRPM7 and T-type \(Ca^{2+}\) channels caused \(Ca^{2+}\) signaling disturbances, mitochondrial activity and subsequent oocyte activation and embryonic development.

### Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| GV           | Germinal Vesicle |
| GVBD         | Germinal Vesicle Breakdown |
| MII          | Metaphase II |
| \([Ca^{2+}]_i\) | Intracellular \(Ca^{2+}\) Concentration |
| \([Ca^{2+}]_m\) | Mitochondrial Matrix \(Ca^{2+}\) Concentration |
| SERCAs       | sarcoplasmic/endoplasmic reticulum \(Ca^{2+}\) ATPases |
| TRPM7        | transient receptor potential (TRP) ion channel subfamily member 7 |
| ER           | Endoplasmic reticulum |
| ART          | assisted reproductive technologies |

### Declarations

**Consent for publication**

The manuscript is approved by all authors for publication, and it is original research that has not been published previously, and is not being submitted to any other journal in whole or in part.

**Availability of Supporting Data**

Some or all data used during the study are available from the corresponding author by request.

**Competing Interests**
I would like to state that there is no conflict of interest or financial disclosure to declare for this manuscript.

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**Author contributions**

Feng Wang, Qing-Yuan Sun and Xiang-Hong Ou conceived and designed the experiments. Feng Wang and others conducted experiments. Feng Wang and Qing-Yuan Sun analyzed the data. Feng Wang, Heide Schatten, Qing-Yuan Sun and Xiang-Hong Ou wrote the manuscript.

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**References**

1. **Chow KM, Cheung MC, Cheung IK.** Psychosocial interventions for infertile couples: a critical review. *J Clin Nurs* 2016; 25: 2101-13.

2. **Schatten H, Sun QY, Prather R.** The impact of mitochondrial function/dysfunction on IVF and new treatment possibilities for infertility. *Reprod Biol Endocrinol* 2014; 12: 111.

3. **Stricker SA.** Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol* 1999; 211: 157-76.

4. **Wakai T, Zhang N, Vangheluwe P, Fissore RA.** Regulation of endoplasmic reticulum Ca(2+) oscillations in mammalian eggs. *J Cell Sci* 2013; 126: 5714-24.
5. **Yazawa H, Yanagida K, Sato A.** Human round spermatids from azoospermic men exhibit oocyte-activation and Ca2+ oscillation-inducing activities. *Zygote* 2007; 15: 337-46.

6. **Carvacho I, Piesche M, Maier TJ, Machaca K.** Ion Channel Function During Oocyte Maturation and Fertilization. *Front Cell Dev Biol* 2018; 6: 63.

7. **Marchi S, Patergnani S, Missiroli S, Morciano G, Rimessi A, Wieckowski MR, Giorgi C, Pinton P.** Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. *Cell Calcium* 2018; 69: 62-72.

8. **Miao YL, Stein P, Jefferson WN, Padilla-Banks E, Williams CJ.** Calcium influx-mediated signaling is required for complete mouse egg activation. *Proc Natl Acad Sci U S A* 2012; 109: 4169-74.

9. **Wang F, Yuan RY, Li L, Meng TG, Fan LH, Jing Y, Zhang RR, Li YY, Liang QX, Dong F, Hou Y, Schatten H, Sun QY, Ou XH.** Mitochondrial regulation of [Ca(2+)]i oscillations during cell cycle resumption of the second meiosis of oocyte. *Cell Cycle* 2018: 1-16.

10. **Shimizu H, Schredelseker J, Huang J, Lu K, Naghdi S, Lu F, Franklin S, Fiji HD, Wang K, Zhu H, Tian C, Lin B, Nakano H, Ehrlich A, Nakai J, Stieg AZ, Gimzewski JK, Nakano A, Goldhaber JL, Vondriska TM, Hajnoczky G, Kwon O, Chen JN.** Mitochondrial Ca(2+) uptake by the voltage-dependent anion channel 2 regulates cardiac rhythmicity. *Elife* 2015; 4.

11. **Minge CE, Bennett BD, Norman RJ, Robker RL.** Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone reverses the adverse effects of diet-induced obesity on oocyte quality. *Endocrinology* 2008; 149: 2646-56.

12. **Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, McConnell J.** Maternal diet-induced obesity alters mitochondrial activity and redox
status in mouse oocytes and zygotes. *PLoS One* 2010; 5: e10074.

13. **Jungheim ES, Schoeller EL, Marquard KL, Louden ED, Schaffer JE, Moley KH.** Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. *Endocrinology* 2010; 151: 4039-46.

14. **Wang F, Chen Z, Ren X, Tian Y, Liu C, Jin P, Li Z, Zhang F, Zhu B.** Hormone-sensitive lipase deficiency alters gene expression and cholesterol content of mouse testis. *Reproduction* 2017; 153: 175-185.

15. **Shankar K, Zhong Y, Kang P, Lau F, Blackburn ML, Chen JR, Borengasser SJ, Ronis MJ, Badger TM.** Maternal obesity promotes a proinflammatory signature in rat uterus and blastocyst. *Endocrinology* 2011; 152: 4158-70.

16. **Luzzo KM, Wang Q, Purcell SH, Chi M, Jimenez PT, Grindler N, Schedl T, Moley KH.** High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal growth retardation/brain defects. *PLoS One* 2012; 7: e49217.

17. **Wang F, Ren X-F, Chen Z, Li X-L, Zhu H-J, Li S, Ou X-H, Zhang C, Zhang F-X, Zhu B-C.** The N-terminal His-tag affects the triglyceride lipase activity of hormone-sensitive lipase in testis. *Journal of Cellular Biochemistry* 2019; 0.

18. **Jungheim ES, Schon SB, Schulte MB, DeUgarte DA, Fowler SA, Tuuli MG.** IVF outcomes in obese donor oocyte recipients: a systematic review and meta-analysis. *Hum Reprod* 2013; 28: 2720-7.

19. **Gesink Law DC, Maclehose RF, Longnecker MP.** Obesity and time to pregnancy. *Hum Reprod* 2007; 22: 414-20.

20. **Rittenberg V, Sobaleva S, Ahmad A, Oteng-Ntim E, Bolton V, Khalaf Y, Braude P, El-Toukhy T.** Influence of BMI on risk of miscarriage after single blastocyst transfer. *Hum Reprod* 2011; 26: 2642-50.

21. **Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R,
Madoux S, Ozil JP. Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. Dev Biol 2002; 250: 280-91.

22. Foskett JK, White C, Cheung KH, Mak DO. Inositol trisphosphate receptor Ca2+ release channels. Physiol Rev 2007; 87: 593-658.

23. Bonora M, Giorgi C, Bononi A, Marchi S, Paternagnani S, Rimessi A, Rizzuto R, Pinton P. Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes. Nat Protoc 2013; 8: 2105-18.

24. Bogeski I, Al-Ansary D, Qu B, Niemeyer BA, Hoth M, Peinelt C. Pharmacology of ORAI channels as a tool to understand their physiological functions. Expert Rev Clin Pharmacol 2010; 3: 291-303.

25. Carroll J. The initiation and regulation of Ca2+ signalling at fertilization in mammals. Semin Cell Dev Biol 2001; 12: 37-43.

26. Thastrup O, Dawson AP, Scharff O, Foder B, Cullen PJ, Drobak BK, Bjerrum PJ, Christensen SB, Hanley MR. Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents Actions 1989; 27: 17-23.

27. Kline D, Kline JT. Thapsigargin activates a calcium influx pathway in the unfertilized mouse egg and suppresses repetitive calcium transients in the fertilized egg. J Biol Chem 1992; 267: 17624-30.

28. Carvacho I, Ardestani G, Lee HC, McGarvey K, Fissore RA, Lykke-Hartmann K. TRPM7-like channels are functionally expressed in oocytes and modulate post-fertilization embryo development in mouse. Sci Rep 2016; 6: 34236.

29. Jin J, Wu LJ, Jun J, Cheng X, Xu H, Andrews NC, Clapham DE. The channel kinase, TRPM7, is required for early embryonic development. Proc Natl Acad Sci U S A 2012; 109: E225-33.

30. Bernhardt ML, Padilla-Banks E, Stein P, Zhang Y, Williams CJ. Store-operated
Ca(2+) entry is not required for fertilization-induced Ca(2+) signaling in mouse eggs. *Cell Calcium* 2017; 65: 63-72.

31. Lee DS, Goodman S, Dean DM, Lenis J, Ma P, Gervais PB, Langer A, Investigators P. Randomized comparison of T-type versus L-type calcium-channel blockade on exercise duration in stable angina: results of the Posicor Reduction of Ischemia During Exercise (PRIDE) trial. *Am Heart J* 2002; 144: 60-7.

32. Li P, Rubaiy HN, Chen GL, Hallett T, Zaibi N, Zeng B, Saurabh R, Xu SZ. T-type Ca(2+) channel blocker mibefradil blocks ORAI channels via acting on extracellular surface. *Br J Pharmacol* 2019.

33. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG. Orai1 is an essential pore subunit of the CRAC channel. *Nature* 2006; 443: 230-3.

34. Prakriya M, Lewis RS. Store-Operated Calcium Channels. *Physiol Rev* 2015; 95: 1383-436.

35. Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 2006; 441: 179-85.

36. Bergmeier W, Stefanini L. Novel molecules in calcium signaling in platelets. *J Thromb Haemost* 2009; 7 Suppl 1: 187-90.

37. Rice LV, Bax HJ, Russell LJ, Barrett VJ, Walton SE, Deakin AM, Thomson SA, Lucas F, Solari R, House D, Begg M. Characterization of selective Calcium-Release Activated Calcium channel blockers in mast cells and T-cells from human, rat, mouse and guinea-pig preparations. *Eur J Pharmacol* 2013; 704: 49-57.

38. Ferrer-Buitrago M, Bonte D, De Sutter P, Leybaert L, Heindryckx B. Single Ca(2+) transients vs oscillatory Ca(2+) signaling for assisted oocyte activation: limitations and benefits. *Reproduction* 2018; 155: R105-R119.
[Ca2+]i and mitochondrial membrane potential of wildtype activated oocytes. (A)

Cytoplasmic calcium concentrations ([Ca2+]i) showing dynamic changes in wildtype oocytes during activation. (B) Oocyte mitochondrial membrane potential of wildtype oocytes during activation. (C) Membrane potential fluorescence intensity during oocyte activation. The green and red curves represent labeling with JC-1, indicating relative fluorescence intensities of low membrane potential (488nm excitation, 525nm emission) and high membrane potential (561 excitation, 590 emission), respectively. (D) The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.
Effect of SERCAs inhibitor Thapsigargin on oocyte activation. (A) Cytoplasmic ([Ca2+]i) dynamic changes of Thapsigargin-inhibited oocyte during activation. (B) Mitochondrial membrane potential of 1μM Thapsigargin-inhibited oocytes during activation. (C) Mitochondrial membrane potential fluorescence intensity of 1μM Thapsigargin-inhibited oocytes. The green and red curves represent labeling with JC-1, indicating relative fluorescence intensities of low membrane potential and high membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.
Figure 3

Effect of TRPM7 inhibitor NS-8593 on oocyte activation. (A) [Ca2+]i oscillations of NS-8593-inhibited oocytes. (B) Mitochondrial membrane potential dynamic changes of 1μM NS-8593-inhibited oocytes. (C) Mitochondrial membrane potential fluorescence intensity of 1μM NS-8593-inhibited oocytes. The green and red curves represent low and high membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.
Figure 4

Effect of T-type Ca2+ channel inhibitor Mibefradil on oocyte activation. (A) [Ca2+]i oscillations of Mibefradil-inhibited oocytes. (B) Mitochondrial membrane potential dynamic changes of 5μM Mibefradil-inhibited oocytes. (C) Mitochondrial membrane potential fluorescence intensity of 5μM Mibefradil-inhibited oocytes. The green and red curves represent low and high membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.
Figure 5

Effect of Orai1 inhibitor GSK-7975A on oocyte activation. (A) [Ca2+]i oscillations of GSK-7975A-inhibited oocytes. (B) Mitochondrial membrane potential dynamic changes of 100μM GSK-7975A-inhibited oocytes. (C) Mitochondrial membrane potential fluorescence intensity of 100μM GSK-7975A-inhibited oocytes. The green and red curves represent low and high membrane potential, respectively. The black curve shows the ratio of high membrane potential to low membrane potential indicating relative mitochondrial membrane potential.
Inhibitor effects on long-lasting [Ca\(^{2+}\)]\(_i\) oscillations. (A) Cytoplasmic [Ca\(^{2+}\)]\(_i\) dynamic changes after inhibitor addition. (B) Development of inhibitor addition following [Ca\(^{2+}\)]\(_i\) oscillations initiation. PA indicates survival of embryos of all MII oocytes. PN indicates pronuclear embryos of surviving oocytes. 2-Cell indicates cleaved embryos of pronuclear embryos. RR: 10\(\mu\)M Ruthenium Red; Tha: 1\(\mu\)M Thapsigargin; GSK: 100\(\mu\)M GSK-7975A; Mib: Mibebradil; N: 1\(\mu\)M NS-8593. The significance of differences between groups was analyzed by the Chi-square test and \(p<0.05\) (*) was considered statistically significant. (X) indicates data unavailable.
