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The establishment of appropriate methods for egg-activation by human PLCZ1 RNA injection into human oocyte

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ABSTRACT

Phospholipase C-zeta (PLCZ1), a strong candidate of egg-activating sperm factor, can induce Ca^{2+} oscillations and cause egg activation. For the application of PLCZ1 to clinical use, we examined the pattern of Ca^{2+} responses and developmental rate by comparing PLCZ1 RNA injection methods with the other current methods, such as cytosolic aspiration, electrical stimulation and ionomycin treatment in human oocytes. We found that the pattern of Ca^{2+} oscillations after PLCZ1 RNA injection exhibited similar characteristics to that after ICSI treatment. We also determined the optimal concentration of human PLCZ1 RNA to activate the human oocytes. Our findings suggest that human PLCZ1 RNA is a better therapeutic agent to rescue human oocytes from failed activation, leading to normal and efficient development.

1. Introduction

At fertilization, mammalian oocytes show repetitive transient increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$), known as Ca^{2+} oscillations. Each of which is due to Ca^{2+} release from the endoplasmic reticulum (ER) mainly through type 1 inositol 1,4,5-trisphosphate receptor (InsP3R) [1–4]. Each $[Ca^{2+}]_i$ rise lasts about 1 min and the Ca^{2+} transients occur at intervals of 5–30 min [1,5]. The Ca^{2+} oscillations are a pivotal signal for egg activation and embryo development [6,7]. They cause resumption of the second meiosis and subsequent formation of male and female pronuclei (PN).

Repetitive Ca^{2+} release is induced by a cytosolic sperm factor driven into the ooplasm upon sperm-egg fusion [8]. Several lines of evidence indicate that a sperm-specific isozyme "zeta" of InsP3-producing enzyme phospholipase C (PLCZ1) is a strong candidate to be the sperm factor [1,9–12]. Depleting PLCZ1 from sperm extract by anti-PLCZ1 antibody abolished the Ca^{2+} oscillation-inducing activity [9]. Expressing PLCZ1 in the oocyte by RNA injection induced Ca^{2+} oscillations and the egg activation [9,12]. Recombinant PLCZ1 protein injected in the oocyte could elicit Ca^{2+} oscillations [13,14]. Knocking down PLCZ1 in transgenic mice resulted in the deficiency of Ca^{2+} oscillation inducing activity of the sperm and no offspring [15].

At present, one of the most powerful therapeutic procedures for male factor infertility is to inject a single sperm directly into the egg, known as ICSI. Ca^{2+} oscillations have been observed in human oocytes after ICSI [16]. However, 1–5% of all ICSI treatments resulted in failure, and the main cause for this was shown to be deficiencies in the egg activation process [17,18]. Several cases of male factor infertility are probably results of dysfunctional isoforms or reduced expression levels of PLCZ1 [19–21]. Egg activation failure can be treated by methods to elevate $[Ca^{2+}]_i$, such as applying Ca^{2+} ionophore, as currently used in most clinics, but such chemicals cannot mimic the pattern of $[Ca^{2+}]_i$ rises at normal fertilization and can be potentially cytotoxic or mutagenic for eggs and embryos [22]. On the other hand, PLCZ1 is a native physiological egg activating factor. When expressed by the injection of *in vitro* transcribed RNA, PLCZ1 can induce fertilization-like Ca^{2+} oscillations, resulting in parthenogenetic development up to blastocysts in mice, cows,

Abbreviations: APC/C, anaphase-promoting complex/cyclosome; $[Ca^{2+}]_i$, intracellular calcium ion concentration; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; ER, endoplasmic reticulum; ICSI, intracytoplasmic sperm injection; IMAC, immobilized metal ion affinity chromatography; InsP3R, inositol 1,4,5-trisphosphate receptor; iPS cells, induced pluripotent stem cells; IPTG, isopropyl β-D-1-thiogalactopyranoside; IVF, *in vitro* fertilizaition; MAPK, mitogen activated protein kinase; Micro-TESE, microsurgical testicular sperm extraction; PLCZ1, phospholipase C-zeta; PN, pronucleus; ROSI, round spermatid injection.

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pigs, monkeys and humans [9,23–25]. In addition to ICSI, round spermatid injection (ROSI) is developing as an alternative treatment for patients who have defects in spermatogenesis. Recently, it has been shown that ROSI is applicable to men with azoospermia, who possess only round spermatids; this application resulted in the birth of healthy babies [26]. The expression of egg-activating sperm factor could be detected at primary spermatocyte and round spermatid in monkeys and humans, respectively [27–29]. On the contrary, it had also been shown that round spermatids injected into mouse eggs could not induce egg activation [30]. In human, the precise stage of spermatogenesis from which PLCZ1 starts to express remains unknown. Moreover, the expression level of PLCZ1 or Ca^{2+} oscillation-inducing ability is considered to be different among the men with azoospermia. From these reason, stimulation for egg activation should be conducted to resume the cell cycle progression after ROSI. Thus, the effective egg activation method is also useful for ROSI as well as ICSI failure.

In this study, to establish the best egg activation method and improve development rates after ICSI or ROSI, we compared the pattern of $[Ca^{2+}]_i$ elevation after stimulation by cytosolic aspiration, electrical stimulation, ionomycin treatment and human PLCZ1 (hPLCZ1) RNA injection. We found that the pattern of Ca^{2+} oscillations after hPLCZ1 RNA injection exhibited similar characteristics to that after ICSI treatment. And, in terms of the developmental rates, we determined the optimal concentration of hPLCZ1 RNA to activate the human oocytes. Our data provide the basis for future studies to apply hPLCZ1 RNA injection to clinical use to rescue human oocytes from failed activation.

2. Materials and methods

2.1. Ethical aspects

This study was conducted with the informed consent of all participating patients. The Institutional Review Boards of the Saint Mother Obstetrics and Gynecology Clinic approved this study on January 17, 2016. This study was registered and adhered to International Committee of Medical Journal Editors criteria. The University Hospital Medical Information Network Clinical Trials Registry is UMIN000020860.

2.2. Preparation of human PLCZ1 RNA

cDNA encoding human PLCZ1 (hPLCZ1; GenBank accession number NM_033123) was prepared using PCR techniques from human testis cDNA library (PCR Ready First Strand cDNA; C1234260; BioChain Institute, Hayward, CA), and cloned into pTNT vector (Promega, Madison, WI). The 30 nucleotides of poly (A) region of pTNT vector was substituted with 168 nucleotides of poly (A) tail. The constructed plasmid (pGLS-hPLCZ1) was purified with NucleoBond Xtra Midi Plus EF kit (Takara, Shiga, Japan) and digested with *Bam*HI, and resulting fragment was used as template for *in vitro* transcription ([31] for details). Briefly, RNA was synthesized by T7 polymerase using mMessage mMachine Kit (Thermo Fisher Scientific, Waltham, MA) and purified by RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Dried RNA was resolved in 150 mM KCl solution (final concentration, ~1.5 μg/μl) and checked the quality by electrophoresis. RNA was diluted to the range between 0.01 and 1000 ng/μl and injected into oocytes.

2.3. Preparation of recombinant human PLCZ1 protein

hPLCZ1 ORF was amplified using PCR techniques from pGLS-hPLCZ1 and cloned into pE-SUMOstar vector (LifeSensors, Inc., Malvern, PA). The constructed plasmids (pE-SUMO-hPLCZ1) were sequence-verified and transformed into E. coli strain C41(DE3) (Lucigen Corporation, Middleton, WI). To express the recombinant 6xHis-SUMO-hPLCZ1 protein, bacterial culture was incubated at 37 °C in LB medium with 50 μg/mL ampicillin and 0.2% (w/v) glucose overnight. Next day, the culture was inoculated to 400 mL of LB medium containing 50 μg/mL ampicillin and grown to log phase (A600 = 1.0). The target protein expression was induced at 37 °C with 1 mM IPTG for 8 h. Induced bacterial cultures were then centrifuged and total cell lysate was prepared by xTractor Buffer (Clontech Laboratories, Inc., Mountain View, CA) containing protease inhibitor mixture (EDTA-free; Roche Diagnostics, Switzerland). His-tagged protein was purified from the cell lysate by using TALON metal affinity resin (Clontech Laboratories) at 4 °C. To cleave the linkage between 6xHis-SUMO and hPLCZ1, elution fraction (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole) was incubated with His-tagged SUMOstar protease 1 (LifeSensors) at 37 °C for 5 h in the presence of 5 mM dithiothreitol (DTT). After incubation, the solution was purified by Amicon Ultra 10 K filters (Merck Millipore Corporation, Darmstadt, Germany) and TALON metal affinity resin to remove the 6xHis-SUMO and His-tagged SUMOstar protease 1. The flow-through that contained recombinant hPLCZ1 protein was concentrated and the buffer was exchanged with PBS using Amicon Ultra 10 K filters. Protein concentration was estimated by Pierce BCA protein assay kit (Thermo Fisher Scientific). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses and Coomassie Brilliant Blue (CBB) staining were performed to confirm the expression and purification of the recombinant hPLCZ1 protein.

2.4. Procedure for egg activation

Human M-II oocytes were obtained from IVF patients who had consented to participate in this study. The number of provided oocytes was limited to two at most. Mature oocytes (at metaphase II), collected from ovaries 36 h after hCG injection [32], were maintained for 5–7 h in human tubal fluid (HTF) medium with 10% serum protein substitute (SPS; SAGE In Vitro Fertilization, Cooper Surgical, Malov, Denmark) [33] before their activation. HTF medium contains 2.04 mM calcium chloride. Oocytes were activated by one of the following four procedures.

1 PLCZ1 RNA injection and recombinant PLCZ1 injection

PLCZ1 RNA or recombinant PLCZ1 protein was injected into oocyte by using a Piezo manipulator (Prime Tech Ltd, Tsuchiura, Japan). To minimize damage to the egg, the solution was injected very mildly. Piezo settings were intensity 2 and speed 2. In the dish, the pipette was washed in 12% PVP drop, PLCZ1 RNA was aspirated, and approx. 4 pl of PLCZ1 were injected into the oocyte in the HTF medium with 10% SPS covered with mineral oil. Same volume of PLCZ1 solution was injected into oocyte at parthenogenetic activation and ROSI.

- Cytosolic aspiration and injection

2–3 times of cytosolic aspiration as the same way of ICSI was performed following Tesarik's method [34]. Then oocytes were cultured in the HTF medium with 10% SPS.

- Electrical stimulation

Oocytes were placed in 295 mM mannitol solution with 0.1 mM $CaCl_2$ (Sigma-Aldrich) and 0.05 mM $MgCl_2$ (Sigma-Aldrich), and stimulated with an alternating current of 5 V/cm at 1 MHz for 10 s followed by a single 1.2-kV/cm pulse of direct current for 99 ms using an electro-cell fusion generator (LF201; Nepa gene, Chiba,