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GAMETE BIOLOGY

Phospholipase C-zeta deficiency as a cause for repetitive oocyte fertilization failure during ovarian stimulation for in vitro fertilization with ICSI: a case report

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Abstract
Purpose The purpose of this study is to describe impaired oocyte fertilization from phospholipase C-zeta (PLC-ζ) deficiency in normal-appearing sperm that was successfully treated using calcium (Ca^{2+}) ionophore with intracytoplasmic sperm injection (ICSI) of oocytes matured in vitro.
Methods An infertile couple undergoing in vitro fertilization (IVF) experienced failed oocyte fertilization following ICSI with normal-appearing sperm. A semen sample collected from the patient was used to assess the expression of sperm PLC- ζ protein by Western blot analysis and immunofluorescence and PLC-ζ bioactivity by an in vitro model of Ca^{2+} release. A second IVF cycle was performed using Ca^{2+} ionophore with ICSI to enhance Ca^{2+}-induced oocyte activation of oocytes matured in vitro.
Results Sperm PLC-ζ protein deficiency was demonstrated by Western blot analysis and immunofluorescence and confirmed by reduced PLC-ζ bioactivity using an in vitro model of Ca^{2+} release. Nevertheless, with this sperm and supplementation of Ca^{2+} ionophore following ICSI, fertilization of four of six oocytes matured in vitro was obtained. In addition, four embryos underwent cleavage and two of them reached the blastocyst stage. Transfer of these blastocysts into the uterus led to a single pregnancy and live birth.
Conclusions Deficiency of PLC-ζ in normal-appearing human sperm is associated with impaired Ca^{2+}-dependent oocyte activation during ICSI. Under this condition, use of Ca^{2+} ionophore following ICSI of oocytes matured in vitro improves embryo developmental competence, possibly through the activation of Ca^{2+}-dependent mechanisms governing fertilization and preimplantation embryogenesis.

Capsule In an IVF couple with impaired oocyte fertilization during ICSI from deficient PLC-ζ in normal-appearing sperm, Ca^{2+} ionophore use following ICSI of oocytes matured in vitro improved oocyte competence, leading to the birth of a healthy term female offspring.

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Keywords Sperm · Phospholipase C-zeta · Oocyte activation · Assisted reproductive technology (ART) · Calcium ionophore · In vitro maturation

Introduction

Oocyte fertilization failure during in vitro fertilization (IVF) occurs in approximately 4 % of couples [1]. When it occurs, intracytoplasmic sperm injection (ICSI) is usually performed to bypass sperm-oocyte membrane fusion by injecting sperm directly into the mature oocyte, thereby inducing calcium (Ca^{2+})-dependent oocyte activation. Central to this process is phospholipase C-zeta (PLC- ζ) [2], a sperm-specific protein, which when released into the oocyte upon sperm entry induces Ca^{2+} release in the ooplasm [2]. In vitro studies confirm that injection of human PLC-ζ cRNA as well as recombinant human PLC-ζ protein into human oocytes induces release of Ca^{2+} from the endoplasmic

reticulum (ER) leading to oocyte activation and zygote formation [2–6]. Furthermore, absence of PLC-ζ from sperm extracts, or inhibition of PLC-ζ expression in sperm by RNA interference, impairs cytosolic Ca^{2+} release and reduces the amplitude and duration of Ca^{2+} oscillations [2, 3, 7].

Clinical observations further suggest that PLC-ζ deficiency in sperm with normal morphology can underlie oocyte fertilization failure after ICSI [8]. Furthermore, use of Ca^{2+} ionophore with ICSI using PLC-ζ-deficient sperm has been shown to improve oocyte fertilization [9–12]. The present report describes the clinical outcome of an infertile couple with normal-appearing sperm but with PLC-ζ deficiency that was discovered after oocyte fertilization failure following ICSI [8]. It introduces the use of Ca^{2+} ionophore (A23187) following ICSI of oocytes matured in vitro in combination with embryo vitrification to successfully induce oocyte activation, fertilization, and preimplantation embryogenesis, leading to the birth of a healthy term female offspring.

Case

A 32-year-old nulliparous woman presented with a 5-year history of infertility. Her past medical history was significant for hypothyroidism corrected with Synthroid (50 μg orally daily) and hyperandrogenic anovulation due to polycystic ovarian syndrome (PCOS). Her hysterosalpingogram showed a normal intrauterine cavity and bilaterally patent fallopian tubes. Her husband was a 31-year-old male with type 1 diabetes mellitus adequately controlled with Humalog 60 units and Lantus 30 units subcutaneously (sc) daily. His semen analysis was normal by previously established criteria [5] (volume, 4.2 mL; pH, 7.2; sperm concentration, 41.3 million per mL; motility, 49 %; normal forms by strict morphology, 6.0 % according to strict Kruger criteria). The couple did not conceive despite ten cycles of successful ovulation induction with clomiphene citrate administered to the female and adequately timed sexual relations.

In vitro fertilization was subsequently performed using a standard GnRH-antagonist ovarian stimulation protocol (Ganirelix; Merck & Co.) [13]. Recombinant human follicle-stimulating hormone (rhFSH) and urinary gonadotropins were started at a dose of 300 IU sc daily for 2 days and then changed thereafter as clinically indicated. Serial estradiol (E2) levels and transvaginal sonographic measurements of ovarian follicles were performed until at least three follicles reached ≥17 mm in diameter, at which time serum E2 levels reached 1167 pg/mL. Choriogonadotropin alfa (500 μg sc, Ovidrel, EMD Serono, Inc., Rockland, MA) was then administered, and transvaginal oocyte retrieval was performed 35.5 h later.

Nine metaphase II (MII) oocytes were retrieved and were subjected to ICSI (N=4) or inseminated with motile sperm (N=5). All four MII oocytes subjected to ICSI failed to fertilize, consistent with a fertilization problem. Only one of five oocytes inseminated with motile sperm fertilized, as previously reported [8], resulting in the embryo transfer of one cleavage-stage embryo that did not implant.

Sperm deficiency of PLC-ζ was considered as a possible cause for oocyte fertilization failure so that additional semen samples were collected from the male for further analysis. Compared to fertile men, sperm from the patient were deficient in PLC-ζ protein, as demonstrated by Western blot analysis and quantification (Fig. 1a, b), and contained PLC-ζ in aberrant locations or none at all [8], as shown by immunofluorescence (Fig. 2). Furthermore, sperm from the patient, but not from fertile men, failed to induce robust Ca^{2+} release following sperm injection into mouse oocytes and in the majority of cases failed to induce oscillations (Fig. 3) [8].

A second GnRH-antagonist ovarian stimulation with IVF/ICSI was performed using Ca^{2+} ionophore with ICSI to enhance Ca^{2+}-induced oocyte activation, as previously reported [9, 14, 15]. This time, rhFSH and urinary gonadotropins were started at a higher dose of 375 IU sc daily for 2 days and then changed as clinically needed. Serial E2 levels and transvaginal sonographic ovarian measurements again were performed until at least three follicles reached ≥17 mm in diameter when serum E2 levels reached 1991 pg/mL. Choriogonadotropin alfa was then administered, and transvaginal oocyte retrieval was performed as before.

Eleven MII oocytes were subjected to ICSI followed by exposure to Ca^{2+} ionophore (A23187: 5 μmol/L, Sigma Scientific) in G-1 media (Vitrolife) for 30 min [15]. Oocytes were then washed and placed in the incubator. All 11 MII oocytes failed to fertilize. In the same IVF cycle, Ca^{2+} ionophore and ICSI were used in six oocytes matured in vitro (*four progressed from MI to M2 and two from GV to M2).* These oocytes, matured in vitro, were subjected to delayed ICSI 27 h after oocyte retrieval and exposed to Ca^{2+} ionophore for 30 min after ICSI. Delayed ICSI resulted in clearly defined, normal two-pronuclear fertilization of two of the six oocytes. Twenty-four hours later,

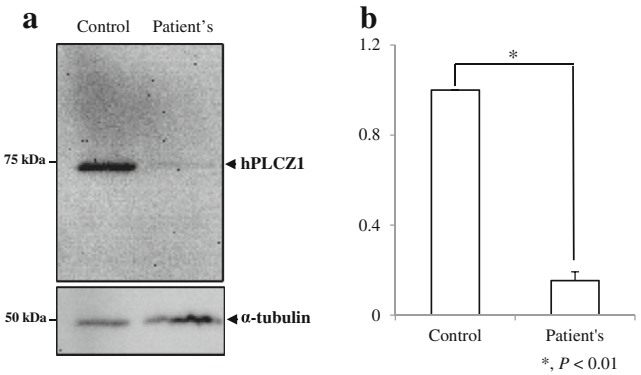

Fig. 1 **a** Western blot analysis of PLC- ζ protein in sperm of reported male (i.e., patient) and fertile male (i.e., control). **b** Bar graph displaying the relative intensity of the PLC-ζ. Only 15 % of PLC-ζ protein was present in the patient's sperm as compared to the fertile male

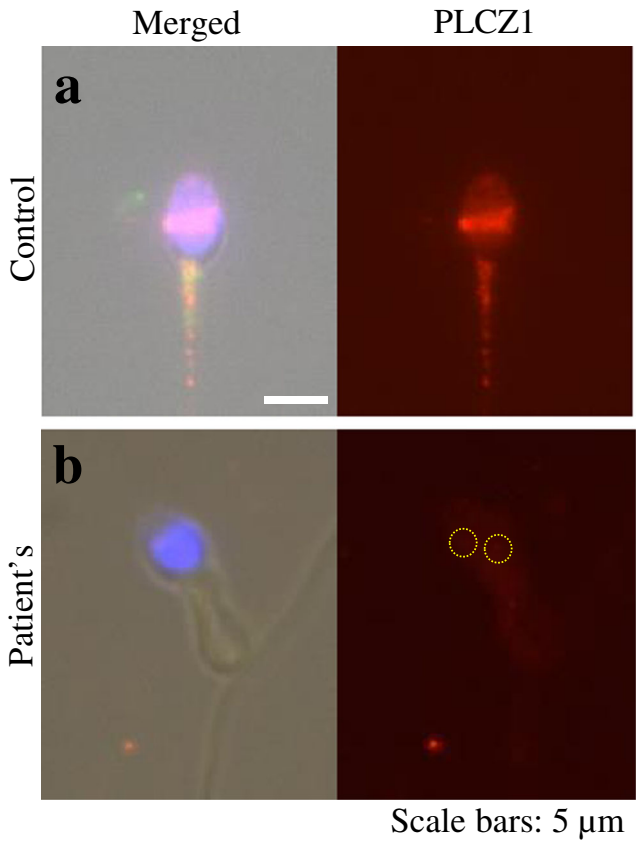

Fig. 2 Immunofluorescence analysis of PLC- ζ located in the male sperm. As compared to the proven fertile control male (**a**), the patient's sperm demonstrates decreased quantity and abnormal localization of PLC- ζ in the post-acrosomal region (**b**). Note that normal location of PLC- ζ is in the equatorial region of the sperm, although some variation can be observed

four cleaving embryos were observed despite only observing normal fertilization in two of them, and two of the cleaving embryos progressed to the blastocyst stage. The blastocysts were vitrified 6 days after egg retrieval [16]. Approximately 6 weeks later, the two embryos were thawed and transferred as good-quality expanded blastocysts into the patient's uterus, resulting in a singleton pregnancy.

A female infant weighing 7 lb 10 oz was subsequently delivered vaginally at 37.5 weeks gestation following labor induction for preeclampsia. The infant was born healthy, except for mild prematurity, and she has reached all of the normal milestones of growth and development at her current age of 10 months.

Discussion

Calcium-dependent oocyte activation is a critical step in oocyte fertilization and preimplantation embryo development [8, 14, 17]. As a crucial sperm protein governing Ca^{2+}-dependent oocyte activation, PLC-ζ released into the oocyte during sperm-oocyte membrane fusion [2] acts on phosphatidylinositol 4,5-biphosphate (PIP_2) [18] to induce its hydrolysis forming the second messengers inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) [18]. Subsequent IP_3 binding to its own receptor on the endoplasmic reticulum (ER) [19] opens Ca^{2+} channels, which release Ca^{2+} from the ER into the cytoplasm of the oocyte [19]. The resulting Ca^{2+} oscillations within the oocyte [18, 19] disseminate from the cortical region throughout the ooplasm [20, 21] at 10- to 20-min intervals [22], triggering oocyte activation [23] to initiate oocyte fertilization and preimplantation embryogenesis [8, 14, 17].

The present report provides the clinical outcome of one of three previously reported men who had normal-appearing sperm despite reduced PLC-ζ protein and bioactivity as well as poor oocyte fertilization capacity [8]. Specifically, the sperm from these men showed PLC- ζ protein levels that were 40–80 % lower than those of normal-appearing control sperm that were capable of oocyte fertilization [8]. Interestingly, PLC-ζ in the sperm of our patient was either absent or located in the post-acrosomal region and showed a punctate pattern rather than a uniform band in the equatorial region, which is the most consistent location in fertile males [5, 24, 25]. Nevertheless, it is worth noting that several distribution patterns of PLC-ζ have been reported within sperm without a history of failed oocyte fertilization [25].

From a clinical perspective, the present case report emphasizes the difficult clinical scenario that develops when oocyte fertilization failure follows ICSI with the use of normal-appearing gametes. Recurrent ICSI-related oocyte fertilization failure occurs in 13 % of successive IVF cycles [26], raising a dilemma regarding the use of homologous versus non-homologous gametes in subsequent IVF cycles. Under this circumstance, detecting sperm PLC-ζ deficiency implicates the sperm, rather than the oocyte, as the cause of failed oocyte fertilization, thereby avoiding unnecessary oocyte donation. A critical amount of PLC-ζ in sperm may be required for optimal Ca^{2+}-dependent oocyte activation, below which low rates of oocyte fertilization after ICSI are observed (i.e., <30 %) [14], although variable sperm PLC-ζ expression between samples and patients may limit quantitative analysis of PLC-ζ as a diagnostic indicator of oocyte activation [25].

As a divalent cation, calcium ionophore A23187 enhances Ca^{2+} transport across the oolemma (from the culture media to the oocyte) to increase intracellular Ca^{2+}, thereby mimicking the first Ca^{2+} release caused by PLC-ζ [27]. This A23187-enhanced Ca^{2+} transport within the oocyte is mediated through a single, large Ca^{2+} rise rather than the smaller, naturally repeating Ca^{2+} oscillations [10, 18]. Although previous studies have shown the benefit of using Ca^{2+} ionophore with ICSI to enhance Ca^{2+}-induced activation of mature oocytes fertilized with

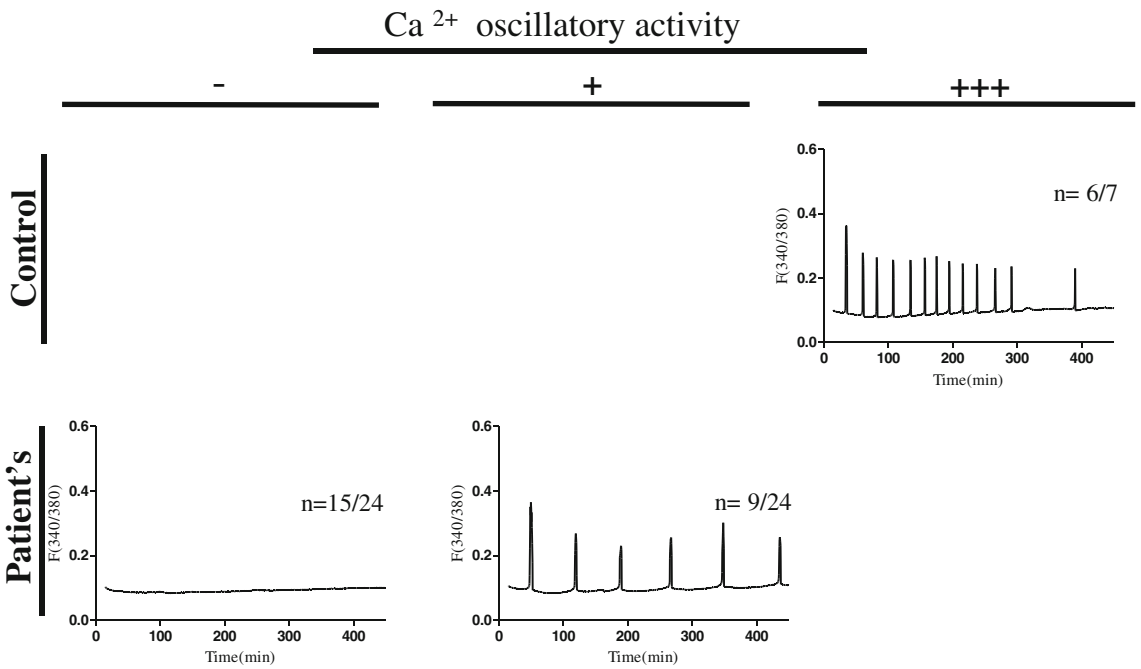

Fig. 3 Calcium release assay demonstrating the functional capacity of PLC- ζ. Patient's sperm does not initiate as robust a Ca^{2+} response as compared to the proven fertile control male. Nine out of 24 sperms initiated a lower than normal Ca^{2+} response. The remaining 15 sperms failed to induce a Ca^{2+} response entirely

PLC-ζ-deficient sperm [11, 14, 15, 28–32], our use of Ca^{2+} ionophore with in vivo matured PCOS oocytes following ICSI did not overcome the failed fertilization, raising caution with the use of Ca^{2+} ionophore and ICSI in this way. Instead, Ca^{2+} ionophore was successful in overcoming PLC-ζ-deficient sperm fertilization failure when used after ICSI of PCOS oocytes matured in vitro, supporting a report of successful pregnancy occurring after ICSI with artificial activation of in vitro matured oocytes [33]. It is possible that androgen-induced impairment of Ca^{2+} oscillations in PCOS oocytes matured in vivo reduced the benefit of artificial oocyte activation, without diminishing the value of using Ca^{2+} ionophore after ICSI of PCOS oocytes matured in vitro [34–36].

The finding that one blastocyst arose from an oocyte stimulated with Ca^{2+}-induced activation from a PCOS patient, which led to a term infant female, raises the additional possible benefit of Ca^{2+} ionophore on preimplantation embryogenesis. Although beyond the scope of this report, Ca^{2+} ionophore has been shown to improve embryo development and pregnancy outcome in patients with a history of previous impaired embryo development [17]. Therefore, it is also possible that successful pregnancies after ICSI supplemented with artificial activation of oocytes matured in vitro [33] might be the result of enhanced embryo competence from both improved fertilization and preimplantation embryogenesis [10, 15, 17].

Although the term female offspring of our couple was normal at birth and has grown appropriately at 10 months of age, the safety of using of Ca^{2+} ionophore with ICSI remains unclear. Data regarding Ca^{2+} ionophore use with ICSI and its relationship to human oocyte competence, embryo development, and neurodevelopmental outcomes in children appear reassuring [17, 37, 38], but immature oocytes or mature oocytes failing to fertilize may harbor inherent defects that potentially harm the embryo [39]. Also relevant is the potential impact of Ca^{2+} ionophore use with ICSI on the rates of miscarriage, genetic abnormalities, and minor as well as major congenital malformations [15, 40]. Theoretically, non-physiological patterns of calcium oscillation in artificially activated oocytes might perturb calcium-mediated signal transduction, mitochondrial respiration, developmental programming, or epigenetic events in ways that alter post-implantation embryo development [41, 42].

Thus, normal-appearing human sperm can be deficient in PLC-ζ and unable to induce Ca^{2+}-dependent oocyte activation during ICSI. If sperm PLC-ζ deficiency is detected, successful oocyte fertilization and preimplantation embryogenesis is possible through ICSI and artificial activation with Ca^{2+} ionophore of oocytes matured in vivo and in vitro. Future challenges with the management of this scenario are the usefulness of quantifying PLC-ζ in sperm as a diagnostic indicator of oocyte activation, the efficacy of Ca^{2+} ionophore use with ICSI in enhancing IVF-related pregnancy outcome following failed oocyte fertilization and/or impaired embryogenesis, and the implications of Ca^{2+} ionophore exposure on pregnancy complications and the long-term health of offspring [40].

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