PLC ζ disruption with complete fertilization failure in normozoospermia

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Abstract

Purpose Intracytoplasmic sperm injection (ICSI) is widely used to achieve fertilization in the presence of severe male factor, resulting in high fertilization rates. Nevertheless, 1–3 % of couples experience complete fertilization failure after ICSI. When a male factor is identified, assisted oocyte activation (AOA) can help overcome fertilization failures. The objective of this study is to describe a case of repeated complete fertilization failures after ICSI with donor oocytes, and to investigate the molecular and functional aspects of phospholipase C zeta (PLC ζ) protein in the patient semen.

Methods The patient was a normozoospermic male who had previously fathered, through natural conception, four children by a different partner. Molecular and functional analysis of sperm-specific PLC ζ in the patient and control samples by means of gene sequencing, immunocytochemistry, Western blot, mouse oocyte activation test (MOAT), and mouse oocyte calcium analysis (MOCA) were used.

Capsule Normozoospermic males can have disrupted expression and distribution of PLC ζ , and reduced activation ability after ICSI in human oocytes, despite their normal activation potential in functional testing using mouse oocytes.

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Results PLC ζ expression levels and distribution were significantly disrupted, although MOAT and MOCA did not indicate a decrease in activation ability.

Conclusions Normozoospermic males can have disrupted expression and distribution of PLC ζ , and reduced activation ability after ICSI in human oocytes, despite their normal activation potential in functional testing using mouse oocytes. Discrepancy among molecular and functional data might exist, as mutations in the gene sequence may not be the only cause of alteration in PLC ζ protein related to activation failures.

Keywords ICSI · Normozoospermia · PLC ζ · Assisted oocyte activation · Fertilization failure

Introduction

Following gamete fusion, oocytes are activated by an initial rise (trigger) and subsequent oscillations of intracellular calcium (Ca²⁺) [1]. Intracytoplasmic sperm injection (ICSI) is widely used to achieve fertilization in the presence of severe male factor, resulting in high fertilization rates. Nevertheless, 1–3 % of couples experience complete fertilization failure after ICSI [2], mostly caused by an activation failure [3–5]. Assisted oocyte activation (AOA) with a Ca²⁺ ionophore (A23187 or ionomycin), or strontium chloride (SrCl₂), direct microinjection of calcium, or electrical stimuli, have been shown to overcome fertilization failure after ICSI [6]. Ca²⁺ ionophore is most widely used for AOA [7], whose most common indication is globozoospermia, with only seven cases of AOA reported with normozoospermic patients worldwide [8–13].

During natural fertilization, oocyte activation is driven by sperm-specific phospholipase C zeta (PLC ζ) released by the spermatozoon when it fuses with the oolemma. In the oocyte,

PLC ζ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol triphosphate (IP₃) and diacylglycerol (DAG), eliciting a downstream response, which ultimately leads to the release in the cytoplasm of Ca²⁺ sequestered in the endoplasmic reticulum. A few functional tests are available in order to identify the cause of fertilization failure after ICSI, such as the injection of a spermatozoon in the oocyte of either mice (mouse oocyte activation test, MOAT) or cow (bovine oocyte activation test, BOAT) to check for pronuclei formation, or the tracking of Ca²⁺ oscillation elicited in mouse oocytes (mouse oocyte calcium analysis, MOCA). We present here a case of a normozoospermic man with recurrent complete fertilization failure after ICSI with oocytes from fertile donors, and a functional and genetic analysis of his sperm activation capacity.

Materials and methods

Patient characteristics

A 50-year-old man and his 45-year-old wife, of black and mulatto phenotype respectively, presented to the clinic reporting a voluntary pregnancy termination 12 years before and a gestational desire of several years. The man had previously fathered, through natural conception, four children (22, 18, 16 and 12 years old at the first medical visit) by a different partner. The woman presented with ovarian failure. Five unrelated semen samples were collected at different stages of the Assisted Reproductive Technique procedure, and were analyzed according to World Health Organization (WHO) recommendations [14]. Sperm count and motility were assessed by Sperm Class Analyzer[®] (Microptic, Spain).

Donor stimulation

Donors underwent a Gonadotropin-releasing hormone (GnRH) antagonist stimulation protocol (Cetrotide[®] 0.25 mg, Merck Serono Europe Limited, U.K.) with either recombinant follicle-stimulating hormone FSH (GONAL-f[®], Merck Serono Europe Limited, U.K.) or highly purified human menopausal gonadotropin HMG (Menopur[®], Ferring S.A.U., Spain). Ovulation was triggered with 0.2 mg GnRH agonist (Decapeptyl[®] 0.2 mg, Ipsen Pharma S.A., Spain). Transdermal estradiol (Vivelledot[®], France) was used for endometrial preparation of the recipient (two 75 μ g patches/3 days plus 2 mg of estradiol valerate vaginally). From the day of the oocyte retrieval, 800 mg/day of micronized vaginal progesterone (Utrogestan[®], Laboratorio Seid, Spain) were also provided. Oocyte donors were different in each of the 5 cycles performed, all with proven fertility.

AOA technique

AOA was performed according to Heindryckx et al. [12]. Briefly, Ca²⁺ is introduced into the oocyte at ICSI, followed by a double Ionomycin (10 μ mol/l MP Biomedical, USA) treatment. The semen sample was prepared by swim up and 10 μ l of washed spermatozoa were placed in the ICSI[™] droplet. In three of the cycles, spermatozoa were selected by intracytoplasmic morphologically selected sperm injection (IMSI; Nikon Instruments). The ICSI pipette, with the selected spermatozoon, was moved to a drop containing 10 % PVP/0.1 mol/l CaCl₂. The spermatozoon was aspirated into the injection needle together with the solution, and ICSI was performed. Injected oocytes were allowed to recover for 30 min and then incubated for 7 min in freshly prepared solution of Ionomycin. Oocytes were washed in culture medium (G1[™] PLUS, Vitrolife) and incubated for 30 min. Finally, injected oocytes were exposed for a second time to the activation solution for 7 min and after thorough washing, and oocytes were incubated under 37 °C, 6%CO₂, 5%O₂ for embryo culture.

Genomic analysis of PLC ζ

After thawing, sperm was washed and at least 3×10^6 spermatozoa were collected and centrifuged at 15.000 g for 2 min at room temperature. Genomic DNA (gDNA) was isolated with QIAMP DNA Blood Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer instructions. Amplicons of PLC ζ were amplified by polymerase chain reaction (PCR) with Phusion High-Fidelity DNA polymerase (NEB, Ipswich, MA, USA) and primer pairs previously described [15, 16] (Suppl. Table 1).

PCR fragments were purified with Gel Extraction Kit (QIAGEN) and the sequence was determined by using BigDye Terminator v3.1 at Sanger ABI 3730xl (GATC Biotechnologies AG, Germany), and analyzed with Chromas Software (Technelysium Ltd., Australia). BLAST analysis was performed against the published sequence of the genomic PLC ζ locus (Homo sapiens 12 BAC RP11-361I14 (Roswell Park Cancer Institute Human BAC Library) complete sequence.

Expression and localization of PLC ζ

Protein expression and localization of PLC ζ were analyzed following previously described protocols [17]. At least 10^7 spermatozoa were centrifuged at 15.000 g for 2 min. The cell pellet was re-suspended in 100 μ l of complete Laemmli Buffer [18] and lysed by three freeze/boil cycles (−20, 98 °C). Two different amounts of protein extract (50 and 100 μ g) were applied to a 10 % sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE); separated proteins were transferred onto PVDF membranes (Millipore, USA).

For immunoblotting, membranes were treated with 10 µg/ml of an anti-human-PLCζ antibody (pab0367P, Covalab, Villeurbanne, France) in blocking buffer and incubated at 4 °C overnight, followed by a secondary antibody (NA934, Amersham, USA) diluted at 1:10,000. An anti-tubulin antibody (T6199, Sigma, USA) was used to normalize the signal.

For immunofluorescence, we followed a previously reported protocol [17]. Briefly, at least 10⁶ spermatozoa were washed and fixed in 4 % paraformaldehyde (PFA)/PBS (Sigma). Fixed sperm cells were incubated overnight at 4 °C with 25 µg/ml of anti-human-PLCζ antibody (pab0367-P, Covalab, France). Samples were subsequently incubated with 5 µg/ml of secondary antibody (Alexa Fluor 568 F [Ab']₂ fragment goat anti-rabbit IgG; Invitrogen, Paisley, UK). Slides were stained with 20 µg/ml FITC-PNA and 2 µg/ml Hoechst-33342 (Sigma) for 15 min at 37 °C in the dark. The localization of PLCζ in relation to acrosomal status and the position of the nucleus were determined by confocal laser scanning microscopy (DM 5000B Leica, Germany).

Mouse oocyte activation test (MOAT)

Seven to 10-week-old B6D2 F1 hybrid females were stimulated using 5–7.5 IU of Folligon® (Intervet, Boxmeer, The Netherlands) and ovulation was induced 46–48 h later by 5–7.5 IU of Chorulon® (Intervet, Boxmeer, The Netherlands). Mice were sacrificed 13–14 h after human chorionic gonadotropin hCG administration to collect MII oocytes. Protein-free potassium simplex optimized medium (KSOM) and HEPES-buffered KSOM (KSOM-HEPES) supplemented with 0.4 % bovine serum albumin (MP Biochemicals, Belgium) were used for culture and manipulation, respectively. Sperm was thawed at room temperature for 15 min. Gamete Buffer® (Cook Medical, USA) was added for washing and the sample was centrifuged at 1.800 g for 10 min. After preparation, mouse oocytes were injected by Piezo-driven ICSI [12], which when carried out resulted in four groups: (i) test sperm: injection of patients' sperm, (ii) positive control (oocytes injected with sperm with proven activation potential), (iii) negative control—sham (oocytes injected with media only), and (iv) medium control (non-manipulated oocytes to exclude spontaneous activation). Activation rate is defined as the number of oocytes showing signs of activation 24 h after ICSI over the number of oocytes that survived injection.

Mouse oocyte calcium analysis (MOCA)

Before ICSI, oocytes were incubated with 7.5 mM fura-2 acetoxymethyl ester (fura-2 AM, Invitrogen, Belgium) for 20 min under standard culture conditions (37 °C, 6 % CO₂, 5 % O₂). Sperm cells were treated with Lysolecithin (0.02 %)

before injection [19]. Intracytoplasmic calcium oscillations were read out via radiometric measurements applying FURA-2AM excitation ratio (lambda 340 and 380 nm). Imaging was performed over a period of 2 h starting within 10 min after ICSI. Only intact oocytes were included for diagnosis. Outcome was analysed according to Vanden Meerschaut [19] and scoring of the following parameters was performed: total number of oocytes showing oscillations, number of calcium peaks per oocyte within 2 h (frequency), relative amplitude (change in the fluorescence ratio divided by the baseline ratio ($\Delta R/R$)). Calcium patterns were classified as score 0 (no calcium spikes), score + (1–2 spikes), score ++ (3–10 spikes) and score +++ (>10 spikes) within 2 h immediately subsequent to ICSI. The product of frequency and amplitude represents the summed relative amplitude of the calcium changes in 2 h (Suppl. Fig. 1).

Statistical analyses

Immunolocalization pattern quantification was statistically analyzed by Fisher's test. A p-value lower than 0.05 was considered statistically significant.

Results

Semen characteristics and clinical parameters of ICSI cycles with and without AOA

The patient was normozoospermic in all five samples analyzed. Sperm characteristics are presented in Suppl. Table 2. During the first cycle, a frozen sample was used; in all subsequent cycles, fresh semen was employed (Table 1). Moreover, a summary of ICSI cycles, including donor characteristics, is presented in Suppl. Table 3.

In the first ICSI cycle, there was total fertilization failure (0/5) of fresh MII donor oocytes inseminated using a frozen semen sample. Oocytes were examined for evidence of fertilization 16–18 h after ICSI. In the second ICSI cycle, 0/6 fresh MII donor oocytes were fertilized using a fresh semen sample. All oocytes in both cycles showed one polar body and no pronuclei. In the three subsequent cycles, we applied IMSI [20] combined with AOA using fresh semen (Table 1). In the third cycle, 7/8 fresh MII donor oocytes were fertilized, and five developing embryos were obtained; two embryos were transferred at Day 3 (one with nine cells of unequal size, <10 % fragmentation; and one with six cells of unequal size, 0 % fragmentation); one non-compacted embryo was vitrified at Day 4 (eight cells of equal size, 0 % fragmentation). No pregnancy was achieved in either the fresh transfer, or the following cryotransfer.

In the fourth cycle, a combination of fresh and vitrified/warmed oocytes from the same donor was used. A total of

Table 1 Overview of ICSI cycles included in this study

Cycle	Donor	MII	Semen	IMSI	AOA	ET day	embryos
1	A	Fresh	Frozen	No	No	na	na
2	B	Fresh	Fresh	No	No	na	na
3	C	Fresh	Fresh	Yes	Yes	3	2
4	D	Fresh/vitrified	Fresh	Yes	Yes	2	2
5	E	Fresh/vitrified	Fresh	Yes	Yes	5	2

na not applicable

15 oocytes (five fresh and ten vitrified) were injected using fresh sperm sample combined with IMSI/AOA. With vitrified/warmed oocytes, we located the MII spindle with polarized light microscopy (Oosight™ Imaging System-Researchs Intruments Inc.) to avoid damaging the metaphase plate with the injection needle. In this cycle, none of the five fresh oocytes were fertilized and 2/10 vitrified/warmed oocytes were fertilized. Embryos were observed 43 h post-insemination, and two embryos were transferred on Day 2 (both with five cells of unequal size, 10 % fragmentation); no pregnancy was achieved.

In the fifth cycle, a total of 12 oocytes (seven fresh and five vitrified/warmed) were inseminated by IMSI combined by AOA, also using fresh sperm sample. Seven oocytes were fertilized (4/7 and 3/5, respectively). Additionally, we opted to let the embryos develop to the blastocyst stage. Two blastocysts (4BA and 3BB) [21] were transferred on Day 5 and four embryos were vitrified (one blastocyst 3DB, two early blastocysts and one morula). A pregnancy was established with a positive beta hCG result 14 days after transfer and confirmed by ultrasonography at 12 weeks of pregnancy. A healthy girl was born by cesarean section at week 37 of pregnancy; the baby was 46 cm long and weighed 2.550 kg.

Gene sequence, expression and localization of PLCζ in the patient semen

Alterations in protein expression and/or functionality of PLCζ could be associated with the presence of polymorphisms in the PLCζ gene (SNP) [15, 16]. Thus, we focused on exons and intronic regions surrounding them. The sequencing of PCR products revealed three polymorphisms (Table 2). One of them, located at intron 1 (rs5796766), was also observed in

samples with proven fertility [15], however, the additional SNPs variants have neither been detected in previous PLCζ reports nor associated with a disease (SNP database; <http://www.ncbi.nlm.nih.gov/SNP/>).

The amount of PLCζ protein in sperm was decreased in comparison with a normozoospermic sample of proven fertility (Fig. 1a and Suppl. Fig. 2). Immunohistochemistry analysis for PLCζ localization on sperm head was performed on sperm samples from four fertile men and the patient. Cell count was performed in four fertile men (cells analyzed: $n=247$, 272, 274, and 282 respectively) and patient ($n=215$) (Suppl. Table 4) showing an alteration of the localization of PLCζ across the spermatozoa heads from the patient when compared to fertile controls (Fig. 1b, c and d and Suppl. Fig. 3). Three different localizations were observed: acrosomal (A), equatorial (Eq) and midpiece (Mp) (Fig. 1d). The percentage of spermatozoa with a reacted acrosome was higher in the case vs controls ($p=0.0015$). When an acrosome could be detected by PNA staining, predominant PLCζ distribution patterns were different in the patient vs. controls both acrosomally (A; 10.8 vs. 77.1 %, $p<0.0001$) and midpiece (Mp; 75.4 vs. 12.2 %, $p<0.0001$). Once the PNA signal in the acrosome is lost, differences in the localization of PLCζ among samples tends to disappear (Fig. 1, b and c)

Functional analysis of patient semen by $[Ca^{2+}]_i$ oscillation induction in mouse oocytes

The MOAT revealed a normal activation capacity of the patients' sperm compared to a positive control. Survival and activation rates were evaluated 24 h after ICSI and are shown in Table 3. A total of 24 dye-loaded MII mouse oocytes were injected for calcium analysis (MOCA), 17 of which were

Table 2 Overview of polymorphisms detected in this case report

Genome coordinates	Position	10 bp 5' and 3'	SNP	Alleles affected	Known SNP	1000G MAF
12:18738145	Intron 1	ATGTCCTATCCATGAAAGCAGG	A/G	2	rs1075421	$G=0.3751$
12:18737539	Intron 1	AAGAACAGGCAAAAAAACCA	A/-	1	rs5796766	nd
12:18694664	Intron 13	TTCTCCTCTCAAGTCTACT	A/G	1	rs11044253	$G=0.3623$

nd not determined

Fig. 1 Expression and localization of PLC ζ protein: **a** Western immunoblotting for PLC ζ (*upper panel*) and tubulin (*lower panel*). Relative intensity of PLC ζ to tubulin in fertile (*white bar*) and patient sperm (*black bar*) is presented; **b** PLC ζ localization patterns in a representative fertile (*white bar*) vs patient (*black bar*) in spermatozoa presenting intact acrosome; **c** PLC ζ localization patterns in a representative fertile (*white bar*) vs patient (*black bar*) in spermatozoa presenting reacted acrosome; % of acrosomal (A), equatorial (Eq) and midpiece (MP) signal are presented. * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.0001$. **d** immunolocalization of PLC ζ in fertile (*upper panels*) and patient (*lower panels*) spermatozoa. Arrows indicate PLC ζ localization: acrosomal (A), equatorial (Eq) and/or midpiece (MP). PLC ζ (red), acrosome (FITC-PNA; green), and DNA (Hoechst33342; blue). Scale bars, 10 μ m

intact after imaging. Ten out of 17 oocytes (58.82 %) were scored as +++, 2 out of 17 (11.76 %) as ++, 2 out of 17 (11.76 %) as + and 3 out of 17 (17.65 %) as 0. The amplitude-frequency

product result was 12.95, which is above the optimal-threshold of 9, for normal activation capacity of human sperm using mouse oocytes [13].

Table 3 Results from the Mouse Oocyte Activation Test (MOAT). Activated oocytes: oocytes which cleave to two cells 24 h after injection. For sham injection or negative control the oocytes are injected without sperm. For media control the oocytes are left in culture media without further manipulation

MOAT	Injected oocytes	Survived oocytes	Activated oocytes	Activation rate
Patient	40	32	29	91 %
Control	41	35	30	86 %
Sham	20	6	0	0
Media control	10	10	0	0

Discussion

We report here a case of secondary infertility in a normozoospermic patient who fathered four children 2 decades ago, now presenting recurrent complete fertilization failures with oocytes from donors of proven fertility after ICSI. The patient ultimately conceived by AOA, and a healthy baby girl was born. One of the most agreed upon factors related to fertilization failure of male origin is PLC ζ [22, 23]. PLC ζ alterations can be due to gene mutations, altered protein expression or cellular localization, absence (globozoospermia), and functionality [16, 15, 17, 24]. The molecular analysis of PLC ζ revealed significantly lowered amount and altered distribution of the protein, with no mutations detected. MOAT and MOCA analysis revealed overall normal [Ca²⁺]_i response induction, although about 40 % of sperm did not elicit strong Ca²⁺ oscillations. Alteration in PLC ζ abundance and distribution, even in the absence of morphological alteration in the sperm and mutations in the gene, can be related to failures to elicit [Ca²⁺]_i oscillations in human oocytes.

ICSI failures in normozoospermia

Complete fertilization failure due to male factor has been mostly related to severe teratozoospermia, and specifically to globozoospermia [25, 26], while it is very rare in normozoospermia. There are seven cases reported of AOA after complete fertilization failure in normozoospermia [8–12, 19]; in some the cause was not clearly identified [8, 10], or was due to an oocyte factor [9]. Among those, six cases were treated using Ca²⁺ ionophore [8, 10, 19].

In a case report of a normozoospermic patient [10], most sperm heads that appeared normal under light microscopy, exhibited abnormal nuclear vacuolization when analyzed by transmission electron microscopy (TEM). For this reason, after two fertilization failures, we proposed IMSI to select sperms without vacuoles and with an intact acrosome. We did not find any evidence of alternations at 6000 $\times$ magnifications; moreover, the proportion of spermatozoa with an intact acrosome was similar in both case and controls. Although TEM analysis would clarify further the presence of alterations

in the sperm head, which in turn might affect the localization of PLC ζ , it is unlikely that such alterations were present in our case.

ICSI failure and PLC ζ presence/distribution

Two point mutations in the *PLC ζ* gene have been described so far, located at exon 6 and 11, coding for active domains, both producing a non-functional, albeit full-length, protein [16], however, no mutations were detected in our patient. Another study reported single nucleotide polymorphisms (SNPs) in intronic regions that correlate with low fertilization rates even though the patient was normozoospermic [15]. We found three SNPs (rs1075421, rs5796766, and rs11044253), one previously described [15] in one man with normal fertilization rates and in two patients presenting activation failure after ICSI.

We found, however, a significantly lower expression of the PLC ζ protein compared to fertile controls. This finding is consistent with a recent report where PLC ζ was significantly reduced in patients with previous fertilization failures, even taking into account the variability in PLC ζ levels among controls [24]. The source of lower expression of PLC ζ remains to be established; altered transcription, translation, or turnover are all possible mechanisms, which so far have not been addressed. Together with its abundance, PLC ζ localization can also be related to fertilization failures [17, 24]. In consistence with these reports, we found altered localization of PLC ζ in the patient sperm. Specifically, a high proportion of spermatozoa presented midpiece PLC ζ localization in acrosome-intact sperm in our patient, in contrast to controls.

We cannot exclude the presence and relevance for the phenotype observed of alteration in other candidate activation molecules, such as PAWP [27, 28], however, due to the limited amount of patient sample available for study, we elected to focus on PLC ζ .

ICSI failure and MOAT/MOCA tests

The MOAT evaluates the capacity of human spermatozoa to activate mouse oocytes [29], and MOCA the specific pattern

of $[Ca^{2+}]_i$ oscillations, allowing for the categorization of spermatozoa in low, intermediate or high oocyte activation capability [12]; in our case, a normal activation capacity was found in MOAT and MOCA, which was probably due to the fact that human PLC ζ has greater activation strength than mouse PLC ζ , as recently reported [23].

A recent report compares three normozoospermic patients that failed ICSI with the partner's oocytes [30]; 1/8, 2/10 and 0/24 of injected sperms for MOAT in the three men, respectively, were capable of initiating robust $[Ca^{2+}]_i$ responses, another 3/8, 5/10, and 13/24 were able to initiate moderate to low $[Ca^{2+}]_i$ responses, whereas 4/8, 3/10, and 11/24 of the sperm were unable to initiate $[Ca^{2+}]_i$ responses. In our case, we found 10/17 robust $[Ca^{2+}]_i$, 4/17 medium-low $[Ca^{2+}]_i$ and 3/17 absence of responses, which is lower than those produced in normozoospermic fertile samples.

Although there are differences in the degree of activation that is elicited by normozoospermic but infertile patients in both the cited work and our case, a common feature among the two sets of patients and controls is that normozoospermic infertile patients consistently show some degree of failure to activate mouse oocytes, whereas fertile patients invariably activate mouse oocytes [30]. This can be explained by the fact that human PLC ζ has a greater activation capacity than mouse PLC ζ , so that a diminished amount of functional protein, which are unable to elicit $[Ca^{2+}]_i$ oscillations in a human oocyte, might still activate mouse oocytes. Consistently, we postulate that the MOAT/MOCA test is probably more suited to identifying structural alterations in human PLC ζ such as protein truncation or point mutations affecting the protein activity, than to evaluating the ability of a small amount of active PLC ζ to activate human oocytes.

Secondary activation failure

A puzzling aspect of our case is the fact that the patient fathered four children (12 to 22 years old) at the time of treatment, and that the anamnesis revealed no significant change in medical or environmental conditions. To the best of our knowledge, there have been no reports in the literature so far of an acquired, apparently idiopathic, activation failure in normozoospermia. Two alternative explanations for this finding could be hypothesized: on the one hand, this event could originate in the clonal expansion, in time, of a subset of spermatogonia and/or spermatogonia stem cells that have acquired alterations giving them a competitive advantage during spermatogenesis; although such alterations have so far been demonstrated in cases of point mutations in the human species [31, 32], other selective mechanisms cannot be excluded. On the other hand, a diminished amount or altered distribution of PLC ζ could be the result of ageing, with an attending dysregulation of transcription, translation and/or turnover of the protein. The patient in the presented case was 50 at the time of

fertilization failure; however, no such data on other cases is reported in the available literature. In our own facility, complete fertilization after ICSI with donor oocytes has occurred in 15 men in the last year, with an average age of 43.5 ± 6.5 years; however, it remains to be determined if this age is significantly related to the phenotype observed.

In conclusion, we identify changes in PLC ζ presence and distribution, which are associated with, though not necessarily the cause of, severely diminished activation ability in normozoospermic sperm. We suggest that the presence and distribution of PLC ζ should be investigated in cases of unexpected male-related fertilization failure in normozoospermia, and that MOAT/MOCA tests might be of limited diagnostic and prognostic value in cases of unaltered genetic sequence of the protein. Further work should be aimed at identifying other molecules involved in the activation trigger and cascade, in order to improve our understanding of oocyte activation, and develop clinically relevant diagnostic tests.

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Compliance with ethical standards

Informed consent Informed consent was obtained from the couple involved in the study in order to analyze the semen samples and to report the case.

Ethical approval All procedures performed were in accordance with the ethical standards of the institutional research committees and with the 1964 Helsinki declaration and its subsequent amendments.

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Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Tesarik J, Sousa M. Mechanism of calcium oscillations in human oocytes: a two-store model. *Mol Hum Reprod*. 1996;2(6):383–6.
2. Mahutte NG, Arici A. Failed fertilization: is it predictable? *Curr Opin Obstet Gynecol*. 2003;15(3):211–8. doi:10.1097/01.gco.0000072858.73466.aa.
3. Sousa M, Tesarik J. Ultrastructural analysis of fertilization failure after intracytoplasmic sperm injection. *Hum Reprod*. 1994;9(12):2374–80.
4. Tesarik J, Mendoza C. In vitro fertilization by intracytoplasmic sperm injection. *Bioessays*. 1999;21(9):791–801. doi:10.1002/(SICI)1521-1878(199909)21:9<791::AID-BIES11>3.0.CO;2-Z.
5. Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA, Vitullo AD. Cytoskeletal organization defects and abortive activation in

- human oocytes after IVF and ICSI failure. *Mol Hum Reprod*. 2000;6(6):510–6.
6. Nasr-Esfahani MH, Deemeh MR, Tavalaee M. Artificial oocyte activation and intracytoplasmic sperm injection. *Fertil Steril*. 2010;94(2):520–6. doi:10.1016/j.fertnstert.2009.03.061.
7. Vanden Meerschaut F, Nikiforaki D, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI fertilization failure. *Reprod BioMed Online*. 2014;28(5):560–71. doi:10.1016/j.rbmo.2014.01.008.
8. Eldar-Geva T, Brooks B, Margalioth EJ, Zylber-Haran E, Gal M, Silber SJ. Successful pregnancy and delivery after calcium ionophore oocyte activation in a normozoospermic patient with previous repeated failed fertilization after intracytoplasmic sperm injection. *Fertil Steril*. 2003;79 Suppl 3:1656–8.
9. Murase Y, Araki Y, Mizuno S, Kawaguchi C, Naito M, Yoshizawa M, et al. Pregnancy following chemical activation of oocytes in a couple with repeated failure of fertilization using ICSI: case report. *Hum Reprod*. 2004;19(7):1604–7. doi:10.1093/humrep/deh294.
10. Chi HJ, Koo JJ, Song SJ, Lee JY, Chang SS. Successful fertilization and pregnancy after intracytoplasmic sperm injection and oocyte activation with calcium ionophore in a normozoospermic patient with extremely low fertilization rates in intracytoplasmic sperm injection cycles. *Fertil Steril*. 2004;82(2):475–7. doi:10.1016/j.fertnstert.2004.01.038.
11. Chen J, Qian Y, Tan Y, Mima H. Successful pregnancy following oocyte activation by strontium in normozoospermic patients of unexplained infertility with fertilisation failures during previous intracytoplasmic sperm injection treatment. *Reprod Fertil Dev*. 2010;22(5):852–5. doi:10.1071/RD09268.
12. Heindryckx B, De Gheselle S, Gerris J, Dhont M, De Sutter P. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reprod BioMed Online*. 2008;17(5):662–8.
13. Vanden Meerschaut F, Nikiforaki D, De Roo C, Lierman S, Qian C, Schmitt-John T, et al. Comparison of pre- and post-implantation development following the application of three artificial activating stimuli in a mouse model with round-headed sperm cells deficient for oocyte activation. *Hum Reprod*. 2013;28(5):1190–8. doi:10.1093/humrep/det038.
14. WHO. WHO laboratory manual for the examination and processing of human semen. 5th Edition, 2010. Chapter 5.6. Preparing HIV-infected semen samples “Prepared samples should be tested by RT-PCR before use, and only HIV-free samples used for ART”. 2010.
15. Yoon SY, Jellerette T, Salicioni AM, Lee HC, Yoo MS, Coward K, et al. Human sperm devoid of PLC, zeta 1 fail to induce Ca(2+) release and are unable to initiate the first step of embryo development. *J Clin Invest*. 2008;118(11):3671–81. doi:10.1172/JCI36942.
16. Heytens E, Parrington J, Coward K, Young C, Lambrecht S, Yoon SY, et al. Reduced amounts and abnormal forms of phospholipase C zeta (PLCzeta) in spermatozoa from infertile men. *Hum Reprod*. 2009;24(10):2417–28. doi:10.1093/humrep/dep207.
17. Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative oocyte activation factor, phospholipase C zeta, in uncapacitated, capacitated, and ionophore-treated human spermatozoa. *Hum Reprod*. 2008;23(11):2513–22. doi:10.1093/humrep/den280.
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680–5.
19. Vanden Meerschaut F, Leybaert L, Nikiforaki D, Qian C, Heindryckx B, De Sutter P. Diagnostic and prognostic value of calcium oscillatory pattern analysis for patients with ICSI fertilization failure. *Hum Reprod*. 2013;28(1):87–98. doi:10.1093/humrep/des368.
20. Berkovitz A, Eltes F, Yaari S, Katz N, Barr I, Fishman A, et al. The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Hum Reprod*. 2005;20(1):185–90. doi:10.1093/humrep/deh545.
21. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. *Curr Opin Obstet Gynecol*. 1999;11(3):307–11.
22. Amdani SN, Jones C, Coward K. Phospholipase C zeta (PLCzeta): oocyte activation and clinical links to male factor infertility. *Adv Biol Regul*. 2013;53(3):292–308. doi:10.1016/j.jbior.2013.07.005.
23. Nomikos M, Yu Y, Elgmami K, Theodoridou M, Campbell K, Vassilakopoulou V, et al. Phospholipase C zeta rescues failed oocyte activation in a prototype of male factor infertility. *Fertil Steril*. 2013;99(1):76–85. doi:10.1016/j.fertnstert.2012.08.035.
24. Kashir J, Jones C, Mounce G, Ramadan WM, Lemmon B, Heindryckx B, et al. Variance in total levels of phospholipase C zeta (PLC-zeta) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. *Fertil Steril*. 2013;99(1):107–17. doi:10.1016/j.fertnstert.2012.09.001.
25. Dam AH, Feenstra I, Westphal JR, Ramos L, van Golde RJ, Kremer JA. Globozoospermia revisited. *Hum Reprod Update*. 2007;13(1):63–75. doi:10.1093/humupd/dml047.
26. Yanagida K. Complete fertilization failure in ICSI. *Hum Cell*. 2004;17(4):187–93.
27. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, et al. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. *FASEB J Off Publ Fed Am Soc Exp Biol*. 2014;28(10):4434–40. doi:10.1096/fj.14-256495.
28. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, et al. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. *Fertil Steril*. 2014;102(2):440–7. doi:10.1016/j.fertnstert.2014.05.003.
29. Rybouchkin A, Dozortsev D, de Sutter PD, Dhont M. Factors affecting the role of the spindle during early response of mouse oocytes to ethanol stimulation. *J Exp Zool*. 1996;275(6):469–75. doi:10.1002/(SICI)1097-010X(19960815)275:6<469::AID-JEZ9>3.0.CO;2-M.
30. Lee HC, Arny M, Grow D, Dumesic D, Fissore RA, Jellerette-Nolan T. Protein phospholipase C Zeta1 expression in patients with failed ICSI but with normal sperm parameters. *J Assist Reprod Genet*. 2014;31(6):749–56. doi:10.1007/s10815-014-0229-9.
31. Giannoulitou E, McVean G, Taylor IB, McGowan SJ, Maher GJ, Iqbal Z, et al. Contributions of intrinsic mutation rate and selfish selection to levels of de novo HRAS mutations in the paternal germline. *Proc Natl Acad Sci U S A*. 2013;110(50):20152–7. doi:10.1073/pnas.1311381110.
32. Maher GJ, Goriely A, Wilkie AO. Cellular evidence for selfish spermatogonial selection in aged human testes. *Andrology*. 2014;2(3):304–14. doi:10.1111/j.2047-2927.2013.00175.x.