Evaluation of PAWP and PLCζ Expression in Infertile Men with Previous ICSI Fertilization Failure

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Purpose: The aim of this study was to evaluate postacrosomal sheet WW domain binding protein (PAWP) and phospholipase C ζ (PLCζ) protein expression in patients with fertilization failure.

Materials and Methods: Semen samples were collected from 15 fertile men (control group) and 15 patients with previous fertilization failure following ICSI (FF group) and were analyzed according to World Health Organization (WHO) criteria. The mean percentages of PAWP and PLCζ positive sperm and the total level of PAWP and PLCζ proteins were assessed using immunofluorescence staining.

Results: A significantly lower level and lower percentage of PAWP positive sperm in patients with fertilization failure was found compared to the control group ($P = 0.01$ and $P = 0.03$, respectively). The mean percentage of PLCζ positive sperm and level of PLCζ protein were significantly lower in FF group compared to the control group ($P = 0.0003$ and $P = 0.04$, respectively). Significant positive correlations was observed between PAWP and PLCζ positive sperms ($r = 0.4$, $P = 0.008$) and also total level of expression of PLCζ and PAWP proteins ($r = 0.4$, $P = 0.02$) in all participants in the study.

Conclusion: This is the first study that evaluates two main candidates for sperm-borne oocyte activating factors (SOAFs) simultaneously in patients with fertilization failure. Considering lower expression of PAWP and PLCζ proteins in such patients, it seems like both factors might have the potential to be considered as SOAFs and diagnostic markers for the oocyte activation ability.

Keywords: fertilization failure; infertility; intra-cytoplasmic sperm injection (ICSI); phospholipase C ζ (PLCζ); postacrosomal sheet WWI domain binding protein (PAWP).

INTRODUCTION

Infertility is a common clinical problem which affects men and women in the world[1]. Approximately, 60% of infertile couples directly or indirectly suffer from male infertility[2]. Intra-cytoplasmic sperm injection (ICSI) is mainly used for male factor infertility such as suboptimal semen parameters[3] which is considered to be the most successful treatment for male infertility[4]. Although the rate of fertilization as a result of ICSI is high (70–75%)[5], it is reported that 1–3% of ICSI cycles still fail[4,6]. The main cause of fertilization failure following ICSI is suggested to be oocyte activation failure[6,7].

Oocyte activation is a series of programmed events following fertilization that gets ready an oocyte to undergo cell division[4]. The activation impetus normally arises after the entrance of sperm borne oocyte activating factor (SOAF) into the oocyte[8]. Several candidates have been proposed as the SOAF for several years. Phospholipase C ζ (PLCζ) and postacrosomal sheet WW domain binding protein (PAWP) are two main candidates under challenge[9]. PLCζ is the most critical candidate responsible for oocyte activation in several biochemical and clinical evidence.[10] However, PAWP also appears to have the appropriate conditions to be considered as a strong candidate for SOAF[11,12]. Microinjection of PLCζ (mRNA or recombinant protein) into oocyte can elicit calcium oscillations in failed oocyte activation[13,14]. Although injection of PAWP (cRNA or recombinant protein) caused calcium oscillations and pronuclear formation in human and mouse oocytes[15]; other studies showed that mouse and human PAWP recombinant protein and cRNA were unable to stimulate calcium release in mouse oocytes[16,17].

However, several studies have reported that patients with fertilization failure may represent PLCζ deficiency, abnormal localization patterns and low expression of PLCζ protein[13,18-20], there is a scarcity of studies regarding PAWP as another SOAF candidate among those patients. Thus, we evaluate both PLCζ and PAWP proteins in patients with previous failed fertilization following ICSI compared to fertile men.

MATERIALS AND METHODS

Materials

A PLCζ rabbit polyclonal antibody (LS-C144827) and

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Table 1. Descriptive analysis of semen parameters in two groups

Variables	Control group (n=15)	FF group (n=15)
Sperm concentration ($\times 10^6$) mean±SD [range]	60 ± 23.2 [25-95]	28.7 ± 22.4 [5-80]
Sperm motility (%) mean ± SD [range]	56.3 ± 14.6 [40-90]	40 ± 27.2 [0-90]
Normal morphology (%) (mean ± SD [range]	4.4 ± 1.7 [2-8]	2.3 ± 2 [0-8.5]

Abbreviation: FF; fertilization failure.

PAWP rabbit polyclonal antibody were obtained from LifeSpan BioSciences (USA) and Proteintech (UK), respectively. Ham's F-10, normal goat serum, DPBS and para-formaldehyde were purchased from Sigma-Aldrich (Germany). anti-rabbit IgG H&L (Alexa Fluor ®555) (ab150082) was from Abcam Company (UK).

Study population

This study was performed on 30 men undergoing ICSI cycles in the IVF center, Taleghani hospital (RIF clinic), during March 2015 to April 2017. The study was permitted by Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran and written informed consent was obtained from all participants prior to the study.

Inclusion and exclusion criteria

Low fertilization (≤ 25 %) to complete fertilization failure after at least one cycle of ICSI was the inclusion criteria for the patients, independent to sperm parameters (group FF, n = 15). Fertile control men with no history of infertility who had fathered children without the use of assisted reproductive techniques (ART) and referred to the clinic for sex selection via preimplantation genetic diagnosis (PGD) were considered as control group (group control, n = 15). Any history of female factor infertility, age > 35 years in females, and any history of trauma or surgery in testis were exclusion criteria for the participants.

Semen analysis

The semen samples were gathered by masturbation following 2-5 days of abstinence. After complete liquefaction, sperm concentration, motility, and morphology were assessed under light microscopy according to World Health Organization criteria (WHO)[21]. After washing the semen using swim up technique, sperms were used for immunofluorescence staining and PLCζ, and PAWP protein expression were assessed in sperm from control and FF groups.

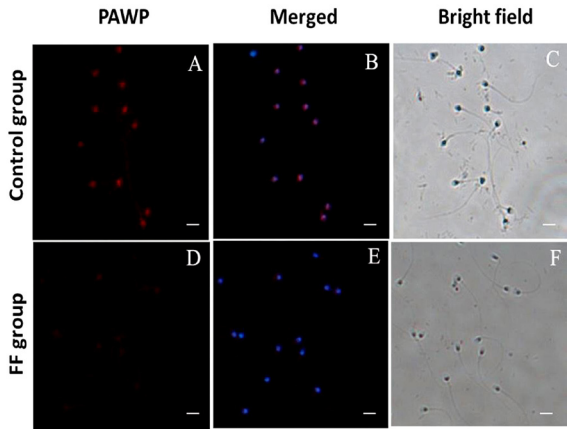


Figure 1. Immunofluorescence staining of PAWP protein and DAPI in sperm cells from control group **(A-C)** and FF group **(D-F)** (A, D= PAWP; **B, E=** merged image of DAPI and PAWP; **C, F** = bright field). Error bar = 10µm.

Swim-up technique

After centrifuging the semen, a pellet was prepared via removal of seminal plasma. The pellet was suspended in Ham's F10 (1 mL) supplemented with 10% human serum albumin. After centrifugation, the pellet was over layered with Ham's F10 supplemented with 10% human serum albumin and incubated in incubator at 37°C for 1 hour. Finally, the supernatant was taken and washed sperms used for further evaluations.

PLCζ and PAWP immunofluorescence staining

PLCζ and PAWP immunofluorescence staining was performed as previously described,[16,18,22] with some modifications. Briefly, after smear preparation on microscopic slide, sperms were fixed with freshly-made paraformaldehyde 4% in PBS for 10 minutes, and permeabilized with 0.2% Triton x-100 in PBS for 10 minutes on ice. After washing, unspecific antigen bindings were blocked with 5% normal goat serum in PBS for 1 hour at 37°C, and incubated in primary antibodies; the PLCζ rabbit anti-human polyclonal antibody and PAWP rabbit polyclonal antibody overnight at 4°C. After washing, sperms were incubated with secondary antibody goat anti-rabbit IgG H&L (Alexa Fluor ®555) at room temperature for 1 hour (light protected), and then washed three times for 5 minutes with washing buffer. After staining of sperm nuclei with DAPI (10µg/mL), the slides were rinsed and mounted.

Immediate after mounting, slides were visualized under a Nikon fluorescence microscope at x20 magnification at an exposure time of 1second and images were captured using Nikon camera. Afterwards, PLCζ and PAWP protein expression were assessed via analysis of the percentages of sperm exhibiting PLCζ and PAWP immunofluorescence (the percentages of PLCζ and PAWP positive sperm), and also quantitative analysis of total PLCζ and PAWP immunofluorescence in the sperm head. Two hundred sperms were analyzed in each subject and results were recorded. **Figures 1 and 2** represent samples of sperms were stained with PLCζ and PAWP immunofluorescence procedure and counterstained with DAPI.

Statistical analysis

Prism 6/Graph-Pad software (San Diego, CA, USA) was used to perform all the statistical analyses. The normality of distribution of variables was tested first and then independent sample *T*-test and non-parametric tests were used for comparison of PLCζ and PAWP expression between groups. In addition, the correlation between PAWP and PLCζ expression was calculated using the Pearson or Spearman correlation tests according to normality of data. The level of significance was $P < 0.05$.

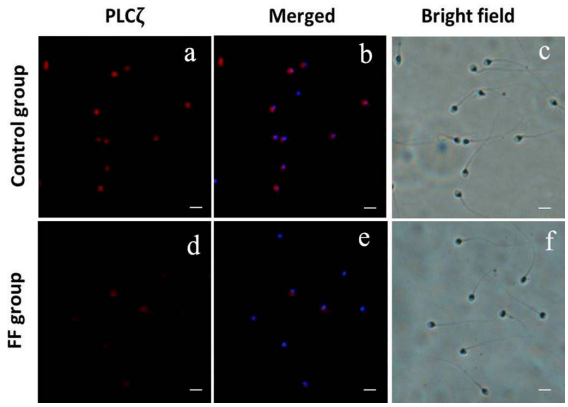


Figure 2. Immunofluorescence staining of PLCζ protein and DAPI in sperm cells. Panels a-c show sperm from control group and Panels d-f represent sperm from FF group (a, d= PLCζ; b, e= merged image of DAPI and PLCζ; c, f = bright field). Error bar = 10µm.

RESULTS

Semen samples were collected from fifteen fertile control men aged 27 to 39 years (32.8 ± 1) (mean ± SEM), and fifteen patients aged 25 to 38 years (31.7 ± 0.9) (mean ± SEM) after at least one cycle with low fertilization or complete fertilization failure. Routine semen analysis was performed according to WHO criteria. **Table 1** shows the basic sperm characteristics of participant samples. The mean of sperm concentration ($\times 10^6$/mL) (28.7 ± 22.4 vs 60 ± 23.2) (mean ± SD), the percentage of total motility (40 ± 27.2 vs 56.3 ± 14.6) (mean ± SD), and the percentage of normal sperm morphology (2.3 ± 2 vs 4.4 ± 1.7) (mean ± SD) were significantly lower in FF group compared to control group. PLCζ and PAWP were detected by immunofluorescence staining and PLCζ, and PAWP protein expression were assessed in sperm from control and FF groups. As shown in **Figure 3A**, the mean percentage of PAWP positive sperm was significantly lower in FF group (62.7 ± 20.1) (mean ± SD) compared to control group (80.3 ± 21.3) (mean ± SD) (P = 0.03). Likewise, we found a significantly lower percentage of PLCζ positive sperm in FF group (58.4 ± 25) (mean ± SD) compared to control group (87.6 ± 9.8) (P = 0.0003) (mean ± SD) (**Figure 3C**).

Quantitative immunofluorescence analysis revealed that total levels of PAWP was significantly lower in group FF compared to group control (P = .01) (**Figure 3B**). As displayed in **Figure 3D**, total levels of PLCζ in FF group was significantly lower when compared to control group (P = .04).

The correlation between percentages of PLCζ and PAWP positive sperm, and total PLCζ and PAWP levels was calculated. Spearman correlation analysis demonstrated a significant positive correlation between the percentages of PLCζ positive sperm and PAWP positive sperm in all individuals (r = 0.4, P = .008) (**Figure 4A**). Moreover, a significant positive correlation was found between total level of expression of PLCζ and PAWP proteins in all participants in the study (r = 0.4, P = .02) (**Figure 4B**).

DISCUSSION

In the present study, we assessed the expression level of PLCζ and PAWP proteins simultaneously in infertile men with previous fertilization failure for the first time and found lower expression of both PAWP and PLCζ proteins in such patients. In addition, we showed positive significant correlations between PLCζ and PAWP expression (both percentage of positive sperm and total level) in all individuals.

The main cause of fertilization failure following ICSI is considered to be the failure of oocyte activation[6].

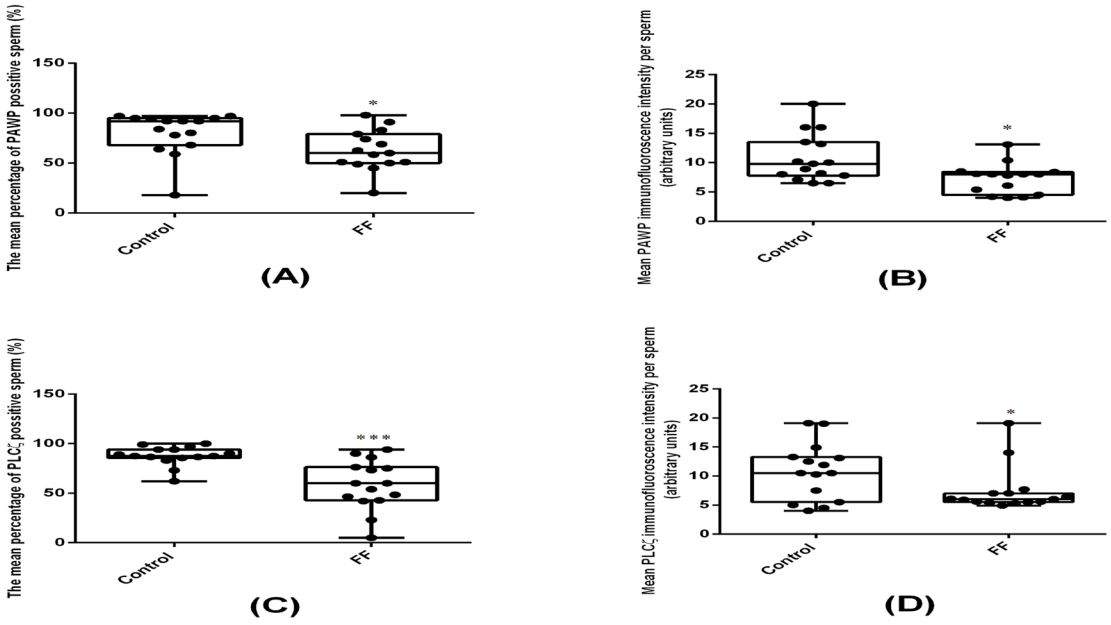
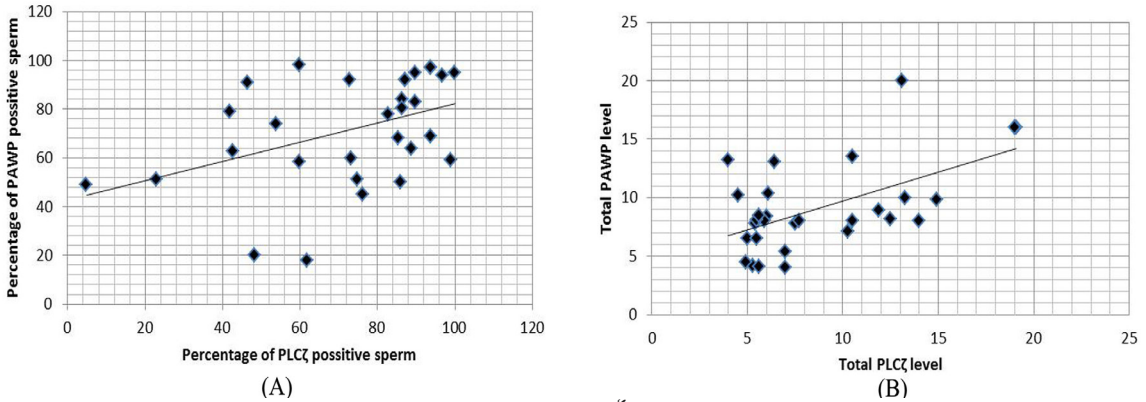


Figure 3. Comparison of the mean percentages of PAWP and PLCζ positive sperm and the total level of PAWP and PLCζ proteins expression between FF and control groups (A-D). *P < 0.05, and ***P < 0.001 significant differences.



(A) (B)

Figure 4. Correlations between the mean percentages of PAWP and PLCζ positive sperm (r = 0.4, *P* = 0.008) **(A)**, and the total level of PAWP and PLCζ proteins expression (r = 0.4, *P* = 0.02) **(B)**.

Oocyte activation occurs when spermatozoa releases an oocyte-activating protein into the oocyte, triggering calcium oscillations via phosphoinositide signaling pathway[23]. Currently, there are conflicting reports about which SOAF (s) is (are) responsible for calcium oscillations. Indeed, identifying the real SOAF (s) has been an important goal for researchers in recent years [12,15-17]. PLCζ and PAWP have been identified as two major SOAF candidates under study[9]; Indeed, identifying the real SOAF as a potential predictor of successful fertilization has been a key aim for researchers especially in the field of clinical research [24]. Although PLCζ deficiency in fertilization failure is demonstrated in several clinical studies,[13,18-20], there is scarcity of data on the expression of PAWP in patients with fertilization failure.

The PAWP protein is a component in the postacrosomal sheath of perinuclear theca (PAS-PT) of sperm head which is accumulated during spermatogenesis and diffused into the oocyte cytoplasm after fertilization. Blocking of calcium oscillations after injection of PAWP competitive peptides indicates PAWP role in oocyte activation[11, 24]. In the current study, assessment of PAWP expression level using immunofluorescence staining demonstrated that the percentage of PAWP positive sperm as well as the total levels of PAWP in FF group were significantly lower than the control group (**Figure 3, A-B**). In line with our study, Aarabi et al. reported significant positive correlations between expression of PAWP with fertilization outcomes in patients undergoing ICSI and suggested PAWP levels in sperm as a predictive value for oocyte activation capability[25]. Recently, a study published by Freour et al. reported no correlation between mRNA and PAWP protein with fertilization rate in patients without previous ICSI cycles[26] which is in contrast to our results. The reason might be the differences in baseline characteristic of people studied in their report compared to the present study.

In this study, we displayed lower percentage of PLCζ positive sperm and lower expression of this protein in patients with previous fertilization failure compared to control group (**Figure 3 C-D**) which was consistent with prior studies[13,18-20]. Accordingly, Yoon et al. showed that sperm from the patients with repeated ICSI failure was deficient in PLCζ protein compared to fertile men[13]. In the other study that was conducted by Kashir et al., lower PLCζ protein, lower proportion of PLCζ positive sperm and abnormal localization patterns were verified in patients suffering from oocyte activation deficiency (OAD). However, due to the significant variance in total levels of PLCζ, some patients with failed fertilization showed similar amount of the protein compared to fertile men which may limit the predictive value of quantitative immunofluorescent analysis for oocyte activation capability[20]. In addition, Patients with history of failed fertilization and normal semen parameters may show PLCζ deficiency.[18,19]

In accordance to our results, Yelumalai et al. found that the rate of fertilization is correlated with PLCζ expression at the level of protein, localization patterns, and the percentage of PLCζ positive sperm[27]. However, in a study was performed by Ferrer-Vaquer et al., no significant difference of PLCζ localization pattern and level of expression was observed between patients with previous fertilization failure and fertile men. The authors explained that normal PLCζ expression is an essential but not a guarantee and sufficient factor for the correct oocyte activation necessarily[28]. Therefore, the assessment of other SOAF candidates in patients with fertilization failure can help us for better understanding of the problem. In the present study, we assessed both PAWP and PLCζ proteins in patients with fertilization failure and found lower expression of both PAWP and PLCζ proteins in those patients. Overall, our results are in agreement with a recent study investigating the correlation between expression of three potential factors including PLCζ, PAWP, and TR-KIT with fertilization rate and found significant positive correlation between them in patients subjected to ICSI. The authors also reported significant positive correlations between the percentage of PAWP positive sperm with PLCζ and TR-KIT[29]. In another study, positive correlation between PLCζ and PAWP at protein levels is demonstrated in globozoospermic men[30]. Similar to these studies, we also showed that total level and percentage of PAWP positive sperm are correlated with PLCζ significantly. In agreement with previous studies indicating similar level of PLCζ expression in some control and patients with fertilization failure[20,28], in this study we observed surprisingly equal PAWP protein expression in some individuals in the control and FF groups (**Figure 3 A-B**). Taken together, to the best of our knowledge, this is the first report evaluating two major SOAF candidates simultaneously in patients with fertilization failure. We showed lower expression of both PLCζ and PAWP pro-

teins and significant correlation between these factors in the patients. It seems both factors might hold the potential to be considered as SOAFs and diagnostic markers for oocyte activation ability.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

REFERENCES

1. http://www.who.int/reproductivehealth/topics/infertility/definitions/en/index.html.
2. Esteves SC, Miyaoka R, Agarwal A. An update on the clinical assessment of the infertile male. [corrected]. Clinics (Sao Paulo). 2011;66:691-700.
3. Terada Y, Schatten G, Hasegawa H, Yaegashi N. Essential roles of the sperm centrosome in human fertilization: developing the therapy for fertilization failure due to sperm centrosomal dysfunction. Tohoku J Exp Med. 2010;220:247-58.
4. Vanden Meerschaut DN, Heindryckx B, De Sutter P. Assisted oocyte activation following ICSI fertilization failure. Reprod BioMed Online. 2014;28:560-71.
5. Palermo GD, Neri QV, Takeuchi T, Rosenwaks Z. ICSI: where we have been and where we are going. Semin Reprod Med. 2009;27:191-201.
6. Flaherty SP, Payne D, Matthews CD. Fertilization failures and abnormal fertilization after intracytoplasmic sperm injection. Hum Reprod. 1998;13 Suppl 1:155-64.
7. 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;6:510-6.
8. Swann K. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development. 1990;110:1295-302.
9. Vadnais ML and Gerton GL. From PAWP to "Pop": opening up new pathways to fatherhood. Asian J Androl. 2015;17:443-4.
10. Amdani SN, Yeste M, Jones C, Coward K. Sperm Factors and Oocyte Activation: Current Controversies and Considerations. Biol Reprod. 2015;93:50.
11. Aarabi M, Qin Z, Xu W, Mewburn J, Oko R. Sperm-borne protein, PAWP, initiates zygotic development in Xenopus laevis by eliciting intracellular calcium release. Mol Reprod Dev. 2010;77:249-56.
12. Aarabi M, Sutovsky P, Oko R. Re: Is PAWP the 'real' sperm factor? Asian J Androl. 2015;17:446-9.
13. Yoon SY, Jellerette T, Salicioni AM, 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;118:3671-81.
14. Nomikos M, Yu Y, Elgmati K, et al. Phospholipase Czeta rescues failed oocyte activation in a prototype of male factor infertility. Fertil Steril. 2013;99:76-85.
15. Aarabi M, Balakier H, Bashar S, et al. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. FASEB J. 2014;28:4434-40.
16. Nomikos M, Sanders JR, Kashir J, et al. Functional disparity between human PAWP and PLCzeta in the generation of Ca2+ oscillations for oocyte activation. Mol Hum Reprod. 2015;21:702-10.
17. Nomikos M, Sanders JR, Theodoridou M, et al. Sperm-specific post-acrosomal WW-domain binding protein (PAWP) does not cause Ca2+ release in mouse oocytes. Mol Hum Reprod. 2014;20:938-47.
18. 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;31:749-56.
19. Chithiwala ZH, Lee HC, Hill DL, et al. Phospholipase C-zeta deficiency as a cause for repetitive oocyte fertilization failure during ovarian stimulation for in vitro fertilization with ICSI: a case report. J Assist Reprod Genet. 2015;32:1415-9.
20. Kashir J, Jones C, Mounce G, 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;99:107-17.
21. WHO. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva, Switzerland: WHO Press; 2010.
22. Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative oocyte activation factor, phospholipase Czeta, in uncapacitated, capacitated, and ionophore-treated human spermatozoa. Hum Reprod. 2008;23:2513-22.
23. Whitaker M. Calcium at fertilization and in early development. Physiol Rev. 2006;86:25-88.
24. Wu AT, Sutovsky P, Xu W, van der Spoel AC, Platt FM, Oko R. The postacrosomal assembly of sperm head protein, PAWP, is independent of acrosome formation and dependent on microtubular manchette transport. Dev Biol.

2007;312:471–83.

25. Aarabi M, Balakier H, Bashar S, et al. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. Fertil Steril. 2014;102:440-7.
26. Freour T, Barragan M, Ferrer-Vaquer A, Rodriguez A, Vassena R. WBP2NL/PAWP mRNA and protein expression in sperm cells are not related to semen parameters, fertilization rate, or reproductive outcome. J Assist Reprod Genet. 2017;34(6):803-10.
27. Yelumalai S, Yeste M, Jones C, et al. Total levels, localization patterns, and proportions of sperm exhibiting phospholipase C zeta are significantly correlated with fertilization rates after intracytoplasmic sperm injection. Fertil Steril. 2015;104:561-8 e4.
28. Ferrer-Vaquer A, Barragan M, Freour T, Vernaeve V, Vassena R. PLCζ sequence, protein levels, and distribution in human sperm do not correlate with semen characteristics and fertilization rates after ICSI. J Assist Reprod Genet. 2016;33:747-56.
29. Tavalaee M and Nasr-Esfahani MH. Expression profile of PLCzeta, PAWP, and TR-KIT in association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intra-cytoplasmic sperm injection and artificial oocyte activation. Andrology. 2016;4:850-6.
30. Kamali-Dolat Abadi M., Tavalaee M, Shahverdi A, Nasr-Esfahani MH. Evaluation of PLCζ and PAWP Expression in Globozoospermic Individuals. Cell J. 2016;18:438-45.