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RESEARCH ARTICLE

# Phospholipase C zeta parameters in sperm from polymorphic teratozoospermic men

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ABSTRACT

Teratozoospermia is a disorder associated with high abnormal sperm morphology which affects fertility in males. In recent years, it has been described that biomarker-based sperm quality evaluation can alleviate male infertility treatment. Phospholipase C zeta (PLCζ) is a sperm-specific factor which appears to be a predicting biomarker for fertilization potential of males. Following fertilization, PLCζ enters into oocyte cytoplasm and induces oocyte activation, a fundamental stage in initiation of embryo development. Currently, PLCζ parameters, including localization patterns, the proportion of PLCζ-expressing sperm and the expression level, are not defined in polymorphic teratozoospermic men. This study aimed to evaluate PLCζ parameters in polymorphic teratozoospermic men, and compare these parameters with fertile normozoospermic men. Semen samples from thirteen normozoospermic fertile men and twenty-three polymorphic teratozoospermic men were included in this study and evaluated using western blotting and immunofluorescence analyses. Our data indicated significantly lower expression of PLCζ in polymorphic teratozoospermic men, as compared with control men; however, there was no significant difference in localization patterns and the proportion of PLCζ-expressing sperm between polymorphic teratozoospermic patients and control men. Collectively, findings from the present study demonstrated that polymorphic teratozoospermic men did not show abnormal localization patterns or the absence of PLCζ, as compared to the control men; nonetheless, lower expression of PLCζ, considering its role in oocyte activation, might be one of the possible causes of infertility in these patients.



## 1. Introduction

Infertility is defined as the inability to conceive after 12 months of regular sexual intercourse without the use of contraception (Zegers-Hochschild et al., 2009). More than 15% of couples of reproductive age suffer from infertility (Boivin et al., 2007). Reduced quality or quantity of sperm in half of the infertile couples indicate male factor infertility alone, or in combination with a female factor (Krausz, 2011).

Teratozoospermia is one of the causes of male infertility (Shabtaie et al., 2016) which is a condition characterized by the presence of high abnormal sperm morphology in semen (more than 96%) (WHO, 2010). Teratozoospermia is divided into 2 types, including polymorphic teratozoospermia with more than one type of sperm morphology abnormalities, and monomorphic teratozoospermia with one type of abnormal sperm morphology such as globozoospermia (round-head sperm morphology) (Perrin et al., 2008, 2013).

In recent years, biomarker-based sperm analysis has been suggested to assess sperm quality for better treatment of male infertility (Sutovsky et al., 2015). It is believed that phospholipase C zeta (PLCζ), a sperm-specific protein, can be a potential biomarker for the prediction of oocyte activation ability in males (Aghajanpour et al., 2011; Park et al., 2015; Yelumalai et al., 2015). Significant evidence supported the role of PLCζ as one of the candidates responsible for oocyte activation, a vital stage for beginning of embryo development (Amdani et al., 2016). Although PLCζ may be a diagnostic, prognostic and therapeutic biomarker of sperm and

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male fertility (Ramadan et al., 2012; Nomikos et al., 2013), there is a strong need to convert scholarly findings to clinical settings for use in infertile patients (Amdani et al., 2013).

Previous studies showed PLCζ deficiency and abnormal localization patterns in globozoospermic infertile men (Heytens et al., 2009; Taylor et al., 2010; Kashir et al., 2012; Kamali-Dolat Abadi et al., 2016). In case of polymorphic teratozoospermia, although possible etiologies of infertility such as sperm aneuploidy, DNA fragmentation, and membrane oxidative damage have been reported in literature (Brahem et al., 2011; Cohen-Bacrie et al., 2009; Agarwal et al., 2014; Hosseinzadeh et al., 2013), PLCζ parameters, including localization patterns, the proportion of PLCζ-expressing sperm and the level of expression, are not identified in polymorphic teratozoospermia. Most of the previous studies on PLCζ parameters performed in infertile men with previous fertilization failure and different semen parameters (Yoon et al., 2008; Yelumalai et al., 2015). Therefore, these studies failed to show how PLCζ parameters are in patients with polymorphic teratozoospermia. The present study aimed to evaluate the PLCζ parameters in polymorphic teratozoospermic patients, and compared these parameters with normozoospermic fertile men.

## 2. Subjects and methods

### 2.1. Materials

A PLCζ rabbit polyclonal antibody (LS-C144827) was purchased from LifeSpan BioSciences (USA). Ponceau S, Ham's F-10, normal goat serum, DPBS (Dulbecco's Phosphate-Buffered Saline), Tween 20, Triton X-100, and para-formaldehyde were bought from Sigma-Aldrich (Germany). Goat anti-rabbit IgG H&E (Alexa Fluor® 555) (ab150082) and horseradish peroxidase-labeled secondary antibodies were obtained from Abcam company (UK; ab6721). ß-actin antibody (mAb 4970) was bought from Cell Signaling (USA). Diff Quick Rapid Sperm Staining kit and ECL western blotting substrate kit were purchased from Avicenna (I.R.I) and Cyto Matin Gene company (I.R.I), respectively. PVDF membranes were obtained from Millipore (USA). Complete™ Protease Inhibitor Cocktail was prepared from Roche company (Switzerland).

### 2.2. Patient selection and ethics

The study was performed between March 2015 and September 2016 and approved by the ethics committee of Shahid Beheshti University of medical sciences and appropriate regulatory bodies within the infertility center. Informed written consents for use of semen and their information were taken from all participants. Sperm samples were obtained from infertile couples (n = 23) who had been referred to the infertility center for primary infertility treatment. Polymorphic teratozoospermic men with sperm concentration >15 million/mL, total motility >40% and normal morphology <4% (WHO, 2010) were included in this study. Normozoospermic donors (n = 13) with proven fertility served as a control group. Any history of trauma, inflammation or surgical history in the testis, varicocele, cryptorchidism, globozoospermia, and the presence of sperm with round-headed morphology of greater than 4% were exclusion criteria in this study.

### 2.3. Semen processing and analysis

All semen samples were collected in sterile container after 2–5 days of abstinence by masturbation. After liquefaction, sperm concentration, total motility [progressive (PR) + non-progressive (NP)] and morphology were evaluated according to the guidelines of the WHO (WHO, 2010). Then, the semen was centrifuged and supernatant was discarded. The pellet was suspended in pre-warmed Ham's F-10 medium, and then used for immunofluorescence staining. To prepare sperm without round cell contamination for western blotting, after semen centrifugation, the pellet was gently covered with pre-warmed Ham's F-10 in the tube with 45°, and kept in an incubator at 37 °C and 5% CO2. After 30–60 min, the supernatant was collected, and used for western blotting.

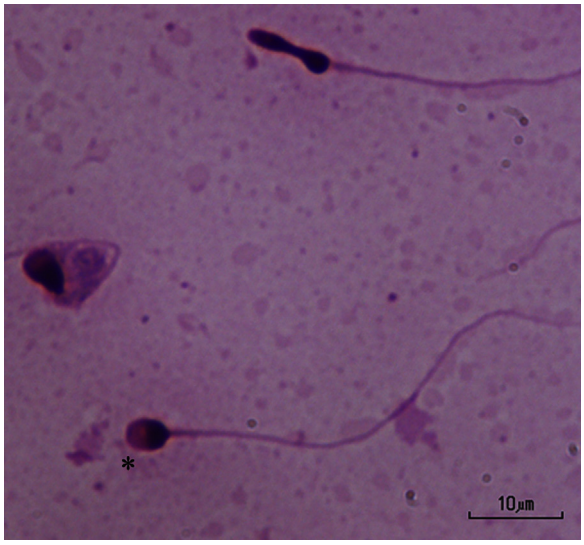


**Fig 1.** Diff quick staining for sperm morphology evaluation. Sperm with an asterisk (*) has normal morphology, and two other sperm have abnormal morphology. *A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM04173.*

### 2.4. Diff-Quick staining for morphology assessment

Slides were made by applying a 10 µl drop of washed semen to a microscope slide. After drying the smear, sperm were stained with Diff-Quick staining kit according to the instructions to evaluate sperm morphology (Fig. 1). Briefly, following sperm fixation with fixative solution for 75 s, sperm were stained with staining solution 1 (eosin Y in phosphate buffer) for 60 s. After draining of staining solution 1 on smear, sperm were stained with staining solution 2 (basophilic thiazine) for 5 s. The slides were then washed with distilled water and allowed to air dry for 1 h. Finally, the slides were analyzed under a bright field microscope (Nikon, Japan) ×100 magnification using immersion oil, and the normal and abnormal sperm morphologies were recorded (n = 200).

The sperm with normal morphology had the following criteria: oval head, acrosomal region comprising 40–70% of the head area, no large vacuoles in head, no vacuoles in the post-acrosomal region, regular borderline and normal length of head, midpiece, principle piece, tail, and residual cytoplasm less than one third of the sperm head (WHO, 2010). Samples showing normal morphology of spermatozoa less than 4% and more than one type of sperm abnormalities were considered as polymorphic teratozoospermia.

### 2.5. Determination of PLCζ immunofluorescence in sperm

PLCζ was detected by immunofluorescent staining as previously described by Grasa et al. (2008) with some modifications. Briefly, sperm were fixed with freshly prepared 4% paraformaldehyde solution/PBS for 10 min, washed twice with 0.1% Tween 20/PBS (washing buffer) for 5 min, and permeabilized with 0.2% Triton X-100/PBS for 10 min on ice. After rewashing with washing buffer, unspecific binding sites were blocked with 5% normal goat serum/PBS for 1 h at 37 °C; the samples were washed, and incubated in the PLCζ rabbit anti-human polyclonal antibody (LS-

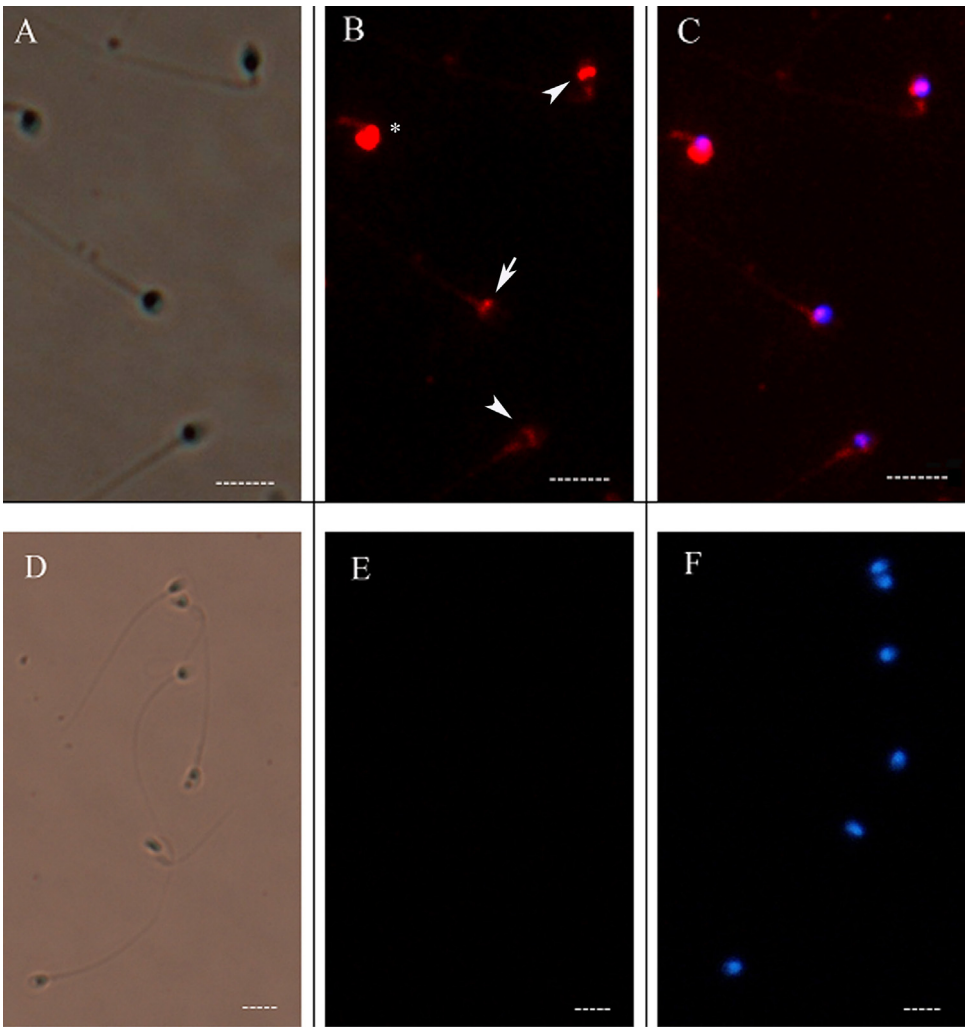


**Fig. 2.** PLCζ immunofluorescence staining (A–C). Sperm were stained with anti-PLCζ (red) and DAPI (blue) (A: brightfield; B: PLCζ immunostaining; C: merged PLCζ and DAPI). Some localization patterns are shown in part B (white arrow: post-acrosomal; white arrowheads: equatorial; asterisk: acrosomal + equatorial). Parts D–F show negative control by eliminating PLCζ primary antibody (D: brightfield; E: negative control; F: merged DAPI and negative control). Scale bars represent 10 μm. *A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM04174.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

C144827; dilution, 1:50) in 5% normal goat serum/PBS overnight at 4 °C. The negative control was prepared by eliminating the primary antibody. Following washing, sperm were incubated with goat anti-rabbit IgG H&E (Alexa Fluor® 555) (dilution, 1:200) in 5% normal goat serum/PBS at room temperature (RT) for 1 h (light protected), and then washed three times for 5 min with washing buffer. Lastly, the sperm were counterstained with DAPI (10 μg/ml), and mounted. The specimens were visualized under a Nikon fluorescence microscope system (TS100, Japan) equipped with a Nikon camera (DS-Fi1c, Japan). The specimen photographs were taken at ×20 magnification at an exposure time 1 s and after merging photos using ImageJ software, the percentage of sperm exhibiting PLCζ immunofluorescence, the level of expression, and localization patterns of PLCζ were recorded for each sample (n = 200). A sample of PLCζ immunofluorescence staining and negative control is shown in Fig. 2. As previously described by Grasa et al. (2008) and Kashir et al. (2013), seven patterns recorded including acrosomal (A), equatorial (E), post-acrosomal (PA), acrosomal and equatorial (A + E), acrosomal and post-acrosomal (A + PA), equatorial and post-acrosomal (E + PA), and acrosomal, equatorial, and post-acrosomal (A + E + PA). Sperm exhibiting no PLCζ immunofluorescence were considered as "none".

### 2.6. Western blotting

PLCζ immunoblotting was performed as previously described by Heytens et al. (2009), with some modifications. A density of $5 \times 10^6$ sperm without round cell contamination was used for protein extraction. Samples were washed by BPS to get rid of albumin contamination and then kept at −80 °C until use. Lysis buffer (urea 8 M, Tris–HCL 40 Mm, 1% Triton X-100) and protease inhibitor were added to the thawed samples, and the mixture was sonicated, gently shaken for 30 min, and then centrifuged for 5 min at 12000 rpm. Lysate from human breast adenocarcinoma cell line (MCF-7) was used as negative control. After the Bradford assay and determination of protein concentration, the supernatant was boiled in 4× sample buffer for 5 min.

Subsequently, protein (~10 μg) was loaded onto 5% and 10% SDS-PAGE gels and resolved polypeptides were transferred to PVDF membranes using a Mini Trans-Blot Cell (Padideh Nojen, I.R.I). After ponceau S staining to ensure effective transfer, the membranes were cut and blocked with 5% non-fat dry milk in 0.1% Tween 20/PBS (washing buffer) and then upper and lower parts of PVDF membrane incubated overnight at 4 °C with the primary antibodies PLCζ (LS-C144827; dilution, 1:1800) and ß-actin (dilution, 1:4000), respectively. After washing with washing buffer, papers were

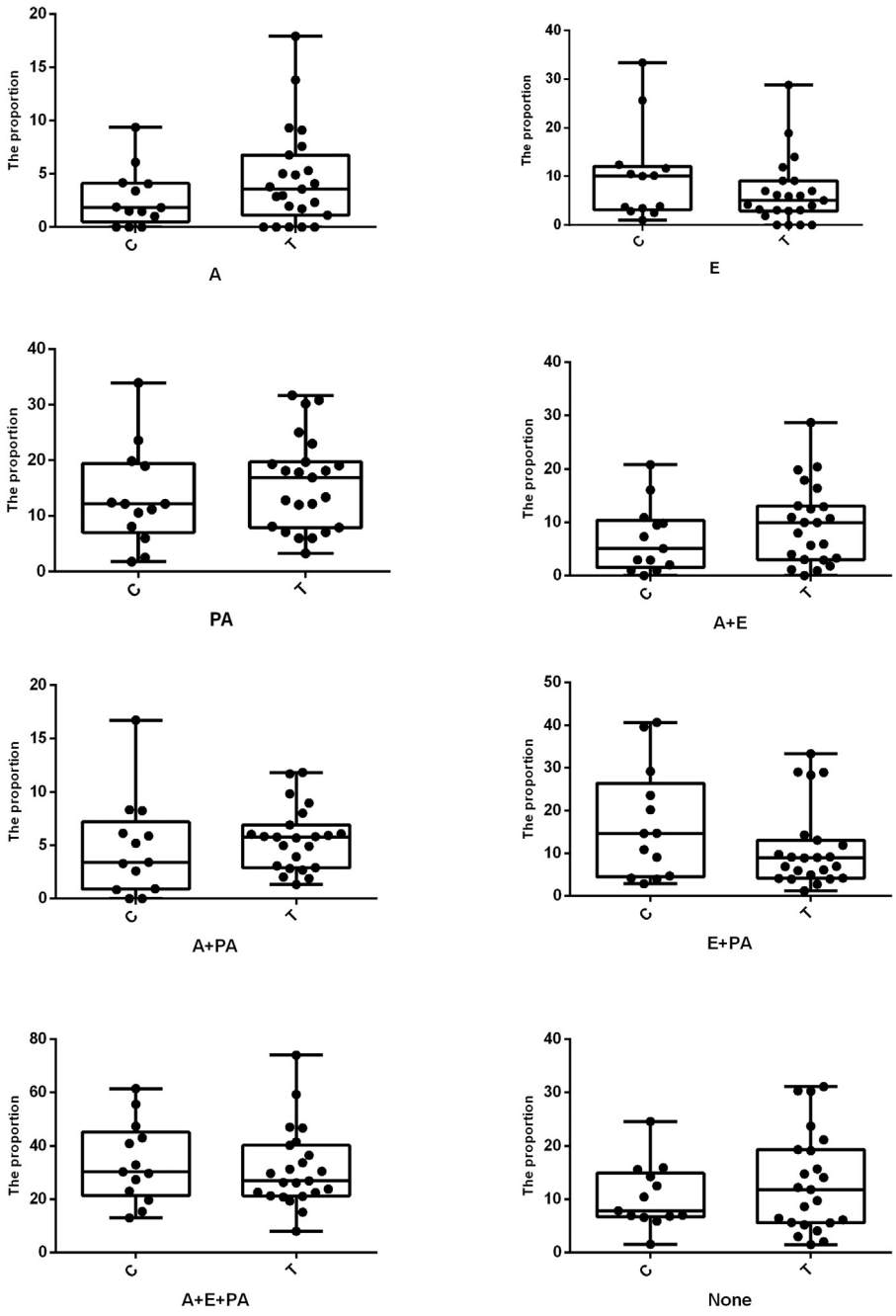


**Fig. 3.** Comparison of different localization patterns showed no significant difference between polymorphic teratozoospermic and control men. C = control, T = polymorphic teratozoospermic men, A = acrosomal, E = equatorial, PA = post-acrosomal, A + E = acrosomal and equatorial, A + PA = acrosomal and post-acrosomal, E + PA = equatorial and post-acrosomal, A + E + PA = acrosomal, equatorial, and postacrosomal.

incubated in secondary antibody (horseradish peroxidase–labeled antibody) for 1 h in RT. Immunoreactivity was detected using ECL staining kit. Densitometric analysis of the images was assessed using imageJ software, and shown as relative protein expression.

### 2.7. Statistical analysis

All data were analyzed by Prism 6/Graph-Pad (version 6.01). For normalizing proportional data, the percentages of PLCζ localization patterns and PLCζ-expressing sperm were transformed using arcsine root transformation ($\arcsin\sqrt{x}$). If, even after transformation, proportional data did not match parametric norms, alternative nonparametric tests were used. Comparison of the expression level, the percentage of localization patterns, and the percentage of PLCζ-expressing sperm were performed using the student t-test (normal distribution of data) or Mann–Whitney U test (non-normal distribution of data) between polymorphic teratozoospermic patients and control men. $P$ values $\leq 0.05$ were considered to be statistically significant.

## 3. Results

### 3.1. Basic and demographic characteristics of polymorphic teratozoospermic patients

Semen was collected from teratozoospermic men (n = 23) aged 23–40 years ($30.8 \pm 1$) (mean ± SEM), and fertile normozoospermic men (n = 13) aged 25–38 years ($32.07 \pm 1.2$) (mean ± SEM).

**Table 1**
Semen parameters in teratozoospermic and control men.

| Parameters | Control men n = 13 | Teratozoospermic men n = 23 | *p* value |
|---|---|---|---|
| Sperm concentration/ml | 77.46 ± 8.03 | 65.56 ± 6.24 | 0.31 |
| Total motility (PR + NP) (%) | 66.92 ± 3.21 | 58.13 ± 2.71 | 0.19 |
| Normal morphology (%) | 7.66 ± 0.48 | 1.79 ± 0.22 | <0.0001 |
| Round head morphology (%) | 1.21 ± 0.52 | 1.42 ± 0.28 | 0.61 |

All data are shown as Mean ± SEM.

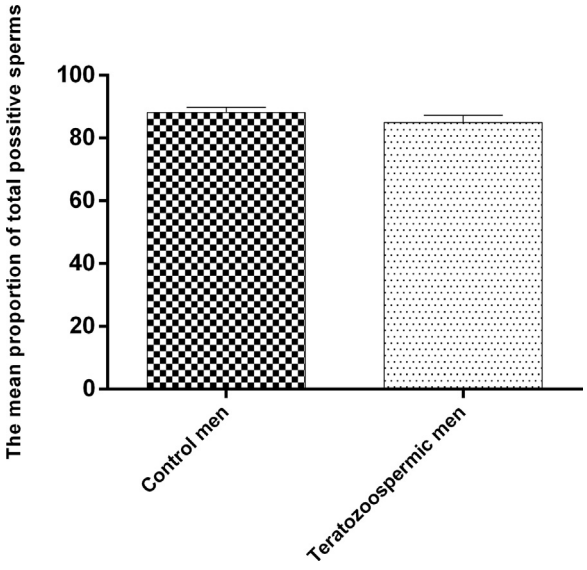


**Fig. 4.** The mean percentage of sperm exhibiting PLCζ immunofluorescence (positive sperm) in control men (n = 13) and polymorphic teratozoospermic men (n = 23). No significant difference was found between two groups. Data are shown as mean ± SEM.

Table 1 represents semen parameters including sperm concentration ($10^6$/mL), total motility (%), normal sperm morphology (%), and round-head morphology (%) in both groups.

### 3.2. Immunofluorescence study

#### 3.2.1. PLCζ localization patterns in sperm

PLCζ localization patterns were evaluated as previously described by Kashir et al. (2013). Different localization patterns, including acrosomal (A), equatorial (E), postacrosomal (PA), acrosomal and equatorial (A + E), acrosomal and post-acrosomal (A + PA), equatorial and post-acrosomal (E + PA), and acrosomal, equatorial, and post- acrosomal (A + E + PA) were evaluated in this study. As shown in Fig. 3, no significant difference in all localization patterns and "none" was detected between polymorphic teratozoospermic and control men.

#### 3.2.2. Proportions of the sperm exhibiting PLCζ immunofluorescence

Comparison of the percentages of sperm exhibiting PLCζ immunofluorescence revealed no significant variance between the polymorphic teratozoospermic patients (84.9 ± 2.2) and control men (88.1 ± 1.7) (Fig. 4).

#### 3.2.3. Mean levels of PLCζ fluorescence

As shown in Fig. 5, quantitative immunofluorescence analysis of mean levels of PLCζ fluorescence using ImageJ software indicated significant differences ($P \leq 0.05$) between teratozoospermic patients (10.05 ± 0.8) and control men (12.9 ± 0.9).

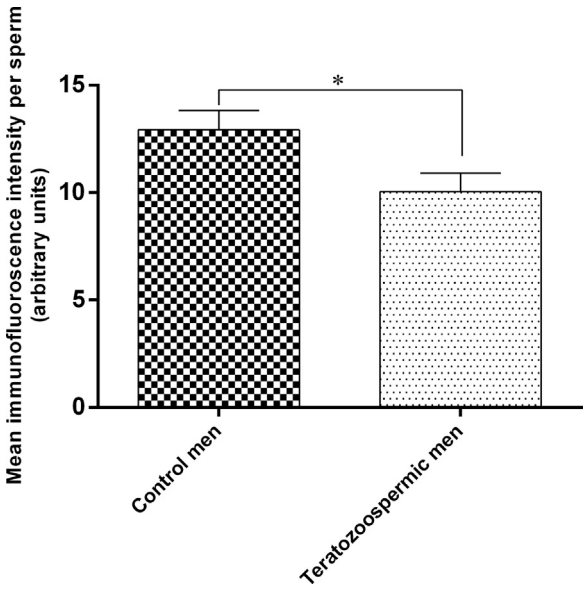


**Fig. 5.** The mean immunofluorescence intensity per sperm (arbitrary units) was detected using Imagej software. Comparison of groups showed a significant difference between them (*$P \leq 0.05$). Data are shown as mean ± SEM.

### 3.3. Western blotting

Relative PLCζ expression in sperm was determined by western blotting (mean ± SEM) and compared between groups. ß-actin levels were used as loading control (Fig. 6A). As shown in Fig. 6B, relative PLCζ expression was significantly lower in polymorphic teratozoospermic patients (0.97 ± 0.2) compared to control men (2.4 ± 0.7).

Western blot analysis also demonstrated an additional band at ~52 kDa in all samples lower than PLCζ band (70 kDa) (Fig. 6A). In addition, the negative control is shown in Fig. 6C.

## 4. Discussion

Nowadays, semen analysis is considered as an essential tool to evaluate the male fertility status (Esteves et al., 2012). However, semen analysis suffers from limitations in the detection of some morphological and physiological sperm defects, resulting in a limited degree of correlations between conventional semen parameters and the actual fertility of infertile patients (Sutovsky et al., 2015). This problem was especially reported in teratozoospermic patients (McKenzie et al., 2004; French et al., 2010). In recent years, it has been suggested that biomarker-based sperm analysis improves the treatment of human infertility (Sutovsky et al., 2015). PLCζ seems to be one of these new diagnostic biomarkers for semen quality (Park et al., 2015; Yelumalai et al., 2015).

In the present study, we evaluated PLCζ parameters in polymorphic teratozoospermic men for the first time and showed a lower amount of the protein in teratozoospermic men. In addition, we found that different localization patterns and the proportion of sperm expressing PLCζ do not differ significantly between teratozoospermic patients and control men.

Considering an association between PLCζ, and sperm count and motility (Yelumalai et al., 2015; Tavalaee et al., 2017), the present study was planned to examine teratozoospermic patients with normal motility and count, so as to minimize interference with other factors. However, it is important to note that other studies were unable to find a correlation between PLCζ, and sperm count and motility (Park et al., 2015; Ferrer-Vaquer et al., 2016).

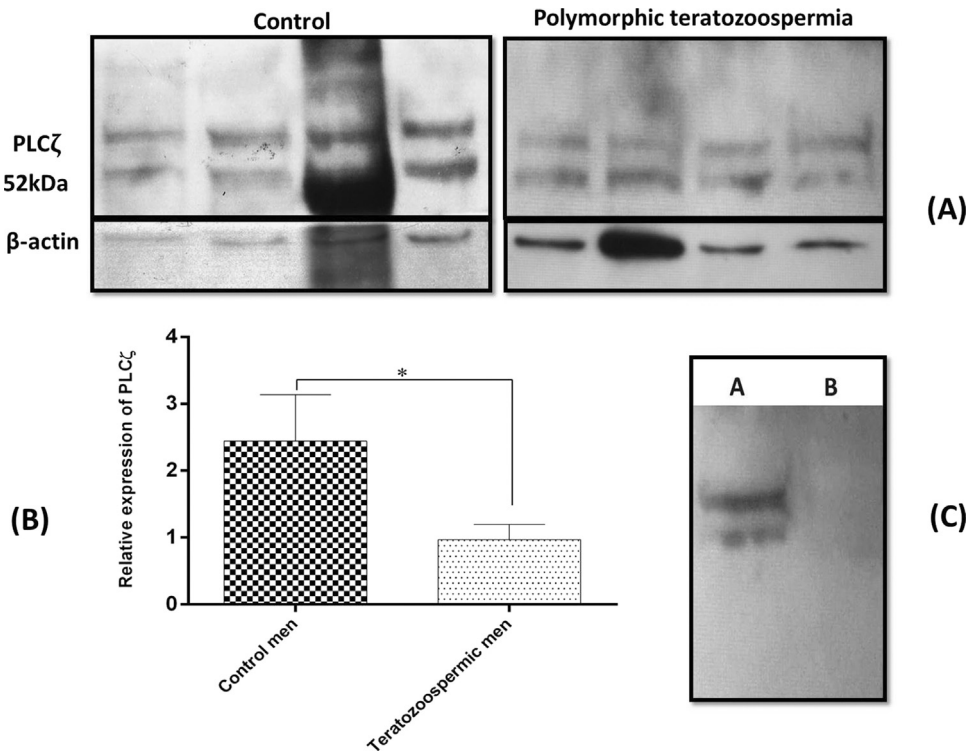


**Fig. 6.** (A) Western blots of PLCζ and β-actin in four control men and four polymorphic teratozoospermic men. The band at ~52 kDa was observed in all samples. (B) Comparison of relative PLCζ expression between normozoospermic control men and polymorphic teratozoospermic men. The asterisk (*) shows statistically significant difference ($P \leq 0.05$) between groups. (C) Western blot of PLCζ (70 kDa) in two samples; column A: Lysate from sperm, and column B: Lysate from MCF-7 line as negative control. Data are presented as mean ± SEM.

In boar and mice, PLCζ mRNA is first expressed in round spermatids and likely to be translated in elongating spermatids (Yoneda et al., 2006; Aarabi et al., 2012**).** The earlier PLCζ mRNA expression during spermatogenesis in hamster was claimed by Young et al. (Young et al., 2009). Aarabi et al. demonstrated that PLCζ is a part of the acrosome during the Golgi phase of human spermatogenesis, and decreases gradually in the acrosome of elongated spermatids. They reported that the transcription of PLCζ mRNA occurs in the principal cell of the epididymal epithelium (Aarabi et al., 2012). Up to the present time, no follow-up study was performed to confirm this hypothesis (Amdani et al., 2015). They also described that human PLCζ is located on the sperm membrane, but not in perinuclear theca (PT) of sperm head where (a) sperm-borne oocyte activating factor(s) is located (Aarabi et al., 2012); however, localization of PLCζ at the PT of the equatorial and post-acrosomal regions of human sperm was shown by Escoffier et al. in 2015 (Escoffier et al., 2015).

PLCζ deficiency or abnormal localization patterns in globozoospermic patients were demonstrated in some studies (Heytens et al., 2009; Kashir et al., 2012; Taylor et al., 2010; Kamali-Dolat Abadi et al., 2016); accordingly, the absence or decreased amount of protein and an abnormal punctate pattern of PLCζ localization was detected in infertile men with globozoospermia (Heytens et al., 2009; Kashir et al., 2012). Taylor et al. also showed the lack of PLCζ in sperm from a globozoospermic patient and the need for ICSI and oocyte activation via calcium ionophore for the treatment of such patients (Taylor et al., 2010). In a recent study performed by Kamali-Dolat Abadi et al. (2016), a reduced amount of PLCζ at RNA and protein levels has been displayed in a larger sample size of globozoospermic patients, as compared to prior studies. In the present study, different localization patterns and the percentage of sperm exhibiting PLCζ immunofluorescence did not differ significantly between teratozoospermic patients and normozoospermic control men (Figs. 3 and 4). This result revealed that polymorphic teratozoospermia, in contrast to globozoospermia, does not demonstrate the absence of PLCζ or abnormal localization patterns as compared with the control men. Consistent with our results, a study carried out by Park et al., revealed that there is no correlation between the percentage of sperm exhibiting PLCζ immunofluorescence and sperm morphology in 44 men (Park et al., 2015).

In the present study, we demonstrated a significant decrease in protein expression in polymorphic teratozoospermic men compared with control men using immunofluorescence staining (Fig. 5), and western blotting (Fig. 6B) techniques. Similar to our study, Villaverde et al. evaluated the level of PLCζ in teratospermic cats, and showed that sperm from teratospermic cats express a reduced amount of PLCζ as compared with normospermic cats (Villaverde et al., 2013). However, reduced amounts of the PLCζ protein in the patients with fertilization failure were reported previously (Yoon et al., 2008; Heytens et al., 2009; Lee et al., 2014); two other studies conducted by Ferrer-Vaquer et al. and Kashir et al. displayed a similar amount of protein in some control men and patients with fertilization failure (Kashir et al., 2013; Ferrer-Vaquer et al., 2016). Moreover, Ferrer-Vaquer et al. described that two donors with a low amount of PLCζ had the normal fertilization rates (Ferrer-Vaquer et al., 2016). Given that the role of PLCζ in male fertility, lower amounts of protein expression in polymorphic teratozoospermic patients might be one of the potential causes of infertility in such patients.

To confirm the previous immunofluorescence results regarding to level of PLCζ expression, we carried out western blotting on sperm samples. Western blot of PLCζ also revealed an additional band at ~52 kDa in all samples lower than PLCζ band (70 kDa) (Fig. 6A). This band has been reported by previous studies using other antibodies (Grasa et al., 2008; Yoon et al., 2008; Heytens et al., 2009). To the best of our knowledge, the band at ~52 kDa

is not recognized conclusively yet; although it may be a proteolytic fragment of PLCζ or truncated endogenous form of human PLCζ (Heytens et al., 2009; Kurokawa et al., 2007; Bi et al. 2009).

Taken together, to the best of our knowledge, this is the first report evaluating PLCζ parameters in the polymorphic teratozoospermic patients. Our data indicated a significant variance in the protein expression between polymorphic teratozoospermic patients and control men while the proportions of PLCζ-expressing sperm and localization patterns did not differ significantly between them.

## 5. Conclusion

The present study revealed that polymorphic teratozoospermic men do not demonstrate the absence of PLCζ or abnormal localization patterns as compared with control men. Considering the role of PLCζ in oocyte activation following fertilization, lower PLCζ protein in sperm from polymorphic teratozoospermic patients compared with control men might affect the fertility potential in these men and might be one of the probable causes of infertility in polymorphic teratozoospermia.

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## Conflicts of interest

The authors declare that they have no conflict of interest.

## Acknowledgement

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2017.09.007.

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