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RESEARCH ARTICLE

Nahid Azad, et al.: PLCζ and PAWP in OAT men

Oligoasthenoteratozoospermic (OAT) men display altered phospholipase C ζ (PLCζ) localization and a lower percentage of sperm cells expressing PLCζ and post-acrosomal sheath WW domain-binding protein (PAWP)

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ABSTRACT

Oligoasthenoteratozoospermia (OAT) is demonstrated to be one of the most common causes of male subfertility. Phospholipase C ζ (PLCζ), a sperm-specific protein, is considered to be one of the sperm-borne oocyte activating factors (SOAFs), which play a vital role in fertilization. The post-acrosomal sheath WW domain-binding protein (PAWP) is another candidate for SOAF. The aim of this study was to compare the PLCζ localization patterns and percentage of PLCζ- and PAWP-positive sperm cells in patients with OAT and fertile men with normozoospermia. A total of 40 men included in this study were classified into two groups: OAT (n = 25) and control group (n = 15). Semen samples were collected and analyzed using conventional semen analysis according to the World Health Organization guidelines. The percentage of PLCζ- and PAWP-positive sperm cells and localization patterns of PLCζ were evaluated using immunofluorescence staining. The mean percentage of sperm cells expressing PAWP and PLCζ was significantly lower in OAT compared to control group (52.8 ± 4.2 vs. 76.8 ± 5 and 63.4 ± 3.5 vs. 86.7 ± 2.1, respectively). In addition, statistically significant differences were found with regard to the PLCζ localization patterns, including equatorial, acrosomal + equatorial, and equatorial + post-acrosomal pattern, between the two groups ($p < 0.01$). The present study showed a lower percentage of sperm cells expressing PLCζ and PAWP, as well as altered localization patterns of PLCζ in men with OAT. Given the role of PLCζ and PAWP in fertilization, as two major candidates for SOAFs, our findings indicate that PLCζ and PAWP impairments may be one of the possible etiologies of decreased fertility in OAT.

KEY WORDS: Oligoasthenoteratozoospermia; OAT; phospholipase C ζ; PLCζ; post-acrosomal sheath WW domain-binding protein; PAWP; semen quality; sperm cells

<H1>INTRODUCTION

Male factor infertility affects 20-70% of couples suffering from infertility [1]. Although the main cause is still unexplained, alterations in semen parameters seem to play a role in male infertility [2]. Oligoasthenoteratozoospermia (OAT), with abnormalities in sperm count, motility and morphology, is considered as one of the most common causes of infertility [3,4]. Some studies indicated that poor sperm quality can negatively affect fertilization outcome following the assisted reproductive technique (ART) [5-7].

Phospholipase C ζ (PLCζ), a sperm-specific protein, is considered to be one of the sperm-borne oocyte activating factors (SOAFs) and a potential biomarker for the prediction of male fertility [8-11]. In addition, significant evidence shows that PLCζ released into the oocyte after the fusion of gametes triggers a series of molecular events named “oocyte activation”, including meiotic resumption, pronuclear formation, and embryo cleavage [11]. Indeed, oocyte activation occurs when PLCζ induces calcium oscillations in the cytoplasm [12-14]. The absence or decreased amount of PLCζ as well as its abnormal localization patterns were reported in infertile patients with oocyte activation deficiency (OAD) and failed intracytoplasmic sperm injection (ICSI) [15,16].

The post-acrosomal sheath WW domain-binding protein (PAWP) is another major candidate for SOAF, and is associated with fertilization outcomes following ICSI [17]. Microinjection of a recombinant PAWP protein or PAWP complementary RNA (cRNA) into humans, mice, and *Xenopus* oocytes induces calcium oscillations similar to those observed following fertilization [18,19].

Nonetheless, no previous studies have simultaneously investigated PLCζ and PAWP proteins in patients with OAT and poor sperm quality.

The aim of our study was to compare the PLCζ localization patterns and percentage of PLCζ- and PAWP-positive sperm cells between patients with OAT and fertile men with normozoospermia.

<H1>MATERIALS AND METHODS

<H2>Chemicals

A PLCζ rabbit polyclonal antibody was purchased from LifeSpan BioSciences, Inc. (USA). A PAWP rabbit polyclonal antibody was obtained from Proteintech Company (UK). Goat anti-rabbit IgG (H + L) [Alexa Fluor ®555] was obtained from Abcam Company (UK). Normal goat serum, Ham's F-10 nutrient medium, human serum albumin (HSA), Tween 20, Dulbecco's phosphate-buffered saline (DPBS), and paraformaldehyde (PFA) were purchased from Sigma-Aldrich (Germany). Diff-Quick rapid stain for sperm morphology was obtained from Avicenna (I.R.I).

<H2>Patients and ethics

The study was carried out between January 2015 and March 2017. We obtained semen samples from 15 fertile men with normozoospermia (control) and 25 patients with OAT, from couples who had been referred to the infertility center for the examination and treatment. Individuals with any history of testicular trauma, surgical intervention, inflammation of the testis, cryptorchidism, and globozoospermia were excluded from the study. The ethics committee of the Shahid Beheshti University of Medical Sciences and appropriate regulatory bodies of the Infertility and Reproductive Health Research Center approved the study. We obtained written informed consents from all participating men. Patients with sperm concentration $<15\times10^6$/mL, total motility <40%, and normal morphology <4%, as confirmed by at least two tests, were considered as having OAT and were included in the study. Fertile men with normozoospermia were sperm donors with proven fertility.

<H2>Semen analysis and processing

All semen samples were collected by masturbation after 2-5 days of sexual abstinence. After the liquefaction (30-45 minutes), semen parameters including sperm concentration ($\times 10^6$/mL), motility (%), and morphology (%) were analyzed according to the World Health Organization (WHO) guidelines [4]. Sperm concentration was manually evaluated using the Makler counting chamber with an inverted microscope (Nikon, Japan) and sperm morphology was assessed by a Diff-Quick staining kit.

Semen processing for the separation of motile sperm cells from dead sperm and other cells was performed using mild centrifugation and several washings. Then, the pellet was gently covered by 0.3 ml Ham's F10 supplemented with 10% HSA and incubated at 37°C for 30-60 minutes for a swim-up. After that, the supernatant was removed and used for immunofluorescence staining.

<H2>Diff-Quick staining

The smears were prepared with a 10-µl drop of semen placed on a microscope slide and, after the fixation, the sperm cells were stained with a Diff-Quick staining kit, to evaluate the morphology. Following the slide examination under a bright-field microscope (Nikon, Japan) at ×40 and ×100 magnifications, photographs were captured using a Nikon camera [Japan] (Figure 1). The morphology of 100 sperm cells was analyzed according to the WHO guidelines, in duplicate. Normal size, shape, and acrosome of the sperm head, without midpiece or tail defects, were considered as normal sperm morphology [4], and the percentage of normal and abnormal sperm cells was determined.

<H2>PLCζ immunofluorescence detection

Immunofluorescent staining of PLCζ was performed as previously described by Grasa et al. [20], with some alterations. Briefly, the washed sperm cells were fixed with a freshly prepared PFA in phosphate-buffered saline (PBS, 4%) for 10 minutes. After permeabilization with 0.2% Triton X-100/PBS on ice for 10 minutes, non-specific binding sites were blocked with 5% normal goat serum/PBS for 1 hour, and incubated with PLCζ rabbit anti-human polyclonal antibody (1:50) in the blocking medium, overnight at 4°C. Then, the sperm cells were incubated with goat anti-rabbit IgG (H + L) antibody (1:200) in the blocking medium at room temperature for 1 hour (light protected). Subsequently, the sperm cells were counterstained with 4', 6-diamidino-2-phenylindole [DAPI] (10 µg/ml) for 5 minutes, and mounted with glycerol. The washing of sperm cells between the described steps was performed using PBST (0.1% Tween 20/PBS). Figure 2A shows different localization patterns of PLCζ in sperm cells stained with DAPI and PLCζ immunofluorescence.

<H2>PAWP immunofluorescence detection

Immunofluorescent staining of PAWP was carried out as previously described by Nomikos et al. [21], with some modifications. Briefly, the washed sperm cells were fixed with 4% PFA/PBS for 10 minutes. Following the permeabilization of sperm cells with 0.5% Triton X-100/PBS on ice for 10 minutes, the non-specific antigens were blocked with 5% normal goat serum/PBS for 1 hour at 37°C, and incubated with PAWP rabbit polyclonal antibody (1:200) in the blocking medium, overnight at 4°C. Then, the sperm cells were incubated with goat anti-rabbit IgG (H + L), diluted 1:100, in the blocking medium at room tepmerature for 1 hour (in the dark). Subsequently, the sperm cells were counterstained with DAPI (10 µg/ml) for 5 minutes, and mounted with a mounting medium. The washing of sperm cells between the described steps was

performed using PBST. Figure 3A shows sperm cells stained with DAPI and PAWP immunofluorescence.

<H2>PAWP and PLCζ immunofluorescence analyses

The microscope slides were visualized under a Nikon fluorescence microscope (TS100, Japan) at ×20 magnification, and photographs were captured using a Nikon camera (DS-Fi1c, Japan). The photos were merged using the ImageJ software (National Institutes of Health).

Different localization patterns of PLCζ in sperm cells from each patient were evaluated as previously described by Grasa et al. [20] and Kashir et al. [15]. The localization patterns, 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) were observed in sperm cells and recorded (n = 200). The percentages of PAWP and PLCζ-positive sperm cells (the cells with PAWP and PLCζ immunofluorescence in the sperm head) were detected in each individual and recorded (n = 200).

<H2>Statistical analysis

All data were analyzed with GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). The results were first analyzed using the D'Agostino-Pearson omnibus normality test. According to the normality test, the percentage of PAWP- and PLCζ-positive sperm cells and different localization patterns of PLCζ were compared between control and OAT group using the t-test or Mann–Whitney U-test. The correlation between PLCζ- and PAWP-positive sperm cells was calculated using the Pearson's correlation coefficient. Data was shown as mean ± standard error of the mean (SEM). The level of significance was considered at $p < 0.05$.

<H1>RESULTS

<H2>Semen analysis

A total of 40 men (25 patients with OAT and 15 controls) were included in this study and their semen parameters were assessed according to the WHO criteria [4]. Table 1 shows the mean age and semen parameters in OAT and control groups. The mean of sperm concentration ($\times 10^6$/mL), total motility (progressive + non-progressive, %), and normal morphology (%) in OAT group were significantly lower compared to control group ($p < 0.0001$).

<H2>Comparative analysis of PLCζ immunofluorescence between the groups

The proportion of sperm cells positive for PLCζ, and different localization patterns of PLCζ (i.e., A, E, PA, A+E, A+PA, E+PA, and A+E+PA) were analyzed using the ImageJ software, and compared between the two groups. As shown in Figure 2B, OAT group displayed a significantly lower proportion of sperm cells expressing PLCζ compared with control group (63.4 ± 3.5 vs. 86.7 ± 2.1, $p < 0.0001$). As depicted in Figure 2C, significant differences were found in the percentage of E, A+E, and E+PA localization patterns between OAT and control groups ($p < 0.01$). However, no significant differences were observed in the other localizations of PLCζ between the two groups.

<H2>Comparative analysis of PAWP immunofluorescence between the groups

The mean percentage of sperm cells positive for PAWP was analyzed and compared between the two groups. As shown in Figure 3B, OAT group showed a lower percentage of PAWP-positive sperm cells compared to control group (52.8 ± 4.2 vs 76.8 ± 5, $p < 0.01$).

<H2>Correlation between PLCζ- and PAWP-positive sperm cells

A significant positive correlation was observed between PLCζ-positive and PAWP-positive sperm cells in all participants, using the Pearson analysis [$r = 0.6$, $p < 0.0001$] (Figure 3C).

<H1>DISCUSSION

To the best of our knowledge, this is the first report simultaneously assessing PLCζ and PAWP in patients with OAT. In this study, men with OAT had PLCζ deficiency and altered localization patterns of PLCζ. In addition, a lower percentage of sperm cells expressing PAWP was found in those patients. A decreased ability of sperm cells for oocyte activation due to alterations in SOAFs may be one of the possible etiologies of decreased fertility in men with OAT.

Biomarker analysis, accompanied by the conventional semen analysis, can improve the treatment of human male infertility [22]. PLCζ, as one of the candidates for SOAFs, was suggested as a diagnostic, prognostic, and therapeutic biomarker in male infertility [8-10,23]. Since the discovery of PLCζ in 2002, a number of studies have demonstrated that PLCζ causes calcium oscillations in oocytes after gamete fusion [23]. PAWP, another major candidate for SOAFs, may also be used as a diagnostic and therapeutic biomarker of infertility [17,22]. In this study, we evaluated PLCζ and PAWP proteins in men with OAT and showed their alterations in the OAT group compared to fertile men with normozoospermia. We observed a significant decrease in the percentage of sperm cells expressing PLCζ and PAWP in patients with OAT. Furthermore, significantly lower proportions of E pattern and its combinations (A+E and E+PA) were found in OAT compared with control group. Finally, a significant positive correlation was observed between the sperm cells expressing PLCζ and PAWP in all individuals.

Grasa et al. [20], demonstrated for the first time that PLCζ is predominantly localized in the E region of the human sperm head. In addition, Yoon et al. [16] showed that PLCζ, while absent in the sperm cells from patients who had experienced fertilization failure, is localized in the E region of the sperm head in fertile men. However, in another study, a variation in PLCζ localization was documented in both control men and infertile patients with OAD; the E

localization pattern alone or in combination with other patterns was predominant in controls, while the PA localization pattern was frequent in OAD patients [15]. Similarly, in our study, a significantly decreased proportion of E pattern and its combinations in patients with OAT indicate altered localization patterns of PLCζ in these patients compared to controls (Figure 2C). Tavalaee et al. [24] reported a significantly lower percentage of PLCζ-positive sperm cells in subfertile patients with OAT compared to fertile men [24]. Consistent with that study, the percentage of sperm cells expressing PLCζ was significantly lower in our patients with OAT, indicating PLCζ impairment in these patients (Figure 2B). Other studies have also demonstrated decreased expression and/or atypical localization of PLCζ in globozoospermic patients [25-28], patients with grade II and III varicocele [29], and patients with previous fertilization failure [16,25,30-32].

Until now, conflicting results have been reported on the correlation between semen parameters and PLCζ. Yelumalai et al. [10] revealed that the total levels of PLCζ are correlated with sperm count and percentage of progressive motile sperm. Recently, Tavalaee et al. [33] have shown a positive correlation between sperm concentration and PLCζ. Moreover, a significant negative correlation between sperm morphology and PLCζ protein has been demonstrated in another study [28]. On the contrary, Park et al. [9] and Ferrer-Vaquer et al. [34] showed no correlation between PLCζ and semen parameters. Collectively, our results are in agreement with the studies reporting a correlation between PLCζ and sperm parameters.

In the present study, we found a lower percentage of PAWP-positive sperm cells in patients with OAT and poor sperm quality compared to control group (Figure 3B), which is in agreement with other two studies showing a significant correlation between PAWP and sperm concentration, motility, and morphology [28,33]. Recently, Freour et al. [35] indicated a significant negative correlation between sperm morphology and PAWP protein expression and positive correlation

between sperm motility and proportion of PAWP-positive sperm cells; however, no correlation was found between PAWP mRNA and semen parameters.

Moreover, similar to our results (Figure 3C), two studies reported a positive correlation between two major candidates for SOAFs [28,33].

Our results suggested that patients with poor sperm quality may be candidates for assisted oocyte activation (a highly specialized method) in infertility clinics, which should be evaluated in the future studies. Due to low sperm count in our samples, only immunofluorescence staining was carried out, with no further analyses.

<H1>CONCLUSION

Our study showed lower percentages of sperm cells expressing PLCζ and PAWP, as well as altered localization of PLCζ in men with OAT, as compared with fertile men. Given the role of PLCζ and PAWP, as two major candidates for SOAFs, in male fertility, our findings indicated that SOAF(s) impairments may be considered as one of the possible etiologies of decreased fertility in patients with OAT.

<H1>ACKNOWLEDGMENTS

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<H1>DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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TABLES AND FIGURES

TABLE 1. Age and sperm parameters in two groups

Parameters	**Control group (n=15)**	**OAT group (n=25)**
Age (years)	33.3±1	32.5±0.7
Sperm concentration (10^6/mL)	63±4.6	9.7±0.8
Total motility (PR+NP) (%)	60±2.3	19.8±2
Normal morphology (%)	6±0.4	1.1±0.2

PR+NP: Progressive + non-progressive; OAT: Oligoasthenoteratozoospermia. Data are shown as mean ± SEM

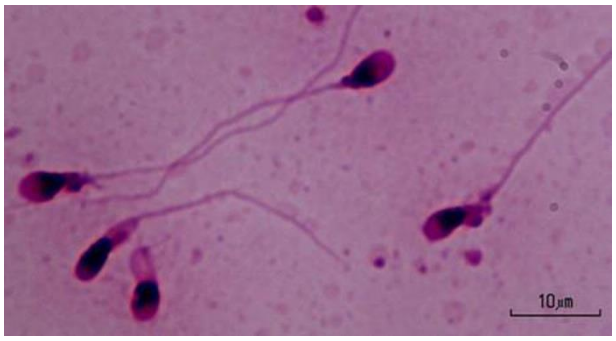

FIGURE 1. Evaluation of sperm cell morphology with Diff-Quick staining. Normal size, shape, and acrosome of the sperm head, without midpiece or tail defects, are considered as normal sperm morphology. Sperm cells that do not show these properties are considered as abnormal. Five sperm cells with abnormal morphology are shown in the figure. Bar = 10 µm.

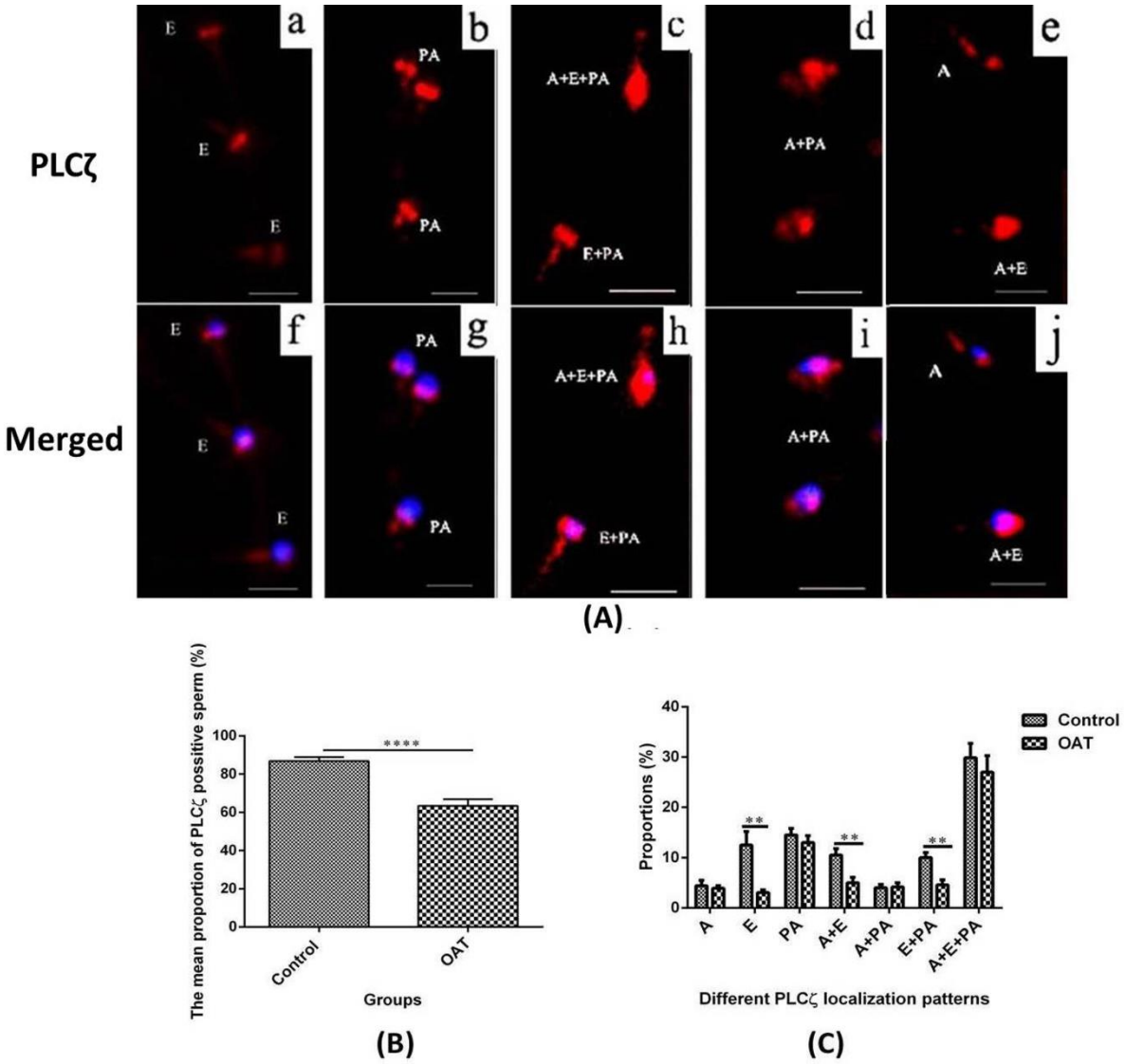

FIGURE 2. Phospholipase C ζ (PLCζ) immunostaining. (A) Different localization patterns of PLCζ in the sperm head. The sperm cells were stained with anti-PLCζ (red, a-e) and 4',6-diamidino-2-phenylindole [DAPI] (blue). Parts f-j represent merged images of anti-PLCζ and DAPI staining [bar = 10 µm]. (B) Comparison of the mean percentages of sperm cells expressing PLCζ between OAT and control group. (C) Comparison of different localization patterns of PLCζ between the two groups. Significant differences: ****p < 0.0001 and **p < 0.01. All data are presented as mean ± SEM. OAT: Oligoasthenoteratozoospermia. PLCζ localization patterns: A = Acrosomal, E = Equatorial, PA = Post-acrosomal, A+E = Acrosomal and equatorial, A+PA = Acrosomal and post-acrosomal, E+PA = Equatorial and post-acrosomal, and A+ E+PA = Acrosomal, equatorial, and post-acrosomal.

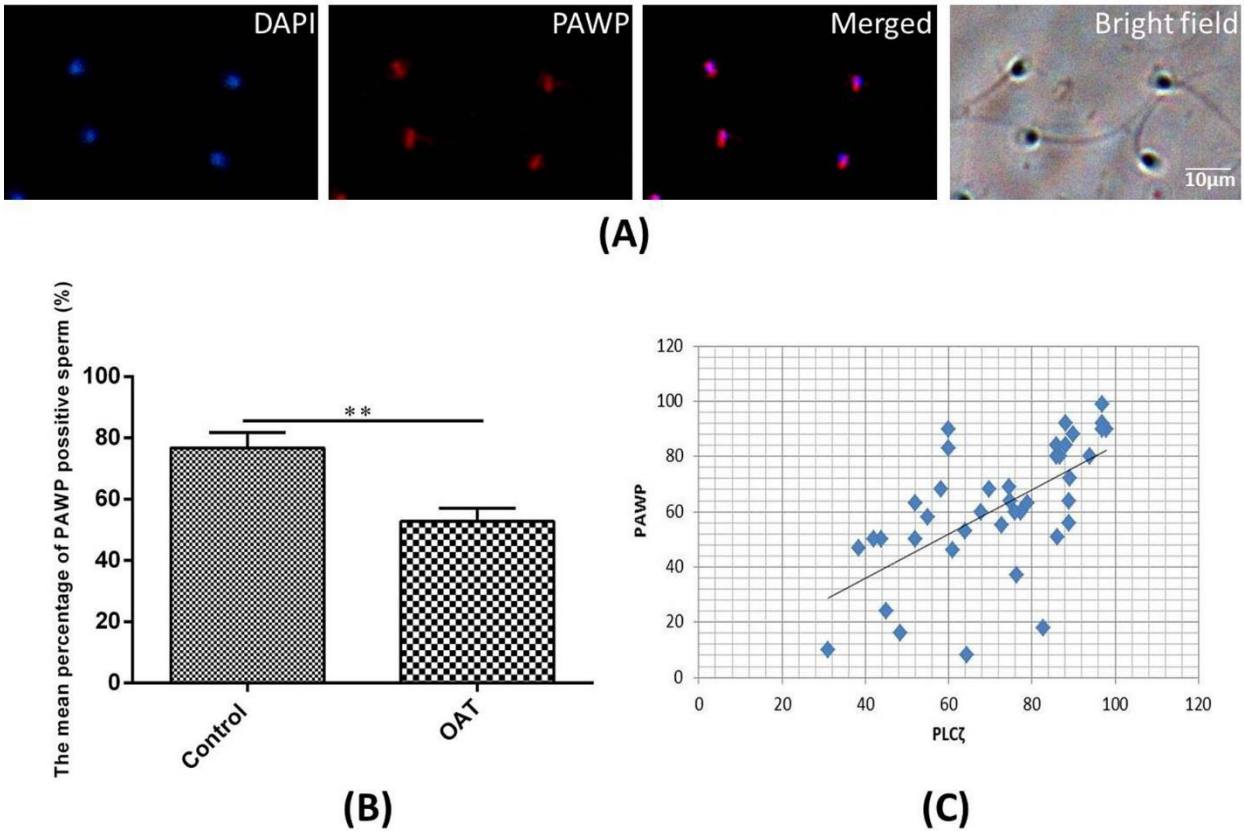

FIGURE 3. Post-acrosomal sheath WW domain-binding protein (PAWP) immunostaining. (A) PAWP immunofluorescence staining (red) in sperm cells and counterstaining with 4', 6-diamidino-2-phenylindole [DAPI] (blue). (B) Comparison of the mean percentages of sperm cells expressing PAWP between OAT and control group. Significant difference: $^{**}p < 0.01$. (C) A correlation between phospholipase C ζ- and PAWP-positive sperm cells in all individuals; $r = 0.6$, $p < 0.0001$. All data are presented as mean ± SEM. OAT: Oligoasthenoteratozoospermia.