Assessment of Sperm PAWP Expression in Infertile Men

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Purpose: The aim of this study was to evaluate expression of Post-Acrosomal WW Binding Protein (PAWP) in infertile men with low and high fertilization post ICSI and also globozoospermic men.

Materials and Methods: Semen samples were collected from 18 infertile men with previously failed or low fertilization (< 25%) post ICSI, 10 men with high fertilization (>50%) post ICSI, 15 globozoospermic men, and 21 fertile individuals. Then, expression of PAWP was assessed at RNA with quantitative Real Time PCR.

Results: Relative expression of PAWP in sperm was significantly ($P < .05$) lower in infertile men with globozoospermia (41.5 ± 5.7) or low fertilization rate (43.3±10.4) compared to fertile (138.8 ± 17.3)or men with high fertilization (211.6 ± 75.6). In addition, a significant positive correlation (r = 0.628; $P = .001$) was observed between percentage of fertilization with the relative expression of PAWP.

Conclusion: Considering solid recent evidences regarding PLC□ as the main sperm factor involved in oocyte activation, therefore co-localization of PLCζ with PAWP in perinuclear theca may account for the above observation and it is likely that PAWP may have other functions and/or it may assist PLCζ.

Keywords: PAWP; fertilization; oocyte activation; globozoospermia.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is recommended to ameliorate fertilization in couples with male factor infertility[1,2]. The average fertilization rate is reported to range from 40% to 70% and total failed fertilization rate is around 2-3%[3]. The etiology of failed fertilization has been ascribed to three main factors;[4,5] defects in oocytes, sperm[6,7,8], or the ICSI procedure[9]. Oocyte and sperm defects are mainly attributed to defects in the process of gametogenesis and gamete maturation. Cytologic analysis of failed fertilized human oocytes after ICSI indicates that lack of oocyte activation accounts for over half of failed fertilized oocytes[3]. It is well documented that upon fusion of sperm with oocyte, unknown component(s) release(s) from sperm head, possibly localized in the perinuclear theca (PT) region, into the oocyte, inducing oocyte activation. These components are collectively termed "sperm-associated oocyte activating factors (SAOAFs)" [10,11]. SAOAF should be [11];1) synthesized during spermiogenesis and localized in sperm head, so that during sperm oocyte fusion, it can diffuse into the oocytes, 2) detectable during the early stages of fertilization[12], 3) elicited oocyte activation events such as calcium oscillations, secondary polar body formation and pronucleus formation[13] and, 4) blocked by use of competitive peptides or antibodies [14,15]. A number of proteins have been proposed as SAOAF candidates, including phospholipase C zeta (PLCζ)[16,17], post-acrosomal WW binding protein (PAWP)[18,19], truncated form of KIT tyrosine kinase (Tr-KIT)[20] and citrate synthase[21]. In this regard, Muciaccia et al (2010) showed that Tr-KIT was mainly localized in the sub-acrosomal and equatorial regions of human sperm[20]. Citrate synthase is a 45 kDa protein and microinjection of recombinant citrate synthase into the newt eggs can cause Ca^2 release, but the exact mechanism of this factor in human and other species remains to be studied [21]. PLCζ, a sperm-specific 70 kDa protein, is predominantly located in sperm head [22]. Previous studies have shown that injection of PLCζ cRNA into mouse oocytes induces calcium oscillation [17]. Study of literature reveal that low expression of PLCζ , mutation in PLCζ, and a variance in distribution and amount of PLCζ are related with male infertility [23-27].

Post-acrosomal WW binding protein (PAWP) is also located in sperm head and is mainly localized in perinuclear theca[18,28]. Despite numerous reports that have proposed PLCζ to be the main SAOAF candidate, Aara-

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Table 1. Primers used for gene expression analysis of PAWP.

Gene Symbol	Primer Sequences (5´-3´)
PAWP	F:CAGATGCCTTGTTCAGTTATTGTC
	R:GCCTTCATTTCCTACGGGTTG
GAPDH	F: CCACTCCTCCACCTTTGACG
	R: CCACCACCCTGTTGCTGTAG

Abbreivations: F: Forward primer; R: Reverse primer.

bi et al.[15] proposed that PAWP could be considered as one of the diagnosis factors for treatment of infertility. They demonstrated that induction of calcium oscillations with PAWP in human and mouse MII oocytes [29]. In addition, a significant correlation was observed between PAWP expression with fertilization rate and embryonic development after ICSI[29]. Concomitantly, a paper published by Nomikos group reported that PAWP cannot hydrolyze phosphatidylinositol 4, 5-bisphosphate (PIP2) in vitro and induce calcium oscillations in mouse MII oocytes[30]. Therefore, the ability of PAWP to induce calcium oscillations in mammals need to be further studied. We have also shown that reduced expression of PLCζ in sperm from infertile men with previous failed fertilization and globozoospermia, and a strong correlation between sperm PLCζ and fertilization rate[31-33]. Therefore, we suggested that PLCζ expression at the mRNA level, could be a suitable marker for predication of semen potency to induce oocyte activation post-ICSI. In the current study, for the first time, sperm PAWP expression was compared between different groups of infertile men with fertile individuals. In addition, the association of this protein with the fertilization rate was reported.

MATERIALS AND METHODS

This study received approval from the institutional review board of Royan Institute and Isfahan Fertility and Infertility Center and the all individual whom provided semen samples signed the consent form.

Inclusion and exclusion criteria

Inclusion criteria: Infertile couples with male factor infertility, lower 45 years, and candidate for ICSI were included for this study. Exclusion criteria: Semen samples with higher than one million per ml somatic cell, men with varicocele, azoospermia, and cryptorchidism were excluded from study.

Semen samples

Semen samples were obtained from males who referred to the Isfahan Fertility and Infertility Center from 2009 to 2013. These samples were collected in sterile containers by masturbation after 3–4 days of sexual abstinence. In this study, we subdivided the individuals into four groups: 1) low percentage of fertilization post-ICSI (LF-ICSI) consisted of 18 individuals who had previous experience of failed or low fertilization (< 25%) post-ICSI; 2) high percentage of fertilization post-ICSI (HF-ICSI) consisted of 10 individuals who had a fertilization rate equal or greater than 50% post ICSI; 3) globozoospermic group consisted of 15 individuals; and 4) fertile or control group consisted of 21 individuals who participated in the embryo donation program and had at least one live child. Percentage of fertilization rate was only reported for HF-ICSI and LF-ICSI groups.
In addition, 20 semen samples from fertile (N=10) and infertile (N=10) men referring to the Isfahan Fertility and Infertility Center were only collected and correlation of RNA expression with protein expression for PAWP were assessed.
Evaluation of semen parameters of all participants were performed by light microscopy according to the World Health Organization [34]. A portion of each sample was used for clinical assessment of semen parameters; the remainder was used for ICSI. The retained portion was washed twice in PBS (pH 7.4). The relative expression level of PAWP mRNA was assessed by real-time PCR.

Intracytoplasmic Sperm Injection (ICSI) Outcomes

Sperm processing, superovulation, oocyte recovery, ICSI procedure and embryo culture were carried out in accordance with our previous study[35]. We assessed the fertilization rate at approximately 16–18 hours post-ICSI, as determined by the presence of two pronuclei. The percent of fertilization for each case was calculated by considering the ratio of fertilized oocytes to the total number of surviving, injected metaphase II (MII) oocytes, multiplied by 100. If percentage of fertilization was between 0 to 25%, these couples were considered for LF-ICSI groups, while percentage of fertilization in the HF-ICSI were between 50 to 100%.

Isolation of total RNA, cDNA synthesis and quantitative real-time PCR

We extracted total RNA from fresh semen samples using the RNX-plus method (Cinnagen) according to the manufacturer's protocol. The concentration of RNA was assessed by measuring absorbance at 260 nm. In order to eliminate possible contamination from genomic DNA, we treated the samples with RNase-free DNase I (Fermentas). Then, 1 µg RNA was subjected to a reverse transcription (RT) reaction using the Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas) and oligo-dT primer (Fermentas) (**Table 1**). RT-PCR for confirmation of cDNA synthesis and quantitative real-time PCR were performed according to the method by Aghajanpour et al (2011) in a thermal cycler Rotor gene 6000 (Corbett) using SYBR green as described in the manufacturer's protocol (TaKaRa). The levels of expression of PAWP were normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Calculation of relative expression was assessed by the Ct method [36], and results were expressed as 2-(ΔΔct) [33].

Protein isolation, Western Blot analysis and quantification of protein

Protein was extracted using TRI Reagent (Sigma) and protein concentration of each sample was estimated by Bradford assay (BioRad) to determine total protein load per each lane. Equal amounts of each sample containing 30 µg of protein was subjected to 12% SDS- polyacrylamide gel electrophoresis (PAGE) and then transferred to PVDF membrane (Bio-Rad). Blocking was achieved with 10% non-fat dry milk overnight incubation followed by incubating with polyclonal anti-PAWP antibody (1:200 dilution; sc-86781; santa Cruz) in 2% Skimmed milk for 90 min at 25°C and washed in TBS–T, three times each for 15 min. Finally, the membranes were incubated with Horseradish peroxidase-conjugated polyclonal rabbit Anti- Goat IgG (1:100000; A5420, Sigma) for 1 h at room temperature. The immune reactivity on western blots was detected

Table 2. Description of semen parameters in the fertile, high fertilization post-ICSI (HF-ICSI), low fertilization post-ICSI (LF-ICSI), and globozoospermic groups (N = 64).

Group	Parameter	Maximum	Minimum	Mean ± SE
Fertile (n=21)	Concentration $\times 10^6$	93.25 ± 10.83	42.00	234.00
	% Sperm motility	58.81 ± 2.23	30.00	70.00
	% Abnormal morphology	96.32 ± 0.61	90	100.00
	Volume(ml)	3.74 ± 0.34	1.00	6.00
High fertilization (HF-ICSI; n=10)	Concentration $\times 10^6$	90.80 ± 23.25	27.00	284.00
	% Sperm motility	59.50 ± 2.83	40.00	70.00
	% Abnormal morphology	96.50 ± 1.50	87.00	100.00
	Volume(ml)	2.95 ± 0.53	1.50	7.00
Low fertilization (LF-ICSI; n=18)	Concentration $\times 10^6$	60.65 ± 22.21	0.25	328.00
	% Sperm motility	34.35 ± 4.64	2.00	65.00
	% Abnormal morphology	99.78 ± 0.15	99.00	100.00
	Volume(ml)	3.29 ± 0.42	1	7.00
Globozoospermic (n=15)	Concentration $\times 10^6$	73.20 ± 7.85	7.00	110.00
	% Sperm motility	38.00 ± 3.99	20.00	70.00
	% Abnormal morphology	100.00 ± 0.00	100.00	100.00
	Volume (ml)	2.57 ± 0.26	1.00	4.00

by GE Amersham ECL Plus Western Blotting Detection Reagent (Amersham) by exposure to X-ray films. The protein bands obtained from western blot were analyzed by IMAGE J (version 1.240) software to determine the band density.

Statistical analysis

We used Microsoft Excel and SPSS (version 17, USA) for data analyses. Data were expressed as mean ± standard error of mean (SEM). Differences in gene expressions between the four groups were determined with one-way ANOVA. Correlation analysis was carried out using Pearson's correlation analysis. Result of PAWP at RNA and protein were expressed as 2-(ΔΔct) [Δct=Ct of PAWP -Ct of GAPDH; ΔΔct= Δct of each infertile – mean of □ct fertile; and lastly data was expressed as 2-(ΔΔct)], and band density [density of PAWP bands were divided by the density of their respective GAPDH band, as housekeeping protein, which represented the expression level of this protein], respectively. $P < .05$ was considered significant.

RESULTS

The mean ages of fertile (n = 21) and infertile individuals (n = 43) were 35.6±1.1 and 36.1 ± 0.9 years, respectively; their partners mean ages were 29.9 ± 0.8 years and 30.2 ± 0.9 years, respectively.

The descriptive analysis of sperm parameters in the fertile, HF-ICSI (> 50%), failed or low fertilization post-ICSI (LF-ICSI; < 25%), and globozoospermic groups were shown in **Table 2**. Sperm concentration and percentage of sperm motility were significantly higher in fertile, HF-ICSI (> 50%) groups compared to LF-ICSI (< 25%), and globozoospermic groups ($P < .05$). In addition, percentage of abnormal sperm morphology was significantly lower in fertile, HF-ICSI (> 50%) groups compared to LF-ICSI (< 25%), and globozoospermic groups ($P < .05$).

In this study, the mean Ct for the house keeping genes in the fertile, HF-ICSI, LF-ICSI, and globozoospermic groups were 19.95 ± 2.4, 19.71 ± 2.05, 19.13 ± 1.97 and 20.06±1.76, respectively. These results show that the quality of RNA between samples were similar.

We assessed the relative expressions of PAWP by quantitative real-time PCR between study groups. There were significantly higher expressions of PAWP in the fertile group compared to the LF-ICSI (138.8 ± 17.3 vs. 43.3 ± 10.4; $p \leq 0.035$) and globozoospermic groups (138.8 ± 17.3 vs. 41.5 ± 5.7; $P \leq .043$). In addition, mean relative expression of PAWP was significantly higher in the HF-ICSI group compared to LF-ICSI (211.6 ± 75.6 vs. 43.3 ± 10.4; $p \leq 0.001$) and globozoospermic (211.6 ± 75.6 vs. 41.5 ± 5.7; $P \leq 0.001$) groups. Furthermore, a significant positive correlation (r = 0.628; $P = .001$) was observed between percentage of fertilization with the relative expression of PAWP (**Figure 1A-B**).

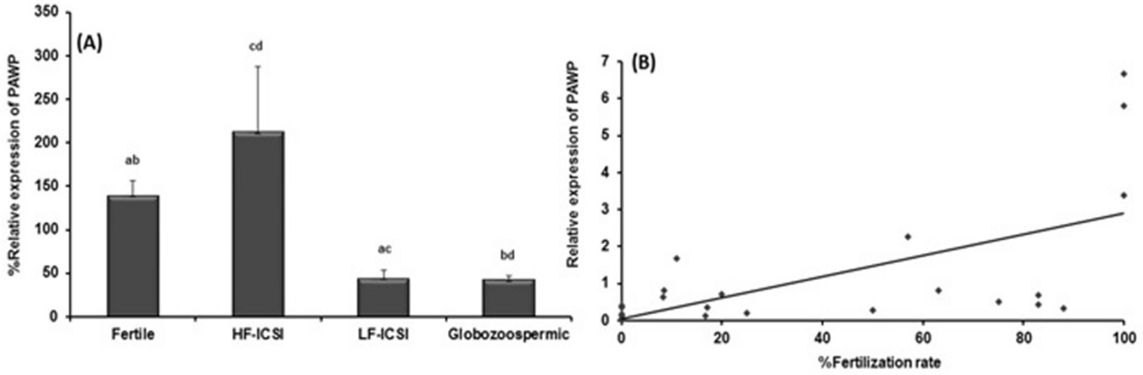

Figure 1 A) Quantitative real-time analysis of PAWP in fertile, high fertilization post-ICSI (HF-ICSI), low fertilization post-ICSI (LF-ICSI), and globozoospermic individuals. (N = 64) **B)** Scatter plot shows significant correlation (r = 0.628; $p = 0.001$) between percent of fertilization and relative expressions of PAWP (N = 25).

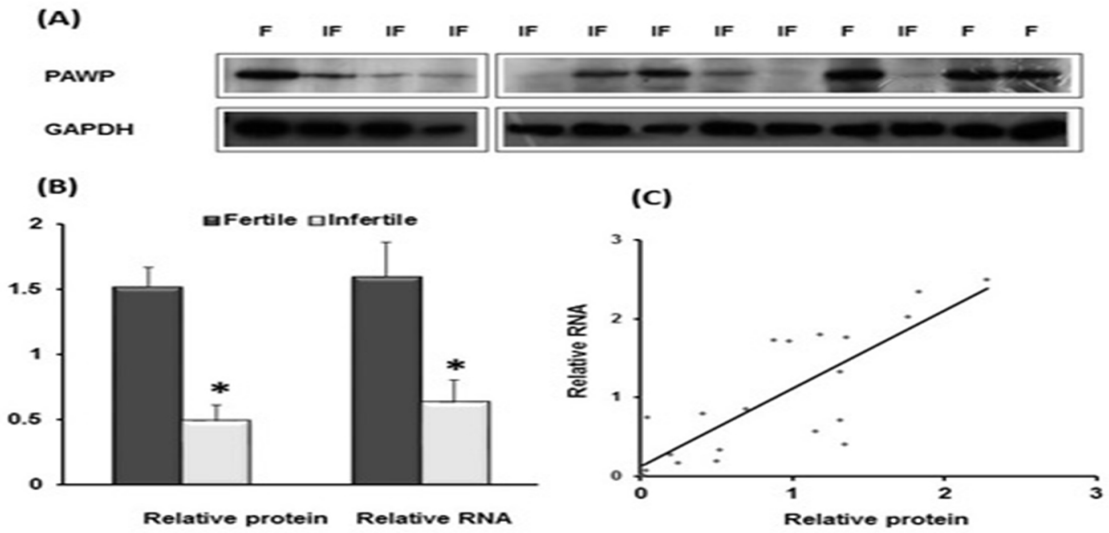

Figure 2 A) Western blot analysis of PAWP in fertile (F) and infertile (IF) individuals. **B)** Comparison of mean relative expression of PAWP RNA and protein level in fertile and infertile individuals and C) Correlation analysis between RNA and protein for PAWP (r = 0.8; $p \leq 0.0001$). Aster indicates significant difference at $p < 0.001$ between fertile and infertile individuals **(B)**.

Western blot analysis of different semen samples from fertile and infertile couples showing that in some of the infertile individuals has low expression level of PAWP (**Figure 2A**). Furthermore, the mean relative expression at both RNA and protein level was significantly lower in infertile compared to fertile couples (**Figure 2B**). In addition, correlation analysis between RNA with protein expression showed a strong significant positive correlation (r = 0.8; $P \leq .0001$) (**Figure 2C**).

DISCUSSION

Understanding the etiology of failed fertilization is essential for counseling and improving the treatment outcome of in infertile individuals. One main reason for failed fertilization is impaired oocyte activation, the mechanism of which is debated within the literature. It is generally accepted that a sperm-associated factor is responsible for initiation of oocyte activation[10]. In this regard, numerous studies have focused on a sperm-specific protein called PLCζ that induces calcium oscillations and, thereby, oocyte activation through the InsP3 pathway[17,37]. It is believed that PLCζ is inactive inside spermatozoa and upon entry into the oocyte; it becomes activated[38].

However, Wu et al. have introduced PAWP as a new candidate of SAOAF that has the ability to induce oocyte activation[18,28]. It has been suggested that both PAWP and PLCζ possess dual roles in oocyte activation mechanism and PAWP-mediated signaling pathway may act upstream or downstream of Ca^{2+} signaling[18]. Our previous studies have shown decreased expressions of PLCζ in LF-ICSI and globozoospermic men[33,39,40]. In the current study, we also showed that the expression of PAWP was significantly lower in LF-ICSI and globozoospermic men compared to fertile or individuals with high fertilization. Therefore, low expression of PAWP, as in globozoospermic individuals, might account for impaired spermiogenesis in these individuals. This conclusion is consistent with previous reports which showed, sperm from individuals with low fertilization rates had a high degree of protamine deficiency as a sign of impaired spermiogenesis[33].

The general consensus in the literature states that the chance for fertilization by injection of round sperm is very low[41], despite very rare report of fertilization and pregnancy following ICSI in globozoospermia[42]. In this study, we observed a low expression of PAWP in globozoospermia cases at RNA level and also protein level. PAWP might be related to acrosomal biogenesis, considering the fact that globozoospermia have different genetic causes and this reason may lead to low expression of PAWP in these individuals[43,44]. Furthermore, localization of PAWP is dependent on microtubule-manchette protein transport, manchette descent and is independent of sub-acrosomal PT formation. Considering the absence of manchette[28] and acrosomes in globozoospermia, this may suggest another reason for low expression of PAWP in globozoospermic individuals.

Sperm, a transcriptionally inactive cell, contains RNA transcripts the role of which remains to be identified.[45] Considering PAWP mRNA could be translated during fertilization and possibly sustain a 'long-lived' Ca2+ response, therefore, significant low expression of PAWP in the LF-ICSI and globozoospermic groups is consistent with the inability of these sperm to induce Ca^{2+} oscillations and fertilization following their insemination. In this study, we showed a significant correlation between expression of PAWP (r = 0.6) with the percentage of fertilization which is in accordance with others reports in this filed[29] and contrary to two previous studies[46,47]. This is likely to be due to different cohort of patients recruited in these studies. In our study, the patients were infertile men with failed or low fertilization. Despite these observations, PAWP null mice show that PAWP is not the main candidate for SAOAF[46]. Therefore, observation and associations observed in this study may be related to co-localization of two proteins in the same region of sperm and correctly emphasizes previous study[11] working on TR-KIT, as they state: "it cannot be excluded that factors present in the perinu-

clear theca (PT) and/or in the equatorial region might equally be absent in spermatozoa that are unable to trigger egg activation". Considering that PAWP null mice do not jeopardized the fertilizing ability of sperm and in human absence of PLCζ alone is adequate to prevent fertilization regardless of presence of PAWP[47], therefore, it is likely that PAWP may have other functional roles and/or it may assist PLCζ. In this study, we also showed that there is a significant correlation between PAWP expressions at RNA level with PAWP protein. We further showed that RNA and protein of PAWP, are lower in infertile compared to fertile individuals and this is likely to be due to their associative expression and co-localized in PT. The causes of spermatogenic defects in infertile men are multifactorial and other factors such as level of oxidative stress, genetic and epigenetic alterations, and environmental factors can affect spermatogenesis and fertility potential[48]. Therefore, one limitation of this study was assessment of only sperm PAWP in low number of infertile men. More researches are needed to confirm these results and factors involved in men infertility.

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CONFLICT OF INTEREST

None of the authors has any conflicts of interest to disclose and all authors support submission to this journal.

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