Quantitative expression of phospholipase C zeta, as an index to assess fertilization potential of a semen sample

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BACKGROUND: Failed fertilization post-ICSI has been mainly attributed to the sperm’s inability to induce oocyte activation. Phospholipase C zeta (PLCζ) is considered to be one of the factors for the induction of oocyte activation. The aim of this study was to quantitatively assess the expression of PLCζ in globozoospermic men or those with previously low or failed fertilization in comparison with fertile men or those with high fertilization potential. In addition, the relationship between expression of PLCζ and that of other sperm markers was evaluated.

METHODS: Real-time PCR was carried out to evaluate relative expression of PLCζ mRNA. Chromatin maturity and acrosin activity were assessed by CMA3 staining and a colorimetric method.

RESULTS: The expression of PLCζ was significantly lower in globozoospermic men (P < 0.01, n = 8) or individuals with previously low or failed fertilization (P < 0.01, n = 36) in comparison to fertile men (n = 24). In addition, a significant difference was observed between globozoospermic (P < 0.01) and individuals with previously low or failed fertilization (P = 0.003) in comparison to high fertilization individuals (n = 17). Expression of PLCζ was not correlated with either chromatin maturity or acrosin activity. However, a significant correlation was observed between the percentage of fertilization and relative expression of PLCζ (r = 0.4, P < 0.01).

CONCLUSION: In this study, for the first time, we have shown that assessment of relative expression of PLCζ may provide a useful marker for the ability of sperm to induce oocyte activation after ICSI.

Key words: failed fertilization / phospholipase C ζ / acrosin activity / protamine / ICSI

Introduction

During successful fertilization, the sperm binds to the zona pellucida and then makes its way through this barrier with the aid of proteases, including acrosin (Saling, 1989). Next, the sperm fuses with the oocyte plasma membrane and initiates oocyte activation by releasing sperm oocyte activating factors (SOAFs; Dozortsev et al., 1995; Parrington et al., 2007). Failed fertilization has been mostly attributed to the inability of sperm to activate the oocyte (Mahutte and Arici, 2003; Nasr-Esfahani et al., 2010a; Kashir et al., 2010). Based on different reports, this phenomenon may account for about 30% of human oocytes that fail to fertilize following ICSI and 2–3% of complete fertilization failure following ICSI (Van Steirteghem et al., 1993; Mahutte and Arici, 2003; Kashir et al., 2010; Nasr-Esfahani et al., 2010a).

Sperm-specific phospholipase C zeta (PLCζ), a gamete-specific 70 kDa protein, is considered to be an SOAF or a main component of SOAFs (Kurokawa et al., 2004; Swann et al., 2006; Kashir et al., 2010). PLCζ is predominantly localized in the equatorial region of sperm and has been shown to induce calcium oscillations and thereby oocyte activation through the IP3 pathway (Swann et al., 2006).
An inability to induce oocyte activation (Yoon et al., 2008; Heytens et al., 2009). This form of failed fertilization can be overcome by artificial oocyte activation (AOA; Heindryckx et al., 2008; Nasr-esfahani et al., 2008a, 2010a,b; Taylor et al., 2010). Yet with the exception of the mouse oocyte activation test (MOAT), there has thus far been no diagnostic test to predict the potential of a semen sample to induce oocyte activation (Nasr-esfahani et al., 2010a). MOAT requires animal facilities and special equipment, which may not be available to many assisted reproductive centers (Nasr-esfahani et al., 2010a). We have recently evaluated the association between the ability of a semen sample to induce fertilization post-ICSI with acrosin activity. These results have shown a slight significant correlation between these two factors, with low prognostic value in a small number of cases included in our study (Nasr-esfahani et al., 2010a). The reason for choosing such an assessment of acrosin activity was the concurrent absence of acrosin and PLCζ protein in globozoospermia and the close association between the locations of proacrosin with PLCζ protein distribution (Florke-Gerloff et al., 1984; Grasa et al., 2008). Therefore, in this study, in addition to acrosin activity, we aimed to evaluate the relative expression of PLCζ mRNA in semen samples of (i) fertile individuals with low fertilization rate or total failure of fertilization following ICSI, (ii) fertile individuals reffereing for embryo donation, (iii) infertile individuals who have a high fertilization rate after ICSI or IVF and (iv) globozoospermic individuals, in order to evaluate whether assessment of levels of PLCζ mRNA in semen samples might assist embryologist in the selection of individuals whom require AOA.

Materials and Methods

Sperm analysis and sperm processing

This study received the approval of the Institutional Review Board of Isfahan Fertility and Infertility Center (IFIC) and Royan Institute. Eighty-five semen samples were collected from men who had been referred to the IFIC. All semen samples were collected by masturbation into sterile containers after 3–4 days of sexual abstinence and were delivered to the laboratory within 45 min after ejaculation. One portion of the semen was used for evaluation of sperm concentration and motility according to World Health Organization guidelines (WHO, 2010). The remaining portion of the semen sample was washed twice in Ca2+Mg2+-free phosphate buffer saline (PBS; pH 7.4) and used for evaluation of relative expression of PLCζ mRNA levels by real-time PCR. Acrosin activity, pronamine deficiency and morphology were assessed by spectrophotometry, chromomycin A3 (CMA3) and Papanicolaou staining, respectively.

Experimental design

The study assessed 85 semen samples from fertile and infertile individuals. Semen samples from individuals were divided into four groups based on the following experimental design—low fertilization (LF-ICSI) group: individuals who had previously experienced failed or low fertilization (<25%) following ICSI were contacted to participate in the study (n = 36); High fertilization post-ICSI or IVF (HF-ICSI/IVF) group: individuals who had been referred for IVF/ICSI treatment and had achieved a fertilization rate equal to or higher than 70% (n = 17); Globozoospermic, (n = 8); Fertile: a control group selected from fertile individuals who had been referred to the IFIC for embryo donation (n = 24).

To reduce female factors, any patient with fewer than four matured MI oocytes that had survived the procedure was excluded from this study. Furthermore, immature, deformed and post-mature oocytes, or any oocyte with certain types of abnormality, were excluded from this study. The number of oocyte ranged from 4 to 17, with a mean of 8.18 ± 0.71 in LF-ICSI group and ranged from 4 to 27, with a mean of 10.00 ± 1.36 in HF-ICSI/IVF group. The mean number of ICSI cycles in the LF-ICSI group was 1.15 ± 0.07.

RNA extraction and first strand cDNA synthesis

Total RNA from semen samples in both fertile and infertile individuals was extracted using RNAX-Plus (Cinnagen). The integrity of extracted RNA was evaluated by agarose electrophoresis and its concentration assessed by measuring absorbance at 260 nm. In order to eliminate possible contamination of genomic DNA, RNA-containing samples were treated with DNaseI (Fermentas). First strand cDNA synthesis was carried out using 2 μg of total RNA with the RevertAid First Strand cDNA Synthesis kit (Fermentas). cDNA synthesis was performed according to the manufacturer’s protocol utilizing a random hexamer primer, RNase inhibitor (20 U/μl), 10 mM dNTP mix, 5X reaction buffer, M-MulV reverse transcriptase (200 U/μl) and DEPC-treated water. The final step of RT–PCR was achieved by the following protocol: one cycle for primary denaturation at 94°C for 4 min, followed by 35 repetitive cycles of denaturation for 45 s at 94°C, an annealing stage for 45 s at 60°C and an extension stage for 1 min at 72°C. Finally, PCR products were qualified by electrophoresis on 2% agarose gel.

Quantitative real-time PCR analysis

Real-time PCR was carried out in a thermal cycler Rotor gene 6000 (Corbett) as described by the manufacturer’s protocol (TaKaRa). The PCR mixture for each reaction contained 10 μl SYBR premix Ex Taq II (TaKaRa), 1 μl of each primer (5 pmol/μl) and 50 ng cDNA adjusted to a final volume of 20 μl using dH2O. All reactions were carried out in duplicate. Real-time–specific primer pairs were designed by the Beacon designer 7.5 (Table I). The real-time PCR protocol was composed of: 4 min at 94°C followed by 40 repetitive cycles for 10 s at 94°C, 30 s at 60°C and 30 s at 72°C. The expression level of PLCζ mRNA was normalized by expression of the housekeeping gene GAPDH. The calculation of relative expression was achieved using the ∆∆Ct method (Livak and Schmittgen, 2001; Vaerman et al., 2004), as followed.

(i) Separate calculation of the delta cycle threshold [∆Ct ≤ mean (Ct PLCζ – Ct GAPDH)] for each fertile and infertile individual.

(ii) Calculation of the mean of ∆Ct of all fertile individuals as the normal value.

<table>
<thead>
<tr>
<th>Table I Primers used to assess PLCζ mRNA levels in human sperm.</th>
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<tbody>
<tr>
<td><strong>Gene symbol</strong></td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>PLCζ</td>
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<tr>
<td></td>
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<tr>
<td>GAPDH</td>
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<td>GAPDH, glyceraldehyde-3-phosphate dehydrogenase.</td>
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</table>
(iii) Subtraction of $\Delta C_t$ of normal value of fertile individuals from $\Delta C_t$ of infertile individual for each infertile ($\Delta C_t = C_t$ infertile $- C_t$ standard).

(iv) Final result was expressed as $2^{-\Delta C_t}$.

**Assessment of protamine deficiency (chromomycin A3 staining)**

CMA3 staining was performed as described by Nasr-Esfahani et al. (2001) using an Olympus fluorescent microscope (BX51, Tokyo, Japan) with the appropriate filters (460–470 nm) at 1000 x magnification. Semen samples were washed with PBS buffer. Sperm suspensions were fixed in Carnoy’s solution (methanol:acetic acid; 3:1) at 4°C for 5 min and smeared on slides. Then slides were treated with 100 µl of CMA3 solution [0.25 mg/ml in McIlvaine’s buffer (7 ml of 0.1 M citric acid, 32.9 ml of 0.2 M Na2HPO4. 7H2O, pH 7.0, containing 10 mM MgCl2)] for 20 min. Next, slides were washed in PBS buffer, mounted with buffered glycerol (1:1) and observed with a fluorescent microscope. CMA3 positive or protamine-deficient spermatozoa were defined as having a light yellow stain, whereas those that were CMA3 negative or with a normal amount of protamine were defined as dark yellow sperm. Two hundred sperm were counted for each case and the percentage of CMA3 negative sperm was recorded as previously described (Nasr-Esfahani et al., 2001).

**Total acrosin activity assay**

Two million spermatozoa were obtained from each semen sample and washed thoroughly with PBS. A detergent buffer containing Triton X-100 (0.01%) and 23 mM $\alpha$-benzoyl-dl-arginine p-nitroanilide hydrochloride (BAPNA) at pH 8.0 was used to allow activation of proacrosin into enzymatically active acrosin. Then, the sperm pellet was suspended into 1 ml of detergent buffer for 3 h at 37°C. In this procedure, BAPNA was hydrolyzed by acrosin and converted to a chromophoric product (CECIL, UK). Total acrosin activity ($\mu$IU/10⁶ sperm) was calculated according to Kennedy et al. (1989).

**Statistical analysis**

The percentage data were modeled by ArcSin transformation and the transformed data were analyzed by one-sample t-test and one-way analysis of variance (ANOVA) using the Statistical Package for the Social Studies (SPSS Science, Chicago, IL, USA). Differences between groups were verified by one-way ANOVA. For relative expression of PLCζ, the one sample t-test was used to compare data with the fertile group. Two-tailed Pearson correlation test was used to assess the correlations between groups. All the data were presented as mean ± SEM, and differences were considered significant at $P < 0.05$.

**Results**

The mean ages of men who participated in the study were 36 ± 5.5 and 36.9 ± 7.1 in the fertile and infertile groups, respectively. The mean ages of their partners were 30.4 ± 5.5 and 31.86 ± 6.16 in both groups, respectively. Semen analysis results in four groups are presented in Table II. The expression of PLCζ mRNA, acrosin activity, percentage of sperm with protamine deficiency (CMA3 positivity) and percentage with abnormal morphology were assessed in the four aforementioned groups.

The expression values of PLCζ relative to fertile individuals were 41.7 ± 7.7, 119.1 ± 22.8 and 27.8 ± 8.1 in the LF-ICSI, HF-ICSI and globozoospermic groups, respectively. The differences between the fertile ($P < 0.01$) and HF-ICSI ($P < 0.01$) and HF-IVF group and HF-ICSI group were significant. In addition, a significant difference was observed between fertile ($P < 0.01$) and HF-ICSI ($P = 0.003$) versus globozoospermic groups. As these comparisons involve relative expression, they are not on a linear scale (Fig. 1).

Figure 2 shows the relative expression of PLCζ in relation to the fertile group with the corresponding fertilization rate in each group. The percentages of fertilization were 20.3 ± 2.9, 83.2 ± 4.5 and 73.6 ± 6.9, in the LF-ICSI, HF-ICSI and HF-IVF groups, respectively. Fertilization rate was significantly higher in the HF-ICSI ($P < 0.01$) and HF-IVF group ($P < 0.01$) compared with the LF-ICSI group. In addition, relative expression of PLCζ in relation to the fertile group was 41.7 ± 7.7, 119.1 ± 22.8 and 102.6 ± 2.1 in the LF-ICSI, HF-ICSI and HF-IVF groups, respectively. Relative expression of PLCζ in relation to the fertile group was higher in the HF-ICSI ($P = 0.003$) and HF-IVF groups ($P = 0.04$) compared with the LF-ICSI group. A significant correlation was observed between the percentage of fertilization and relative expression of PLCζ in relation to the fertile group ($r = 0.4, P < 0.01$) (Figure 3).

**Table II Description of semen parameters in the fertile, HF-ICSI/IVF, LF-ICSI and globozoospermic groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Mean ± SE</th>
<th>Minimum</th>
<th>Maximum</th>
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</thead>
<tbody>
<tr>
<td>Fertile (n = 24)</td>
<td>Concentration × 10⁶</td>
<td>94.69 ± 15.79</td>
<td>21.00</td>
<td>395.00</td>
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<tr>
<td></td>
<td>% Sperm motility</td>
<td>57.50 ± 2.31</td>
<td>45.00</td>
<td>70.00</td>
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<tr>
<td></td>
<td>% Abnormal morphology</td>
<td>94.66 ± 0.59</td>
<td>87.00</td>
<td>96.00</td>
</tr>
<tr>
<td>High fertilization (HF-ICSI/IVF; n = 17)</td>
<td>Concentration × 10⁶</td>
<td>98.11 ± 16.94</td>
<td>27.00</td>
<td>284.00</td>
</tr>
<tr>
<td></td>
<td>% Sperm motility</td>
<td>53.82 ± 3.60</td>
<td>25.00</td>
<td>75.00</td>
</tr>
<tr>
<td></td>
<td>% Abnormal morphology</td>
<td>98.38 ± 0.60</td>
<td>91</td>
<td>100.00</td>
</tr>
<tr>
<td>Low fertilization (LF-ICSI; n = 36)</td>
<td>Concentration × 10⁶</td>
<td>47.03 ± 10.27</td>
<td>0.10</td>
<td>328.00</td>
</tr>
<tr>
<td></td>
<td>% Sperm motility</td>
<td>34.42 ± 3.92</td>
<td>2.00</td>
<td>70.00</td>
</tr>
<tr>
<td></td>
<td>% Abnormal morphology</td>
<td>99.68 ± 0.13</td>
<td>97.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Globozoospermia (n = 8)</td>
<td>Concentration × 10⁶</td>
<td>63.18 ± 15.75</td>
<td>4.00</td>
<td>128.50</td>
</tr>
<tr>
<td></td>
<td>% Sperm motility</td>
<td>37.22 ± 5.65</td>
<td>5.00</td>
<td>50.00</td>
</tr>
<tr>
<td></td>
<td>% Abnormal morphology</td>
<td>100 ± 0.00</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 1 A comparison of relative expression of PLCζ mRNA in relation to fertile individuals with low or total failed fertilization (LF-ICSI, \(n = 36\)), IVF/ICSI cases with high fertilization rate (HF-ICSI/IVF, \(n = 17\)), globozoospermic individuals (\(n = 8\)) and fertile groups (\(n = 24\)). The mean expression of PLCζ in fertile individuals was considered as 100%. Data were analyzed by one-sample t-test. Common letters are significantly different at \(P < 0.05\) (a, b and c < 0.01, d = 0.003).

Figure 2 Relative expressions of PLCζ mRNA in relation to fertile individuals and fertilization rate in infertile individuals with low or total failed fertilization (LF-ICSI), and with high fertilization rate in ICSI (HF-ICSI) and IVF (HF-IVF). Data were analyzed by one-way ANOVA. Inset scatter shows significant correlation between percentage of fertilization rate and relative expression of PLCζ in relation to fertile group (\(r = 0.403, P = 0.009\)). Common letters are significantly different at \(P < 0.05\) (a and b < 0.01, c = 0.003, d = 0.04).

Figure 4 shows acrosin activity, percentage of protamine deficiency (CMA3 positivity) and percentage of abnormal morphology in sperm from infertile individuals with low or total failed fertilization (LF-ICSI), IVF/ICSI cases with a high fertilization rate (HF-ICSI/IVF), globozoospermic individuals and fertile groups. Mean values of acrosin activity were \(77.2 \pm 8.2\), \(61 \pm 7.8\), \(84.4 \pm 12.4\) and \(23.1 \pm 7.8\) in the fertile, LF-ICSI, HF-ICSI/IVF and globozoospermic individuals, respectively (Fig. 4A). The mean acrosin activity was lower in globozoospermic individuals compared with the HF-ICSI/IVF (\(P = 0.04\)) and fertile groups (\(P = 0.04\)). The low acrosin activity observed in sperm from globozoospermic individuals is very likely background activity, since they have absent acrosomal membrane and content. Percentages of protamine deficiency were assessed by CMA3 positivity and were \(24 \pm 2.5\), \(39.1 \pm 3.4\), \(35.4 \pm 2.9\) and \(49.8 \pm 6.6\) in the fertile, LF-ICSI, HF-ICSI/IVF and globozoospermic groups, respectively (Fig. 4B). The mean CMA3 positivity was higher in the globozoospermic (\(P < 0.01\)) and LF-ICSI (\(P = 0.005\)) groups compared with the fertile group. Percentages of abnormal morphology were \(94.6 \pm 0.5\), \(99 \pm 0.6\), \(98.5 \pm 0.6\) and \(100 \pm 0.0\) in the fertile, LF-ICSI, HF-ICSI/IVF and globozoospermic groups, respectively (Fig. 4C). The percentage of abnormal morphology was significantly lower in the fertile group compared with LF-ICSI (\(P < 0.01\)), HF-ICSI/IVF (\(P < 0.01\)) and globozoospermic (\(P < 0.01\)) groups.

Discussion

It is well established that in addition to generating the diploid state of the zygote, the sperm also endows the oocyte with paternal centrioles, proteins and RNAs (Ostermeier et al., 2004; Miller et al., 2005), although the exact roles of mRNA introduced into the oocyte by the sperm are not fully understood (Lalancette et al., 2008). However, some reports have suggested that RNAs may have a vital role in embryo cleavage and may potentially influence phenotypic traits of the offspring (Ostermeier et al., 2004; Miller and Ostermeier, 2006). Therefore, the RNA content of the sperm may be divided into three categories: non-functional, functional and foreign mRNA (Ostermeier et al., 2004). Moreover, the transcriptomes of processed sperm from fertile and infertile samples has been assessed and differential patterns of up or down-regulated genes have been reported, thereby confirming the fact that there are huge mRNA content differences between fertile and infertile individuals (Garrido et al., 2009).
Previous studies have identified the presence of PLCζ mRNA in the sperm, and its functional role in the zygote remains to be established (Boerke et al., 2007; Lalancette et al., 2008). Certainly, injection of PLCζ cRNA into the oocyte results in the production of PLCζ protein (Saunders et al., 2002). In our study, we have assessed the usefulness of quantification of PLCζ mRNA levels in the sperm as an index of the potential for a semen sample to induce oocyte activation. In this study, the relative expression of PLCζ was assessed in infertile individuals with low or total failed fertilization (LF-ICSI), IVF/ICSI cases with high fertilization rate (HF-ICSI/IVF), globozoospermic individuals and fertile individuals. Data were analyzed by one-way ANOVA. Common letters are significantly different at P < 0.05 (a = 0.04, b = 0.04, c, d, e, f and g < 0.01).

Figure 4 A comparison of acrosin activity, percentage of protamine deficiency (CMA3 positivity) and percentage of abnormal morphology in infertile individuals with low or total failed fertilization (LF-ICSI), IVF/ICSI cases with high fertilization rate (HF-ICSI/IVF), globozoospermic individuals and fertile individuals. Data were analyzed by one-way ANOVA. Common letters are significantly different at P < 0.05 (a = 0.04, b = 0.04, c, d, e, f and g < 0.01).

The expression of PLCζ mRNA in individuals with high fertilization rates following IVF and ICSI was as expected, similar to fertile individuals, further confirming that low or high expression of PLCζ mRNA is related to the fertilization potential of the sperm sample or its ability to induce oocyte activation. Figure 2 shows the relative expression of PLCζ in relation to the fertile group with the corresponding fertilization rate in each group and the existence of a significant correlation between these two parameters. Therefore, our results indicate an association between PLCζ mRNA levels and fertilization ability of a semen sample, further emphasizing the value of assessing PLCζ mRNA levels as a method for forecasting fertilization potential or the ability of a semen sample to induce oocyte activation.

Previous studies investigating the pattern of expression of PLCζ during spermatogenesis in different species revealed that PLCζ may be synthesized during the early stages of meiotic spermatocytes, becomes detectable at the spermatid stage, and is translated in the late spermatid stage (Yoneda et al., 2006; Mizushima et al., 2009; Young et al., 2009). Therefore, in addition to sperm morphology, we assessed late spermatogenic markers such as acrosin content and the maturation or protamination status of chromatin by acrosin activity and CMA3 staining, respectively, to determine whether an association between these markers and relative expression of PLCζ mRNA exists. Our results revealed no significant correlation between these parameters (data not shown), and relative expression of PLCζ mRNA. Assessment of mean values of these parameters between different groups revealed low acrosin activity and a higher percentage of CMA3 positive sperm in globozoospermic individuals. Therefore, this suggests that assessment of acrosin content and protamine status are not appropriate markers to foresee the ability of a semen sample to induce oocyte activation.

The only factor that significantly differed between the globozoospermic, low or failed fertilization groups when compared with the fertile group was sperm morphology, even though a significant correlation between these parameters and relative expression of PLCζ was not observed. This suggests that semen samples with high degrees of abnormal morphology have a low potential to induce oocyte activation. The lack of correlation may be attributed to the selection of normal sperm during the ICSI procedure and heterogeneity within a semen sample. These results are in agreement with our previous results suggesting that individuals with severe teratozoospermic semen samples may benefit from AOA (Nasr-Esfahani et al., 2008a).

In this study, as with our previous study, we showed that globozoospermic individuals have higher degrees of chromatin immaturity assessed by CMA3 when compared with fertile individuals or individuals with high fertilization rates (Deemeh et al., 2007; Nasr-Esfahani et al., 2009, 2008b), which suggests that proper histone protamine exchange did not occur in these individuals. This may be attributed to inherited genetic defects.

A previous study of the pattern of localization of PLCζ protein in human sperm demonstrated a primarily equatorial localization, in line with the putative role of PLCζ in oocyte activation (Grasa et al., 2008). However, these authors also demonstrated the additional presence of PLCζ protein in post-acrosomal and acrosomal regions in uncapacitated sperm with an intact acrosome and a change
in the distribution of this protein to post-acrosomal or equatorial region post-capacitation and an induced acrosome reaction, suggesting other potential roles for PLCζ, for instance during the acrosome reaction. In regard to this, it is interesting to note that in our study individuals with no acrosome, as seen with the sperm of globozoospermic individuals, have low PLCζ mRNA levels. Therefore, we assessed the correlation between the percentage of sperm with a small acrosome and relative expression of PLCζ, but found no significant correlation between these parameters. This suggests that localization of PLCζ to the acrosome may be independent of PLCζ mRNA expression. The relationship between the absence of acrosome in globozoospermic individuals and low PLCζ mRNA levels in globozoospermic individuals may be related to inherited genetic factors, which require further investigation.

In conclusion, this study suggests, for the first time, that assessment of relative expression of PLCζ mRNA level in sperm may provide a useful marker for the ability of sperm to induce oocyte activation. Other sperm tests performed in this study such as assessment of chromatin immaturity, acrosin activity and size of acrosome do not provide additional information about the ability of sperm to induce oocyte activation.

Authors’ roles

M.H.N.-E. designed the study, interpreted the results, contributed to the writing of the paper, revised the paper, contributed to the editing of the paper and gave final approval. M.T. contributed to andrology methods; performed the statistical analysis; interpreted the results, and contributed to the writing of the paper. S.A. performed the semen analysis, prepared samples, collected data and carried out real-time analysis. K.G. (supervisor of student) interpretation of real-time results and contributed to the editing of the paper. A.S. carried out real-time analysis and interpreted real-time results. M.R.D. provided clinical information. J.M. (supervisor of student). J.P. was involved in interpretation of results, and contributed to the writing of the paper.

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