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Assessment of Expression Levels and Localization Patterns of Phospholipase C zeta in Different Grades of HOST in Human Sperm

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Abstract

Background: Phospholipase C zeta (PLC-ζ) deficiency in sperm can underlie oocyte activation failure after intracytoplasmic sperm injection (ICSI). The aim of this study was to determine PLC-ζ expression and location in individual spermatozoa in each host score so that a hypo-osmotic swelling test (HOST) may be used to help routine sperm selection for ICSI.

Materials and Methods: In this experimental study, fresh semen samples were randomly obtained from 30 men who were referred to the Andrology Unit of the Infertility Center. Samples were processed by density gradient centrifugation (DGC) and exposed to hypotonic conditions. Seven different tail patterns, classified from 'a' to 'g' can be detected according to World Health Organization (WHO) criteria. Then, the PLC-ζ protein localization pattern was assessed by quantitative Immunofluorescence in individual sperm Host grades. Moreover, the sperm content of PLC-ζ protein was evaluated by flow cytometry correlated with semen analysis parameters.

Results: In the present study, quantitive immunofluorescence analysis indicated that sperm from different host grades exhibited seven localization patterns of PLC-ζ of acrosomal (A); equatorial (EQ), and postacrosomal (PA) patterns. A+EQ=acrosomal and equatorial, A+PA=acrosomal and post-acrosomal, EQ+PA=equatorial and post-crosomal, and A+EQ+PA.

The sperm from HOST grade 'd' exhibited significantly higher PLC-ζ (A+PA) and (A+EQ+PA) staining compared to sperm from other grades (P=0.006). The sperm from grade 'd' exhibited higher PLC-ζ (EQ+PA) compared with other grades (P=0.001). However, grade 'd' was not significantly different from 'c' (P=0.087). Analysis of the combined results confirmed that there was a clear reduction in PLC-ζ immunofluorescence in Host grades 'a', 'f' and 'g' sperms.

Conclusion: Our data suggest that HOST may represent a useful diagnostic tool for the selection of sperms exhibiting a higher level of PLC-ζ expression.

Keywords: Infertility, Intracytoplasmic Sperm Injection, Phospholipase C Zeta, Sperm-Ovum Interactions

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Introduction

Fertilization failure during assisted reproductive technology (ART) occurs in about (1-3%) of couples with a history of infertility (1, 2). Oocyte activation deficiency may be the most frequent cause of fertilization failure (3, 4). The sperm-specific phospholipase C zeta (PLC-ζ) has been identified as one of the possible factors involved in oocyte activation (5). Some studies have related the reduction of protein expression levels and aberrant forms of PLC-ζ to some male infertility such as globospermia and varicocele (6, 7).

Previous research has shown that the expression and localization of PLC-ζ coincide with late spermatogenic events such as histone-protamine remodelling involved in the maintenance of sperm chromatin integrity (8). PLC-ζ with a molecular mass of around 70 KDa is delivered into the ooplasm by sperm, which activates the phosphoinositide pathway by hydrolyzing phosphatidylinositol 4, 5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3), which then induces Ca^{2+} oscillations by binding to its receptor on the endoplasmic reticulum. The activation of the oocyte is caused by Ca^{2+} oscillations. Oocyte stimulation causes cortical granule exocytosis, which prevents polyspermy, the release of oocyte meiotic arrest, and the creation of the female pronucleus (9).

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Same to sperm quality which differs in different men and even in the ejaculates of the same individuals, the basic pattern of PLC-ζ localization has been characterized as varying significantly in sperm from normal fertile males, and it may also vary between ejaculates from the same person (10, 11). This variability may explain the main differences in sperm to activate the oocyte between individuals or within an ejaculate (12). In recent years, seven localization patterns of PLC-ζ were identified in human sperm: acrosomal (A); equatorial (EQ); post acrosomal (PA); A+ PA; A+EQ; EQ+ PA; and (A+ EQ+ PA) (13, 14). Equatorial and post-acrosomal PLC-ζ localization is the most prominent in human sperm. After gamete fusion, equatorial and post-acrosomal regions would permit the rapid dispersion of PLC-ζ into the oocyte (14).

The PLC-ζ deficit in sperm with normal morphology is well-established to cause Oocyte activation failure following ICSI (15, 16). Finding a means to choose the appropriate sperm for ICSI bypasses the natural barrier is critical. According to some data, the hypo-osmotic swelling test (HOST) has the potential to be used as a non-destructive sperm viability test for ICSI sperm selection (17, 18). Upon exposure of spermatozoa to hypo-osmotic conditions, seven forms of sperm tail have been identified that are referred to as 'a' to g' (19).

Previous research has shown that the HOST value can indicate the degree of sperm DNA damage in an ejaculate and that tail swelling can predict the possibility of DNA damage in individual spermatozoa. Therefore, these authors conclude HOST grade 'd' and grade 'c' may be healthier spermatozoa (17, 20). Some sperm selection methods are introduced in studies (21). The clinical value of HOST is supported by other authors but there is less information on the status of PLC-ζ expression in sperm of different HOST scores. This study aimed to determine PLC-ζ expression and location in individual spermatozoa in each host score so that HOST may be used to help with routine sperm selection for ICSI.

Materials and Methods

Ethical Issue

This experimental study was approved by the Research Ethics Committee of Islamic Azad University, Flavarjan, Iran, and (IR.IAU.FAlA.REC.1401.017). All participants signed written informed consent. In this study, fresh semen samples were randomly obtained from 30 men referred to the Andrology Unit of the Infertility Center.

Patients and standard semen analyses

Fresh sperm samples were randomly collected from 30 males aged 24-45 who visited the Shahid Beheshti Hospital Fertility and Infertility Center's Andrology. Male recruitment criteria required a minimum sperm count of 5×10^6 spermatozoa/mL. Semen samples were collected in sterile containers by masturbating after 3-4 days of sexual abstinence. After the liquefaction of the sperm, the World Health Organization (19) guidelines for sperm analysis were used. Computer-assisted semen analysis (CASA) was used to determine sperm count and motility (Test Sperm 2.1; Video test, St. Petersburg, Russia). The Eosin-staining technique was used to test the viability of the sperm. Diff-Quik staining (Idehvarzan, Tehran, Iran) was used to analyze sperm morphology, and one hundred sperms were scored on each slide and graded in duplicate using Kruger's rigorous criteria according to the WHO 2010 guideline.

Sperm preparation and HOST procedure

A density gradient was used to process sperm samples. Two ml of liquefied sperm was stacked on a two-step discontinuous Pure Sperm concentration gradient 40:80 percent (Nidacon International AB), centrifuged at 300 g for 20 minutes at room temperature, then collected and washed twice.

As previously mentioned, each patient's HOST was prepared and performed on semen samples. First, 100 µl of washed sperm was mixed with 1 ml of warmed 150+5 m Osm hypo-osmotic swelling solution (Ham's medium diluted with an equal volume of sterile purified H_2O_2 and heated to 37°C for 5 minutes). The percentage of HOST-positive samples and their grades were then calculated using WHO guidelines (19).

Immunofluorescence

PLC-ζ rabbit polyclonal antibody (LS-C144827) was bought from Life Span BioSciences for this work (USA). Azad et al. (22) used immunoblot to determine the specificity of this antibody. Samples were pelleted by centrifugation at 1500 g for 5 minutes, washed in phosphate buffer saline (PBS), fixed with 4 percent paraformaldehyde in PBS (BDH, Lutterworth, UK), rinsed in PBS, and drawn on slides pre-coated with 0.01 percent poly-L-lysine (Sigma Aldrich, USA). The attached sperm were permeabilized for 30 minutes in PBS (Sigma Aldrich, USA) containing 0.5 percent Triton X-100. After blocking for 1 hour in 3 percent bovine serum albumin (Sigma-Aldrich, USA), the slides were incubated overnight at 4°C with a primary anti-human-PLC antibody diluted in 0.05 percent bovine serum albumin. After that, the samples were washed three times in PBS and incubated for one hour at room temperature with 5 mg/ml of diluted secondary donkey anti-rabbit antibodies conjugated with DYLight-488 (Thermo, USA). Finally, samples were washed three times in PBS and mounted for analysis (Invitrogen's Prolong Gold Antifade Mounting Reagent). A fluorescent microscope was used to study 1000 sperm (Olympus, BX51, Japan).

Statistical analysis

In each example, the data is shown as the mean ± SEM of the number of samples assessed. To compare the distribution of PLC-ζ in HOST grades, a statistical one-way analysis of variance (ANOVA) was used. The

significance level was P≤0.05. Statistical Package for Social Sciences version 22 was used to conduct all data analyses (SPSS Inc., Chicago, IL, USA).

Results

Semen analysis

The mean sperm concentration determined in the semen analysis was 69.56 ± 11.4 million/ml (mean ± SD) ranging from 8 to 233 million/ml means sperm motility was 40.08% ± 11.3. Further, means sperm normal morphology was 7.6 ± 0.39 with a minimum and a maximum of 5 to 12%.

The following are the average percentages for different degrees of sperm tail swelling: 44.2 ± 1.1 (grade a), 17.6 ± 0.35 (grade b), 9.3 ± 0.23 (grade c), 6.05 ± 0.12 (grade d), 5.5 ± 0.16 (grade e), 6.3 ± 023 (grade f) and 10.85 ± 0.25 (grade g).

Proportional analysis of PLC-ζ localization patterns in each HOST grade sperm

The present study reported that individual spermatozoa with PLCζ deficiency and altered localization patterns are identifiable regarding different grades of HOST (Figs.1, 2). As shown in Table 1, no significant difference in (A), and (EQ), localization was detected in sperm from host grades. The sperm from grade 'd' displayed higher PLC-ζ (PA) compared with grade a' (P=0.01) and grade 'f' (P=0.049). The results indicated that the sperm from grade'd' exhibited significantly higher PLC-ζ (A+EQ) staining compared with sperm from grade 'f' (P=0.023) and grade 'g' (P=0.012). Proportional analysis of sperm exhibiting the sperm from grade 'd' exhibited, significantly higher PLC-ζ (PA+EQ) staining compared with sperm from other grades (P=0.001). Grade 'c' was an exception and no significant difference was observed between grade'd' and 'c' (P=0.087). PLC-ζ immunoreactivity indicated that a significantly larger proportion of sperm of 'd' grades exhibited PLC-ζ (A+PA) and (A+EQ+PA) staining, compared with other grades (Table 1).

Analysis of the combined results confirmed that there was a clear reduction in PLC-ζ immunofluorescence in Host grades 'a', 'f ', and 'g' sperms. As shown in Table 1, grade 'f' displayed significantly lower 5 of the 7 localization patterns compared to grade 'd'. Also, grades 'a' and 'g' displayed significantly lower 4 of the 7 localization patterns compared to grade 'd'.

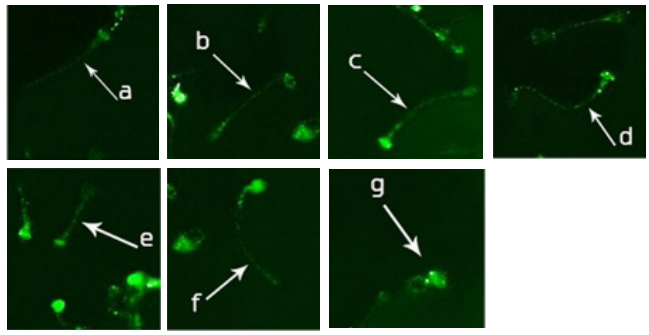

Fig.1: Representative PLC-ζ immunostaining of different PLC-ζ localization patterns obtained by immunofluorescence in Host grade. Grade 'a' sperm are HOST negative whiles grades 'b', 'c', 'd, 'e', 'f', and 'g' are HOST positive (scale bars: 2 µm).

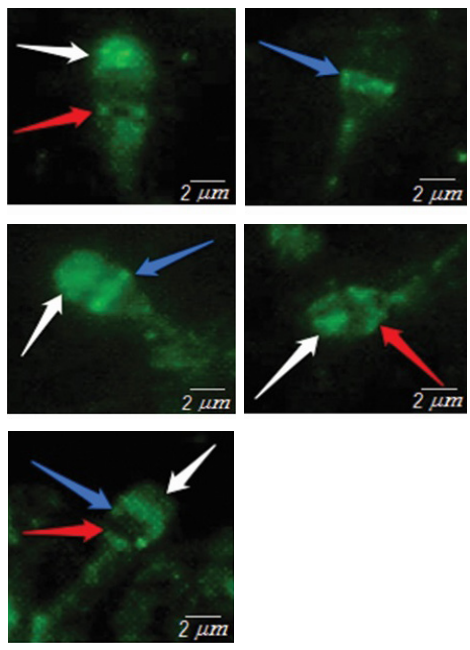

Fig.2: Representative image of phospholipase C zeta (PLC-ζ) immunofluorescence in human sperm. The white arrow indicates acrosomal localization; the blue arrow indicates equatorial localization; and the red arrows indicate postacrosomal localization (scale bars: 2 µm).

Table 1: Comparison of PLC-ζ localization patterns between grade'd' and other HOST grades

HOST grade	A	PA	EQ	A+PA	A+EQ	PA+EQ	A+PA+EQ
'a'	0.46 ± 0.3 P=0.16	0.6 ± 0.23 **P=0.01**	0.96 ± 0.83 P=0.25	3.3 ± 0.5 **P<0.001**	1.2 ± 0.8 P=0.13	3.7 ± 0.9 **P<0.001**	7.4 ± 0.7 **P<0.001**
'b'	0.46 ± 0.3 P=0.16	0.36 ± 0.23 P=0.87	2.17 ± 0.83 **P=0.01**	2.7 ± 0.5 **P<0.001**	1.3 ± 0.79 P=0.08	3 ±.9 **P=0. 001**	6.8 ± 0.7 **P<0.001**
'c'	0.36 ± 0.3 P=0.9	0.32 ± 0.23 P=0.17	0.64 ± 0.83 P=0.4	1.5 ± 0.5 **P=0.006**	0.75 ± 0.79 P=0.34	1.5 ± 0.9 P=0.087	3 ± 0.7 **P<0.001**
'e'	0.46 ± 0.3 P=0.16	0.39 ± 0.23 P=0.09	0.17 ± 0.83 P=0.83	2.6 ± 0.5 **P<0.001**	0.96 ± 0.79 P=0.22	3 ± 0.9 **P=0.001**	4.6 ± 0.7 **P<0.001**
'f'	0.5 ± 0.3 P=0.13	0.46 ± 0.23 **P=0.049**	0.2 ± 0.83 P=0.79	3 ± 0.5 **P<0.001**	1.8 ±.79 **P=0.023**	3.9 ± 0.9 **P<0.001**	6.1 ± 0.7 **P<0.001**
'g'	0.18 ± 0.3 P=0.6.3	0.36 ± 0.23 P=0.13	0.9 ± 0.83 P=0.3	3.5 ± 0.5 **P<0.001**	2 ± 0.79 **P=0.012**	4.3 ± 0.9 **P<0.001**	8 ± 0.7 **P<0.001**

The obtained P value of comparison of PLC-ζ localization patterns between grade 'd' and other HOST grades 'a', 'b', 'c', 'e', 'f', and 'g'. All data are presented as mean ± SE. Statistically significant (P≤0.05) differences are detailed in bold. PLC-ζ localization patterns: A; Acrosomal, E; Equatorial, PA; Post-acrosomal, A+EQ; Acrosomal and equatorial, A+PA; Acrosomal and post-acrosomal, EQ+PA; Equatorial and post-acrosomal, and A+EQ+PA; Acrosomal, equatorial, and post-acrosomal.

Discussion

While sperm staining should be avoided, the WHO has validated the Hypo osmotic Swelling Test as an alternate viability test (23). It has been claimed that the HOST is a supplemental test that is a simple, cost-effective, quick, and non-invasive method to select individual healthy spermatozoa (17, 18).

Some investigators reported that there is a correlation between HOST and other sperm parameters such as motility (24), aneuploidy (25), IVF outcome, and zona-free hamster ovum penetration assay (26). Also, HOST has been used to identify spermatozoa that appear to have minimal DNA damage (17). Recently, it was observed with normal semen parameters, the use of HOST in ICSI-Frozen ET cycles led to increases in the rate of live births in women aged 36-40 (27).

The present study reported that individual spermatozoa with PLCζ deficiency and altered localization patterns are identifiable regarding different grades of HOST. In this sense, all of the specimens investigated in this work were processed by density gradient and then submitted to HOST. It should be noted that the lowest occurrences of spontaneously developed tail swellings (SDTS) were found in DGW sperms (28).

Besides, for the first time, the results of this study showed that sperm HOST grades exhibit various expressions of PLC-ζ protein in the head. In addition, a significant PLC-ζ localization in the midpiece of human sperm was observed. This is in line with some other reports indicating PLC-ζ localization patterns were not restricted to the sperm head, but have also been observed in the sperm tail and midpiece (29, 30).

As previously mentioned PLC-ζ is a prognostic and diagnostic marker for ICSI outcome and repetitive ICSI failure depends on the localization patterns and the amount of PLC-ζ in the sperm head (14). Recent clinical reports showed a relationship between reduced protein expression levels and abnormal forms of PLC-ζ with human male infertility (31). PLC-ζ protein expression was considerably reduced or nonexistent in globozoospermia, which was characterized by low rates of oocyte activation (30). A recent study indicated that oocyte activation and clinical outcomes might not be related to PLC-ζ quantity alone (32).

Aarabi et al point to another sperm protein (PAWP) as a candidate for oocyte activation (33). When PLC-ζ is lacking or non-functional, PAWP is unable to promote the activation of human oocytes (34). Furthermore, in both mouse and human oocytes, injection of the recombinant protein PLC-ζ caused [Ca^{2+}] oscillations (35). Therefore, these studies confirmed the main role of PLC-ζ in the activation of mammalian oocytes.

Lee et al. (16) found that in patients with normal semen parameters that have low fertilization after ICSI, a few sperm expressing PLC-ζ and initiating robust calcium oscillation. Since, Semen samples contain a heterogeneous population of spermatozoa (32, 36) during the ICSI procedure identifying and using sperm that expresses higher-level PLC-ζ may increase the fertilization rate.

Indeed, earlier studies indicated that peripheral localization patterns of PLC-ζ were responsible for acrosome reaction and egg activation, whiles the post-acrosomal localization could modulate some aspect of pronuclear function (12). Higher levels of PLC-ζ (A+PA) were linked to ICSI success by Yelumalai et al. (14). Recent studies suggest successful fertilization was related to higher levels of (A+ EQ) PLC-ζ (29).

The results of the current study investigated seven localization patterns of PLC-ζ in seven Host scores: A=acrosomal, E=equatorial, PA=post-acrosomal, A+EQ=acrosomal and equatorial, A+PA=acrosomal and post-acrosomal, EQ+PA=equatorial and post-acrosomal, and A+EQ+PA. In the present study, grade 'f' displayed significantly lower 5 of the 7 localization patterns compared to grade 'd'. Also, grades 'a' and 'g' displayed significantly lower 4 of the 7 localization patterns compared to grade 'd'. Hence, the selection of sperm based on HOST may prevent insemination of sperm which is poor in PLC-ζ expression and localization.

Several reports showed PLC-ζ deficiency correlation with other defects in sperm. For instance, Kashir et al. study showed that cryopreservation could reduce the level of PLC-ζ expression in spermatozoa. It may be due to a change in membrane function that disturbs PLC-ζ localization and leak the sperm head (37). Park et al. (38) study showed a negative connection between sperm PLC-ζ immunoreactivity and an oxidation marker, 8-hydroxy-2'-deoxyguanosine (8-OHdG), and concluded that reduction of PLC-ζ expression in human sperm could be correlated to oxidative stress. Tavalaee et al. (39) demonstrated that DNA damage could lead to reduced expression of PLC-ζ in human sperm.

As a result, it has been proposed that the reduction of PLC-ζ expression in Host grades 'a', 'f', and 'g' in our study could be a consequence of the other problems in these sperm, such as DNA fragmentation or membrane function. This is in agreement with the Bassiri et al. (17, 20) study, which showed DNA fragmentation is higher in Host grades 'a' and 'g' grade sperms and recommended limiting the used sperm of these HOST grads in ICSI.

One of the limitations of the current study was the lack of evaluation of ICSI outcome for each Host graded sperm.According to the literature, the HOST may be helpful for screening paternal factors connected to repeat embryonic or early fetal loss and it can also be utilized in clinical laboratories (40). Analysis of the combined results confirmed that there was a clear reduction in PLC-ζ immunofluorescence in Host grades 'a', f ', and 'g' grade sperms. For the first time also our study showed that the sperm from grade 'd' exhibited significantly higher PLC-ζ (A+EQ+PA) and (A+PA) staining compared with sperm

from other grades. According to these results the sperm from HOST grade 'd' may be healthier with a greater capacity for inducing oocyte activation.

Conclusion

These findings promote the potential application of HOST as a useful method in selecting the most suitable sperm for ICSI that express PLC-ζ. Future studies could test these conclusions by looking at the particular Ca^{2+} oscillation signatures of each HOST grade sperm.

Acknowledgments

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Authors' Contributions

E.Y.; Contributed to conception, design, data analysis, interpretation, prepared samples, collected data, and manuscript preparation. A.A.; Contributed to manuscript writing and the final review of the manuscript and helped in the statistical analysis. All authors read and approved the final manuscript.

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