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The effect of prolonged incubation and temperature on oocyte activator phospholipase C-zeta activity of sperm

Uzun süreli inkübasyonun ve sıcaklığın oosit aktivatörü fosfolipaz C-zeta aktivitesine etkisi

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ABSTRACT

Objective: Fertilization capacity of capacitated sperm decreases exponentially over time, but the reason is still under investigation. The aim of the study was to analyze the effect of prolonged incubation and temperature on sperm fertilization capacity with motility and staining parameters of phospholipase C-zeta (PLCZ), which is considered to be the oocyte activation factor.

Materials and Methods: Density gradient washing was applied to semen of 11 infertile patients without severe oligoasthenospermia out of 16 patients. The samples were divided and cultured either at room temperature or at 37°C for 3 days. The spermatozoons were evaluated for motility, PLCZ staining and intensity daily.

Results: All parameters decreased both at room and body temperature with increased incubation time. There was a strong correlation between the change in motility and in the percentage of PLCZ stained sperms, but this correlation decreased with incubation time.

Conclusion: Prolonged incubation results show the correlation between PLCZ staining parameters and motility. Routine use of PLCZ staining together with semen analysis, will be useful to predict fertilization capacity of the sperm especially for unexplained infertility and fertilization failure cases, and also can increase the success of assisted reproductive technologies (ART) cycles.

Keywords: Temperature, Phospholipase C-zeta, Fertilization capacity, Sperm, Oocyte activaton, Prolonged incubation

ÖZ

Amaç: Kapasite olmuş spermin fertilizasyon kapasitesinin zamanla üstsel olarak düştüğü bilinmesine rağmen bunun nedeni hala araştırılmaktadır. Çalışmada amaç; uzamış inkübasyonun ve sıcaklığın sperm fertilizasyon kapasitesi üzerinde etkisini hareketlilik ve oosit aktivasyon faktörü olarak kabul edilen fosfolipaz C-zeta (PLCZ) ile değerlendirmektir.

Gereç ve Yöntem: On altı infertil erkek hastadan, ciddi oligoasthenospermisi olmayan 11'inin semenlerine dansite gradyan yıkaması uygulandı. Ardından örnekler bölünerek 3 gün oda sıcaklığında veya 37°C'de kültüre edildi. Örnekler hareketlilik, PLCZ boyanma yüzdesi ve yoğunluğu açısından günlük olarak değerlendirildi**.**

Bulgular: Hem oda sıcaklığı, hem de vücut sıcaklığında her üç parametrede de zamanla azalma gözlendi. Özellikle günler arası hareketlilikteki değişim ile PLCZ boyanan sperm yüzdesindeki değişim arasında güçlü bir korelasyon bulundu, ancak bu korelasyon zamanla azalmakta idi.

Sonuç: İnkübasyon süresinin uzamasıyla, PLCZ boyanma parametreleri ve hareketlilik arasında korelasyon görülmektedir. PLCZ boyanmasının semen analizi ile rutinde kullanımı, özellikle açıklanamayan infertilite ve fertilizasyon başarısızlığı vakalarının spermlerinin fertilizasyon kapasitesinin belirlenmesinde yarar sağlayabilecek ve yardımcı üreme teknileri (YÜT) sikluslarındaki başarıyı artırabilecektir.

Anahtar kelimeler: Sıcaklık, Fosfolipaz C-zeta, Fertilizasyon kapasitesi, Sperm, Oosit aktivasyonu, Uzun süreli inkübasyon

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Introduction

The fertilization capacity of spermatozoon in vivo and in vitro conditions is very important in terms of both providing fertility and infertility treatments, and has been investigated for many years. In routine practice, sperm motility is still in use to predict fertilization capacity in addition to other semen analysis parameters. The sperm is more resistant to environmental conditions than other cells because of its structure, and the washed sperm can maintain its motility for

up to 20 days at room temperature and up to 8 days at 37°C [1]. Besides, unwashed sperm motility decreases rapidly in 3 days at room temperature, but its vitality continues until 11 days [2].

In vivo conditions, the fertile window was reported as 6 days [3]. Furthermore, Weinberg et al. reported, that natural conception rate decreased exponentially from ovulation day to 8 days before, also they estimated the mean viable lifetime for sperm as 1,4 days [1]. The cause of exponentially decrease of fertilization capacity of sperm is still under investigation.

Semen analysis is used routinely for the determination of sperm fertilization capacity. Especially, the sperm concentration or count, motility and morphologic parameters are evaluated. In addition, optional procedures can be performed for research purposes in particular, such as: computer aided sperm analysis (CASA), panleukocyte (CD45) immunocytochemical staining, in vivo and in vitro tests for the interaction between spermatozoa and cervical mucus, measurement of reactive oxygen species, human sperm-oocyte interaction tests, human zona pellucida binding tests, assessment of the acrosome reaction, zona-free hamster oocyte penetration test, assessment of sperm chromatin [4]. Despite all routine investigations, the fact that about 10% of cases applied for conventional in vitro fertilization (IVF) have total fertilization failure, made researchers seek for a new test that can clinically determine the sperm fertilization capacity.

The motility and the fertilization capacity of the sperm are different concepts. With its importance lately drawing attention, a phosphoinositide-specific phospholipase C zeta (PLCZ, PLCZ1, PLCζ) is a protein from the phospholipase C family and is localized at the head of the sperm [5]. PLCZ is crucial to fertilization, because it initiates Ca^{2+} oscillations during fertilization [6]. Because of this function, it is considered to be an important indicator of sperm fertilization capacity. PLCZ has been reported to be as low amounts or as abnormal forms in infertile males [7]. It has been reported that in intracytoplasmic sperm injection (ICSI) cases, there is a significant positive correlation between fertilization rates and the percentage of sperms exhibiting PLCZ [8]. However, there is no study of PLCZ change in vitro and in vivo conditions.

The aim of our study is to investigate the usage of PLCZ staining parameters to predict the fertilization capacity in addition to motility with long-term incubation at room temperature and at 37°C.

Materials and Methods

Routine semen analysis according to the guidelines of the World Health Organization (2010) [4] was applied to those patients that made an application to the clinic with a complaint of infertility and agreed to participate in the study. The samples with initial sperm concentrations of $10x10^6$/ml, motility of >30% and a normal morphology of >1% were accepted for the study. All procedures were performed under sterile conditions, and the samples were evaluated double-blind by 2 experienced researchers.

The density gradient washing was applied to the samples according to the guidelines of the World Health Organization: 1 ml of semen was placed gently above the density gradients of 1 ml 40% over 1 ml 80% density gradient medium (SpermGrad, Vitrolife, Sweden), which was diluted with dilution medium (G-Mops, Vitrolife, Sweden). This was centrifuged at 360 g for 20 minutes. The pellet was transferred in a new centrifuge tube, resuspended with 5 ml culture medium (G-IVF Plus, Vitrolife, Sweden) and centrifuged at 360 g for 10 minutes. The washing procedure was repeated and the obtained pellet was resuspended with culture medium [4].

Sperm concentration of each sample was settled to $5x10^6$/ml. After initial (0th hour) motility assessment was made and first smears for PLCZ were done, each semen sample was allocated into two samples, so that three days incubation of one group was held at room temperature and that of the other one at 37°. Motility was assessed, and pellets were smeared on poly-l-lysine coated slides (p0425, Sigma) for PLCZ staining. The remaining samples were centrifuged at 360 g and pellet was resuspended with fresh culture medium maintaining the sperm concentration of $5x10^6$/ml.

Smears were fixed with -10°C methanol for PLCZ immunofluorescent staining. After the fixation, 60 minutes of blocking with 1:10 donkey serum (Santa Cruz Biotechnology) was applied. Goat anti-PLCZ1 primer antibody (Santa Cruz Biotechnology) diluted to 1:200 was incubated overnight at 4°C. FITC conjugated donkey anti-goat secondary antibody (Santa Cruz Biotechnology) washings were done with 0.1M PBS. The samples were evaluated with the Leica DMLB fluorescent microscope fixed to an intensity of 16% and 470 led nm and with 100x objective.

Motility assessment of the samples was done with the phase-contrast microscope and recorded as percentages. PLCZ1 evaluations were done for 400 sperms on each slide; so that the fluorescent positive sperms were counted first on a field, and thereafter total sperms were counted quickly

on that area with light microscope feature of the same fluorescent microscope. After counting of the stained and unstained sperms of one area, we switched to the next area. The evaluation of fluorescent intensity was done giving arbitrary units as 1:very weak to 9:high intensity.

This study was ethically approved by appropriate authorities: ethical approval (Marmara University, School of Medicine, Ethical Committee of Clinical Researches. Protocol Number 09.2016.185), IVF Units Academic Directory Board and all the patients signed a written informed consent.

Statistical Analysis

Statistical analysis and calculations were done with SPSS 15.00 and MS-Excel 2013 programs. Motility, proportions of sperm exhibiting PLCZ immunoreactivity and PLCZ staining intensity values were tested with the Shapiro-Wilk test to determine normal distribution. To compare daily measurement means or medians; repeated measures ANOVA and post-hoc Tukey-Kramer multiple comparison test was used for normal distributed values, and the Friedman test followed by the Dunn test was used for not normal distributed values. $P<0.05$ was considered to be significant.

Daily percent of change was calculated with the formulae: (Day before-Day after/Day before) x 100. To calculate significance of variance between room temperature and 37°C groups, comparisons of independent samples t-test and the Mann Whitney test were used according to normal distribution of percent of change values. The Spearman correlation test was used because of the small sample size for the correlation between daily motility change and daily PLCZ staining percent of change.

Results

Out of 16 patients that agreed to participate in the study; the density gradient washing technique was applied to the semen samples of 11 patients, which fulfilled study criterias, which are as sperm concentrations of $10x10^6$/ml, motility of >30% and a normal morphology of >1% (Table I). In the light and fluorescent microscopic examinations of the PLCZ staining; parameters such as proportions of sperm exhibiting PLCZ immunoreactivity, the staining localization and the staining intensities could be clearly assessed (Figures 1 and 2).

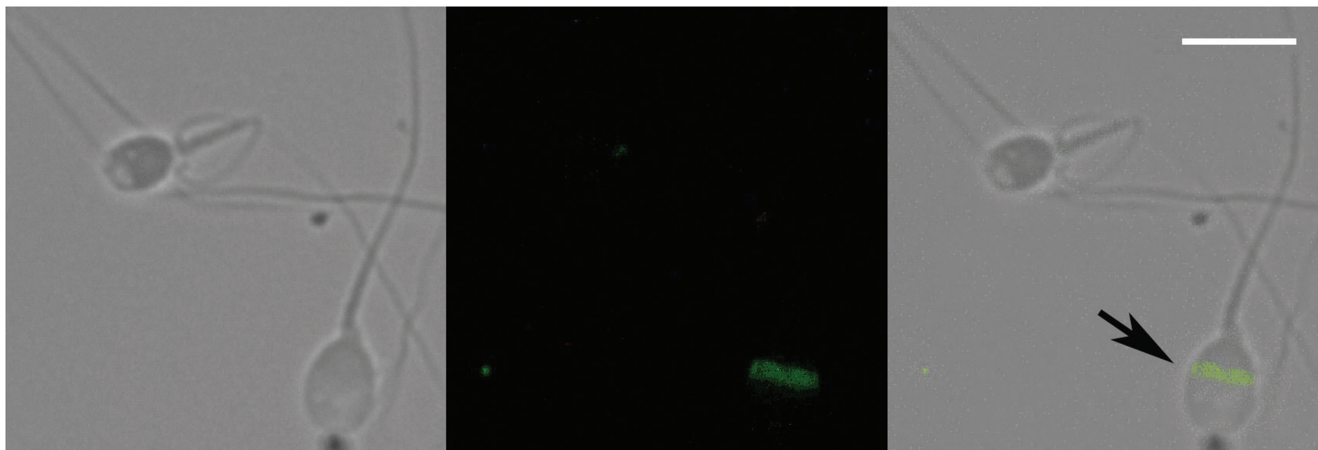


Figure 1. On the left side light microscopic, on the center fluorescent microscopic, on the right side merged micrographs of two sperms. One PLCZ positive stained (arrow) and one negative sperm. Scale bar represents 5µm

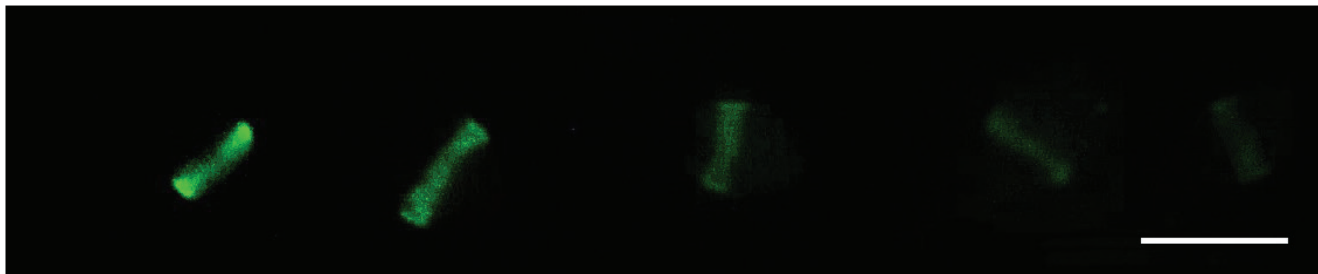
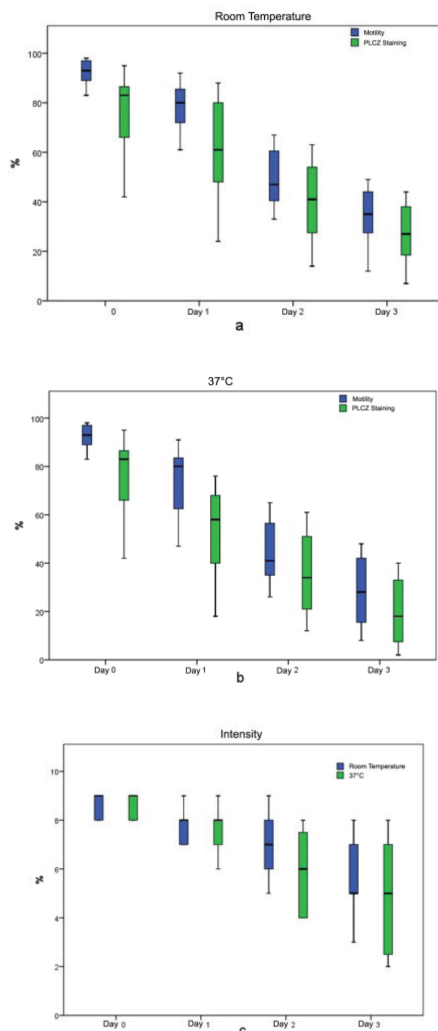


Figure 2. Sperms of different fluorescence intensities. Scored from left to right as 9, 7, 5, 3, 1. Scale bar represents 5µm

Table I. The mean values and standard deviations (SD) of the samples included in the study are shown as the group 1, and the mean values of the samples left out of the study are shown as the group 2.

	Group 1 (SD)	Group 2 (SD)
Age	37.4 (5.7)	27.8 (6.8)
Volume ml	3.1 (1.4)	4.2 (2.2)
pH	7.7 (0.5)	7.6 (0.5)
Sperm Concentration x10^6/ml	36.7 (16)	54.6 (29)
Progressive Motility %	44 (21)	15 (8.3)
Non-progressive Motility %	11 (4.5)	8.2 (4.7)
Normal Morphology %	3.7 (3.7)	1.6 (1.5)
Head Anomaly %	91 (6.9)	95 (4.8)
Midpiece Anomaly %	33 (16)	30 (12)
Principal Piece Anomaly %	11 (5.5)	29 (16)
Excess Residual Cytoplasm %	3 (1.9)	2 (1.2)
Teratozoospermia Index	1.38 (0.22)	1.57 (0.29)

Table II. a) At room temperature: changes of motility rate and PLCZ; b) At 37°C: changes of motility rate and PLCZ; c) At room temperature and at 37°C: changes of staining intensity were shown with box plots.



After the application of the density gradient sperm preparation method, the mean motility of the samples was 92 ± 5%, PLCZ immunoreactivity 76 ± 16% and PLCZ staining intensity 8 ± 0.5. The PLCZ localization patterns were mostly equatorial as expected, with very few postacrosomal and trace amounts of other localizations like acrosomal or combined localizations. Localization was not evaluated, because it did not change with time or temperature. Values after the density gradient sperm preparation method were evaluated as the initial samples for both room temperature and 37°C groups. Table IIa shows the changes of motility and PLCZ at room temperature incubation, table IIb shows the changes of motility and PLCZ at 37°C incubation, and Table IIc shows the change of fluorescent intensities at room temperature and 37°C incubations.

While the data considering distribution of motility and percentage of PLCZ (sperm exhibiting PLCZ immunoreactivity) was normal, but of the PLCZ intensity was not normal. When days were compared, the difference between room temperature and 37°C groups was significant for motility, for PLCZ immunoreactivity and for PLCZ staining intensity. When the PLCZ intensities were compared, there was no significant difference in intensity values between one day intervals. However, the differences in PLCZ staining intensity value at two day intervals were significant. Compared with the fresh sample, the significance of this difference was found to be stronger on the third day.

When the decrease in motility at room temperature and at 37°C were compared, the decrease was more prominent at 37°C incubation group, but it was not statistically significant. Similarly, when PLCZ staining percent of change between 0 to 3 days incubation was compared, there was no significant difference observed between room temperature and 37 °C groups (Table III).

Table III. 0-24, 0-48 and 0-72 hours percentage of change means and standard deviations (SD) at different temperatures.

	Room Temperature Mean (SD)	37°C Mean (SD)
Motility 0 - 24	15.8 (6)	20.9 (12.1)
Motility 0 - 48	46.8 (10.1)	52 (12)
Motility 0 - 72	63.7 (11.8)	69.7 (15)
PLCZ 0 - 24	22.2 (13.4)	33.3 (15.5)
PLCZ 0 - 48	49.2 (13.3)	54.6 15.6)
PLCZ 0 - 72	66.1 (11.5)	76 (15.3)

At room temperature: There was a strong correlation between the change in motility and the change in PLCZ staining (proportions of sperm exhibiting PLCZ immunoreactivity) between the post-wash and 24th hour. The correlation was stronger between 0-1 days motility change and 0-1 days PLCZ staining change than between 0-1 days motility change and 0-2 days PLCZ staining change, which was also stronger than the correlation between 0-1 days motility change and 0-3 days PLCZ staining change. At 37°C: The change of motility at 0-1 days was not correlated with the change of PLCZ staining, but there was a strong correlation between 0-1 days motility change and 0-2 days PLCZ staining change, and this correlation decreased but was significant between 0-1 days motility change and 0-3 days PLCZ staining change (Table IV).

Table IV. a) At room temperature and b) at 37°C: Correlation between 0-1 days percentage of change and 0-1. 0-2 and 0-3 days PLCZ staining change. R_s: Spearman correlation coefficient. *: *P*<0.05; **:*P*<0.01; ***: *P*<0.0001

Room temperature, 0-1 days, Motility Percentage of Change	r_s	***P***
0-1 days, PLCZ Percentage of Change	0.8090909	**0.003940797
0-2 days, PLCZ Percentage of Change	0.7454545	*0.01121548
0-3 days, PLCZ Percentage of Change	0.6545454	*0.03358491

37°C, 0-1 days, Motility Percentage of Change	r_s	***P***
0-1 days, PLCZ Percentage of Change	0.5056961	0.1153
0-2 days, PLCZ Percentage of Change	0.7744895	**0.0070
0-3 days, PLCZ Percentage of Change	0.7334871	*0.0128

Also, 3 days total motility percentage of change and 3 days total PLCZ staining intensity change was significantly correlated at both temperatures (for room temperature (r_s=0.8284; *P*=0.0025) and for 37 °C (r_s=0.9270; *P*<0.0001))

Discussion

Spermatoozoon enters the cervical mucus within the few minutes after the natural intercourse [9] and reaches the fallopian tubes within 10 minutes [10-12] preserving its fertilization capacity up to 8 days. As expected, its fertilization capacity decreases with time [1,13]. Similar results were obtained also in studies of conditions similar to natural environment [14]. In these studies, motility parameters of sperm were examined most frequently, because analysing the motility is both easy to measure and an important parameter in predicting fertilization outcomes [15]. Thijssen et. al and Petrella et al. reported and also we observed in our study, that the motility of washed sperm decreases with prolonged incubation at both room temperature and at 37°C [2,16]. We attribute the more severe motility decrease observed at 37°C to the faster biological reactions as an effect of temperature, and it is a phenomenon consistent with the literature [17]. The reason that this decrease was not that severe in our study was thought to be because of daily medium renewal of sperm.

PLCZ is a protein localized at the head of the sperm, initiates Ca^{2+} oscillations in oocyte during fertilization and has been chosen as a key test of this study since it is undoubtedly crucial in assessing fertilization capacity of the sperm. That, there is a difference between PLCZ staining rates of fertile and infertile men sperms, besides reduced amounts and abnormal forms of PLCZ is observed in sperms from infertile men [7], and furthermore PLCZ stainings significantly correlate with fertilization rates after ICSI but not IVF [8], are supporting studies proving PLCZ's importance.

It has been reported that the amount of PLCZ-stained spermatozoa is different from patients both by semen and by density gradient washed sperm [18]. The percentage of PLCZ staining was observed in our study similarly with a wide range. There is another study reporting, that PLCZ levels are in a wide range in specimens of fertile men, but at lower levels in cases of teratozoospermia, oligoastenozoospermia, and oligoasthenoteratospermia [19]. However, the fact that the PLCZ protein expression and localization parameters do not differ significantly between semen of infertile men and fertile men in the same study suggests, that studies involving this subject should be repeated with a higher number of samples. It is also possible to explain the changes in the percentage of PLCZ stained sperm because of the different amounts of live sperm in the washed semen, but approximately 90% of the sperm are motile and therefore close to all live in the density gradient washed sperm, so the reason for this difference can be explained with the fact that some sperms are motile and live but do not carry PLCZ.

In our study, the number of sperms stained with PLCZ decreased like the sperm motility dependent on the

duration of incubation. This effect was observed both at room temperature and at 37°C. According to the literature, it is known that motility decreases at the body temperature faster than the room temperature [20], and the PLCZ staining giving similar results is therefore an expected finding. This difference was not statistically significant but less; which explains that although sperm has lost its motility, its ability to fertilize can be sustained at least in vitro conditions. We suggest, that the total disappearance of PLCZ staining happens by the sperm death.

The fluorescence intensity of the PLCZ is another issue that attracted attention in recent times. At the beginning of the study, postwashing sperm motility values showed a normal distribution. Kashir et al., reported that the fluorescence intensity of PLCZ was significantly different, when the cases of oocyte activation deficiency and the fertile cases were compared. It was thought, that the differences in these cases may be due to the differences in the amounts of PLCZ or due to the differences in the structure of PLCZ that sperm carried [21]. In our study, the mean fluorescence intensity of the PLCZ decreased slightly with time and temperature changed, while the percentage of motile sperm was severely decreased. At the end of three days, the percentage of change in motility and the percentage of change in intensity was found to be statistically correlating. Therefore, it can be assumed that the activity of PLCZ was reduced.

As a result, PLCZ staining parameters and motility, which both showed sperm fertilization capacity, decreased at room temperature and at body temperature over the prolonged incubation, which was similar to previous in vivo reports [2, 14]. There was a correlation between the percentage of decrease in PLCZ staining parameters and motility. Because motility and PLCZ show different features of sperm, we conclude that in addition to routine semen analysis, PLCZ staining may be better to predict the fertilization capacity. It could be especially helpful for the revealing of the etiology of unexplained infertility cases and of total fertilization failure cases and also can increase the success of ART cycles.

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