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Research Article

## Assessment of the relationship between follicular fluid calcium ion concentration and reproductive outcomes

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### ABSTRACT

**Background:** Quantification of follicular fluid (FF) composition can provide valuable information about the oocyte state which can be helpful in distinguishing high and low quality oocyte. In the absence of appropriate calcium signaling at fertilization, the embryo may fail to implant and/or develop to full term. We herein aimed to investigate whether FF levels of Ca<sup>2+</sup> ion concentrations vary between patients who achieved pregnancy and those who did not following a standard ICSI treatment regimen, and hence to determine whether FF Ca<sup>2+</sup> levels could predict reproductive outcomes in patients undergoing ICSI.

**Methods:** Patients seeking treatment for solely unexplained infertility were recruited in the period between June and December 2013 and were assigned to the groups according to their reproductive outcomes. Group 1 consisted of patients who conceived and Group 2 consisted of those who did not achieve pregnancy following ICSI. Parametric comparisons were performed using Student's t-test. Categorical data were evaluated by using  $\chi^2$  test.

**Results:** Groups 1 and 2 consisted of 34 and 66 patients, respectively. Although FF Ca<sup>2+</sup> ion concentrations were higher in those who did not achieve any pregnancy, the difference did not reach to statistical significance level (7.6±0.4 mg/dl and 7.9±0.9 mg/dl respectively, p=0.070).

**Conclusions:** FF Ca<sup>2+</sup> ion concentrations did not significantly differ between patients who achieved pregnancy and who did not followed ICSI attempts. Thus FF Ca<sup>2+</sup> levels do not appear to be of benefit in predicting reproductive outcomes in unexplained infertility patients undergoing ICSI cycles.

**Keywords:** Follicular fluid, Calcium, Oocyte, ICSI, Reproductive outcomes

### INTRODUCTION

Despite significant advances in the field of reproductive medicine success rate still remain relatively low with clinical pregnancy rates at 33%.<sup>1</sup> All attempts assisted in reproduction to improve pregnancy rates as well as to reduce the possibility of multiple pregnancy is to choose the best quality embryo for transfer and to allow implantation of a single embryo.<sup>2</sup> Despite the importance of oocyte quality, current assessment of oocytes in assisted reproduction is limited and a variety of methods including oocyte morphology studies,<sup>3</sup> molecular and

genetic methods<sup>4</sup> and polar body biopsies<sup>5</sup> are being employed for oocyte selection. However, most of these techniques are not applicable in the current practice since they are complicated, expensive and time-consuming<sup>6</sup> and an optimum technique to determine the best quality oocyte is at this time is still lacking. On the other hand the identification and quantification of follicular fluid (FF) composition can provide valuable information about the oocyte state which can be helpful in distinguishing those oocytes that have a greater capacity to be fertilized and to develop properly.

It has long been known that calcium is an indispensable signal for mammalian fertilization<sup>7</sup> and upon the fusion of the sperm and oocyte membranes the first intracellular signalling event observed is a series of low-frequency oscillations and large transient increase in cytoplasmic free  $\text{Ca}^{2+}$  ions. The initiation of calcium oscillations is considered to be triggered by the sperm factor phospholipase C zeta (PLCz), which is released into the oocyte following oocyte fertilization.<sup>8,9</sup> PLCz triggers a cascade of reactions which finally activates the receptors located on the membrane of the endoplasmic reticulum leading to the cytoplasmic release of calcium.<sup>10,11</sup> It is presumable that the absence or aberrant of calcium oscillations at the time of fertilization could lead to failed or low fertilization following ICSI. In addition, the contribution of external calcium influx to the intracellular calcium release has been evaluated. Some authors reported that in some species eggs are primarily activated by calcium ions which enter the cytosol from the medium and response to depolarization of the egg's plasma membrane.<sup>12</sup> In humans the pathways leading to  $\text{Ca}^{2+}$  increase within the egg are still vigorously debated.

Elevated  $\text{Ca}^{2+}$  plays a vital role as an intracellular messenger in all cells and the  $\text{Ca}^{2+}$  signal occurring in the oocyte at fertilization triggers the subsequent events that mediate early embryo development.<sup>13</sup> In the absence of appropriate calcium signalling at fertilization the embryo may fail to implant and/or develop to full term.<sup>14</sup> Now FF bathes the developing oocytes and the composition of FF may be regarded as the surrogate for the oocyte quality as it appears to be plausible to investigate a possible association between FF  $\text{Ca}^{2+}$  of reproductive outcomes and pregnancy rates. However, there is a paucity of literature reporting this relationship. In the present study, we aimed to investigate whether FF levels of  $\text{Ca}^{2+}$  ion concentrations vary between patients who achieved pregnancy and those who did not followed a standard ICSI treatment regimen and hence to determine whether FF  $\text{Ca}^{2+}$  levels could be used as a predictor of reproductive outcomes in patients undergoing ICSI cycles.

## METHODS

A prospective cross-sectional study was initiated at Assisted Reproduction and Infertility Department of Zeynep Kamil Training and Educational Hospital, Istanbul, Turkey. Ethical Board approval was obtained prior to the initiation of the study. Patients seeking treatment for solely unexplained infertility were recruited in a 6 month period between June and December 2013. Unexplained infertility was diagnosed in patients with normal ovulatory cycles, basal hormone profile, semen analysis, TVS and HSG for >12 months. Inclusion criteria follows age between 23-39 years, body mass index (BMI)  $\leq 28\text{kg/m}^2$ , FSH  $\leq 10$  mIU/mL and unexplained infertility patients who underwent either agonist or antagonist protocol. Patients with male or tubal factor infertility were excluded.

All patients underwent through standardized IVF treatment protocol. Gonadotropine doses were individualized according to age, BMI, basal hormone levels, AFC and previous experiences. Gonadotropin stimulation was achieved either by rFSH or hMG. All patients were administered acetyl-salicylic acid 100 mg daily and folic acid 400 mcg daily simultaneously with the start of the protocol.

Serial ultrasonography controls and E2 level measurements was made until 3 follicles  $\geq 17$  mm and a serum E2 level  $>500$  pg/ml were detected. Choriogonadotropin alpha 250  $\mu\text{g}$  s.c. (Ovitrelle®; Merck Serono, Italy) was administered to induce final follicular maturation. Transvaginal ultrasound-guided oocyte retrieval was performed 35-36 hours after hCG administration. FFs collected from the first punctured MII oocyte containing mature ( $>17$  mm) follicle at the first entry without contamination was analyzed whereas FFs contaminated with blood were not used. All patients were implemented with single dose of cefazolin sodium (Sefazol, Mustafa Nevzat İlaç San., Turkey) 1 gr, i.m at the course of OPU procedure and were given doxycycline 100 mg capsule (Tetradox capsule, Fako İlaç, Turkey) twice daily and methylprednisolone 16 mg capsule (Prednol tablet, Mustafa Nevzat İlaç San., Turkey) once daily orally and continued for 4 days.

The aspirated FFs were centrifuged for 10 minutes at  $2000 \times g$  at room temperature and separated supernatant fluids were assayed for  $\text{Ca}^{2+}$  ion concentrations. All patients underwent ICSI in which a single embryo transfer was performed on either 2nd or 3rd day. Patients were assigned to the groups according to the reproductive outcomes. Group 1 consisted of patients who conceived and Group 2 consisted of those who did not achieve pregnancy following the treatment. Achievement of pregnancy was defined as a positive pregnancy test result ( $\beta$ -hCG levels  $>20$  mIU/ml) 12 days after embryo transfer. The results of  $\text{Ca}^{2+}$  ion concentrations for two groups were compared.

Statistical analyses were performed using the statistical Package for the Social Sciences for Windows 15.0 software (SPSS, Chicago, IL., USA). Descriptive statistics were given as mean and standard deviation. Parametric comparisons were performed using Student's t-test. Categorical data were evaluated by using  $\chi^2$  test. Statistical significance was defined as  $p < 0.05$ .

## RESULTS

From June to December 2013 a total of 100 women with the diagnosis of unexplained infertility were included in the study. Group 1 consisted of 34 and Group 2 consisted of 66 patients. Demographic and induction characteristics are presented in Table 1. None of the demographic characteristics significantly differed between the groups.

Although FF Ca<sup>2+</sup> ion concentrations were higher in those who did not achieve any pregnancy, the difference did

not reach to statistical significance level (7.6±0.4 mg/dl and 7.9±0.9 mg/dl, respectively, p=0.070).

**Table 1: Demographic and stimulation characteristics and treatment outcomes of the groups.**

	Group 1 (n=34)	Group 2 (n=66)	p
Age, y	32.3±4.3	31.7±4.7	0.586 <sup>a</sup>
BMI, kg/m <sup>2</sup>	24.0±3.9	24.7±3.5	0.430 <sup>a</sup>
Infertility duration, y	5.4±2.8	6.0±3.5	0.371 <sup>a</sup>
D3 FSH, IU/l	7.8±2.4	8.0±2.7	0.644 <sup>a</sup>
D3 estradiol, pg/ml	52.2±22.3	50.2±19.2	0.654 <sup>a</sup>
GnRH agonist/antagonist protocol, n	12/22	24/42	0.916 <sup>b</sup>
Total dose of gonadotropin, IU	2616.5±1363.4	2768.6±1124.6	0.521 <sup>a</sup>
Serum E2 on HCG day, pg/ml	2030.2±1581.2	1723.4±787.6	0.216 <sup>a</sup>
Total oocytes retrieved, n	7.6±4.6	7.3±4.4	0.767 <sup>a</sup>
MII oocytes, n	5.9±3.4	5.2±3.8	0.353 <sup>a</sup>
Ca <sup>2+</sup> in follicular fluid,	7.6±0.4	7.9±0.9	0.070 <sup>a</sup>

Data are presented as mean ± SD and number; <sup>a</sup>Student t-test; <sup>b</sup>χ<sup>2</sup> test.

## DISCUSSION

Ca<sup>2+</sup> is universally accepted as the essential trigger of egg activation. The importance of Ca<sup>2+</sup> during the process of oocyte maturation and fertilization has been confirmed in numerous studies conducted in mammalian and non-mammalian animal species. The detailed signal transduction pathways that lead to the intracellular Ca<sup>2+</sup> liberation during hormonal and sperm stimulation have been extensively studied.<sup>15,16</sup> Recently a higher intracellular Ca<sup>2+</sup> concentrations in granulosa cells have been reported to increase the fertilization ratio and oocyte quality in patients undergoing IVF.<sup>17</sup> Hence we postulated that a higher intracellular concentration of Ca<sup>2+</sup> could be represented as a higher FF level of Ca<sup>2+</sup>, which might have help us speculate about reproductive capability of the individual.

The biochemical composition of fluid in mature Graafian follicle is of great importance since this medium constitutes the micromilieu for developing oocytes and the composition of FF may have an impact in the oocyte quality which is a crucial parameter in women undergoing assisted reproduction. FF contains substances implicated in oocyte meiosis rupture the follicular wall and fertilization.<sup>18,19</sup> Numerous metabolites in FF have been investigated in patients undergoing assisted reproduction in recent years.<sup>20,21</sup> FF levels of Ca<sup>2+</sup> ion in IVF/ICSI patients have been evaluated in a limited number of studies. In one of the study Ca<sup>2+</sup> and Mg<sup>2+</sup> was reported as the most abundant elements in FF.<sup>22</sup> In that study it was confirmed that element concentrations in small follicles frequently differed from those of large follicles and that the element concentrations in large follicles more closely resembled those in blood. Azem et al<sup>23</sup> examined the serum and preovulatory FF levels of Ca<sup>2+</sup> and Mg<sup>2+</sup> throughout IVF cycles and sought a

possible relationship to IVF/ET. They also concluded that the presence of these divalent cations in the FF indicated their correlation to folliculogenesis and ovulatory processes. Makki et al<sup>24</sup> reported that they received the highest percentage of invitro maturation and fertilization when they applied the highest concentrations of selenium, calcium and calcium ionophore concentrations. Here by extrapolated that maturation and activation of oocytes may be triggered by changes in intracellular ion concentrations as second messengers in signal transduction pathways. In a more recent study it was reported that treatment with Ca<sup>2+</sup> ionophore improved embryo development and reproductive outcomes in cases with previous developmental problems.<sup>25</sup> Cleavage to 2-cell stage the number of blastocysts formed on day 5 and the rates of implantation, clinical pregnancy and live birth were significantly higher in the ionophore group than in the control group. However to our knowledge there is no study investigating the relationship between FF Ca<sup>2+</sup> ion levels and pregnancy rates in the literature. In what we believe is the first study reporting this relationship. According to the results of our study FF Ca<sup>2+</sup> ion levels do not significantly differ between patients who conceived and those who did not although slightly higher in the latter.

The limitations of the present study include the relatively low patient number and lack of the assessment of intracellular Ca<sup>2+</sup> ion levels for its association with FF levels and pregnancy outcomes. However, further studies with larger patient numbers and designed to evaluate intracellular Ca<sup>2+</sup> levels synchronously are warranted.

## CONCLUSIONS

FF Ca<sup>2+</sup> ion concentrations do not significantly differ between patients who achieved pregnancy and who did

not following ICSI attempts. Thus FF Ca<sup>2+</sup> levels do not appear to be benefit in predicting reproductive outcomes in unexplained infertility patients undergoing ICSI cycles.

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