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A Review of Artificial Oocyte Activation with Calcium Ionophore for Fertilization Failure and a Case Report of a Successful Twin Pregnancy

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Artificial oocyte activation, Fertilization, Embryogenesis.

Introduction

Oocyte activation is required for successful human oocyte fertilization and subsequent embryogenesis. It relies on both sperm and oocyte-borne factors. Once initiated, it drives early fertilization events by eliciting Ca++ (calcium ion) oscillations within the cytoplasm. Cortical granule exocytosis, resumption and completion of meiosis, and formation of pronuclei all rely on these Ca++ oscillations, and without these events, fertilization and embryo development cannot progress normally [1-3].

Natural entry of a sperm into an oocyte in vivo begins, ideally, with fusion of the sperm and oocyte membranes. The sperm releases a testis-specific enzyme called phospholipase C zeta (PLC-z) from its post-acrosomal region [4]. PLC-z diffuses into the cytoplasm, hydrolyzing phosphatidylinositol-4, 5-biphosphate (PIP2) into inositol-1,4, 5-triphosphate (IP3), an important oocyte activation molecule [5-10]. Receptors for IP3 are distributed from cortex to cortex as well as some organelles, all of which are targets for upcoming reactions. This spatial layout helps overcome the typically poor diffusion rate of Ca++ by directing the IP3 to where it is most useful, namely the cortex, endoplasmic reticulum, and mitochondria [6]. IP3 thus offers an efficient means of propagating Ca++ waves across the oocyte.

As IP3 binds to receptors on the endoplasmic reticulum (ER) and triggers Ca++ release from its internal stores, the first wave of calcium propagates across the cytoplasm. Calcium peaks around 30-40 minutes after sperm entry, spiking to 1.1 and lasting the longest of any of the oscillations, roughly 4 minutes before returning to baseline [11]. This acute rise in Ca++ activates protein kinase C molecules (PKCs) which translocate to the oocyte cortex, where the cortical granules are located. PKCs disrupt the actin cytoskeleton and allow exocytosis of granule contents [1,5]. The contents diffuse outward, remodeling the zona pellucida glycoprotein matrix by cross-linking to block additional sperm penetration. This “polyspermy blocking” mechanism takes place within the first hour after initial sperm penetration and is the first noticeable event triggered by the large Ca++ release [5-8].

After that first spike, each subsequent transient has lower amplitude, and the oscillations persist at a frequency of ~2.4 per hour for an average of 4.2 hours. These transients drive two secondary fertilization events: resumption of meiosis and formation of the two pronuclei of fertilization [9,11].

Until now, the oocyte was arrested at the Metaphase II (MII) stage of meiosis by cytostatic factor, or CSF, which is an inhibitor of anaphase-promoting complex (APC). The acute rise in intracellular Ca++ activates a complex set of reactions driven by calcium/calmodulin kinase II, ultimately degrading CSF and relieving APC from inhibition [9]. Sperm decondensation processes unwind the sperm chromosomes, which recombine with the oocyte’s chromosomal complement to complete meiosis II with extrusion of a second polar body [5,12].

Energy requirements in the busily-forming zygote skyrocket temporarily. Ca++ oscillations stimulate mitochondrial respiration, producing the adenosine triphosphate (ATP) necessary to **maintain** the Ca++ waves for the appropriate length of time [3]. Additionally, after each Ca++ oscillation, intracellular Ca++ concentration must be normalized, and ER stores replenished [1].

ATPases power the calcium pumps, which allow this parallel but opposite oscillatory response to occur [11-13]. These energy needs are significant, and without sufficient Ca++ release, there will not be enough ATP produced to effectuate all the necessary processes.

Any early deficiency in the activation pathway, which causes a reduction in PLC-z, PIP2, or IP3, will summarily reduce intracellular Ca++ levels and dampen or prevent oscillations [3]. Deficiencies may arise from the sperm, such as a lack of PLC-z, or the oocyte, which may contain insufficient oocyte stores of Ca++, among other examples [14-16]. Whatever the root cause, deficiencies which result in significant deviations in Ca++ oscillations result in poor outcomes.

Assisted reproduction techniques such as in vitro fertilization (IVF) and the introduction of intracytoplasmic sperm injection (ICSI) transformed the field of assisted reproduction. It allowed couples to overcome numerous barriers to fertilization: severe male factor infertility, sperm coated with a high concentration of antisperm antibodies, immature testicular sperm, posthumously harvested sperm, or idiopathic fertilization failure [16-20]. ICSI bypasses typical sperm-oocyte membrane interaction by depositing a sperm directly into the oocyte cytoplasm. Despite this mechanical assistance, roughly 3% of IVF cases (range 1-5%) will result in total fertilization failure (TFF) despite adequate numbers of oocytes available [14,15].

Fertilization failure despite ICSI can result from the partial or complete inability of the sperm to activate oocytes [14-16]. Without activation, oocytes may be unable to decondense the injected sperm for pronucleus development [22]. Destabilization of the sperm membrane by mechanical means prior to ICSI, during immobilization, is a standard technique used to increase the chance of fertilization. Not only does it render the sperm more controllable during injection, it may allow more efficient PLC-z release into the cytoplasm for activation [8].

Certain cases of TFF may arise from deficient or dysfunctional phospholipase C zeta (PLC-z) due to male chromosome mutations [20]. PLC-z concentration is positively correlated with the frequency and amplitude of Ca++ induced, meaning that the quality and quantity of oscillations depends on the amount of PLC-z released [21-23]. Sperm with insufficient quantity or quality of PLC-z likely cannot activate oocytes properly despite ICSI. This defect is particularly marked in males with globozoospermia, where the sperm acrosome is missing, along with potentially some of the post-acrosomal region as well. ICSI attempts using these sperm produce a variable fertilization rate, ranging from 0-37% [14-16].

Artificial oocyte activation, or AOA, has significant potential to mitigate these barriers to fertilization and early embryo development. Successful fertilization with globozoospermia using calcium ionophore following ICSI has been reported [15,24]. A case was also described where calcium ionophore allowed activation and fertilization of oocytes and a successful pregnancy in a couple with fertilization failure despite normozoospermic motile sperm [25].

In another case, the male partner's sperm had oligoasthenozoospermia but intact acrosomes. The couple had long-term infertility, with a history of 3 IVF cycles and failed fertilization in a total of 17 oocytes. The female partner had mild diminished oocyte reserve (DOR). Five oocytes were retrieved in the first attempt at IVF with ICSI. Two oocytes were exposed to husband's sperm using ICSI only, 2 oocytes with husband's sperm using ICSI with AOA with calcium ionophore, and 1 with donor sperm and ICSI. Fertilization only occurred in the AOA oocytes using the husband's sperm, and a day 3 embryo was transferred [26].

Another case involved a woman with marked DOR, as evidenced by a day 3 follicle stimulating hormone (FSH) level of 64 mIU/mL, with no other factors detected associated with infertility [27]. Her first IVF cycle used conventional insemination despite the DOR for a couple reasons. Most important was that she had limited funds, and if the egg fertilized by conventional insemination, but no pregnancy, she could resume trying to get pregnant naturally with progesterone (P) support and creation of a mature dominant follicle respecting the tenets of the FSH receptor up-regulation technique [28]. The second reason was that if the eggs did fertilize, conventional insemination would lead to a high chance of pregnancy since the oocyte is able to select a good sperm for fertilization better than the embryologist and the process of ICSI may cause some damage to the oocyte.

For cycle 2, ICSI without AOA was used but none of the 3 metaphase II oocytes fertilized. It should be noted that the semen analysis appeared perfectly normal. In her third cycle IVF only 1 egg was retrieved. Not only did it fertilize with AOA by calcium ionophore plus ICSI but transfer of the day 3 embryo led to the delivery of a healthy full-term baby [27].

Presented here is a case of not only a woman with DOR, but one with advanced reproductive age, whose male partner had normal semen parameters, yet they had failed fertilization following IVF with ICSI. Her subsequent IVF cycle had successful fertilization with artificial oocyte activation using calcium ionophore and ICSI, and a viable twin pregnancy ensued.

Case Report

A 42-year-old woman married to a 48-year-old man presented with secondary infertility. She was gravida 3 para 1 (2 miscarriages). She had conceived at age 39 on her first attempt to become pregnant in a completely natural cycle and delivered a healthy full-term baby.

The couple desired a second child, and she returned at age 41. On her first attempt, she again conceived but had a miscarriage. Her third natural pregnancy occurred after 7 months of unprotected intercourse at age 42. During the 7 months preceding the pregnancy, she had tried several cycles of empirical clomiphene

citrate at another center. This third pregnancy was her first attempt after stopping clomiphene citrate. Unfortunately, it ended in another miscarriage.

She was diagnosed with DOR based on her serum anti-mullerian (AMH) level of 0.3 ng/mL [27]. When she consulted us, we discussed the option of natural cycles with correction of follicular maturation (if needed), checking post-coital tests, and treating with supplemental P [28-31]. The other option, based on her age and DOR, was to proceed with IVF-ET [29].

Her husband was 48 years old with normal semen parameters. His sperm concentration was 117.6 million / mL with 63% motility, and sperm morphology 13% based on strict criteria. We also tested his sperm hypo-osmotic swelling (HOS), which measures percentage of tail swelling upon exposure to a hypo-osmotic solution. It is common to develop a low HOS score (<50% swelling) in his age range and it can cause embryo implantation defects [32-34]. His score was normal at 67% for this as well.

The patient followed an FSH receptor up-regulation technique for IVF, and 2 metaphase II (MII) oocytes were retrieved [29]. She opted for ICSI since she felt there was less chance of failed fertilization and had very few oocytes. However, both failed to fertilize despite ICSI.

On her next IVF cycle there were 6 MII oocytes collected. Due to failed fertilization in cycle 1, we decided to try artificial oocyte activation with calcium ionophore, specifically A23187, or calcimycin. Thirty minutes after ICSI was performed, the injected oocytes were exposed to a 10 uM/L solution of A23187 for 7 minutes [27]. They were then rinsed thoroughly and placed in fresh culture drops. Four of the 6 fertilized. On Day 3, four multicell embryos were transferred into the patient. Cell stages were 11, 7, 10, and 6, with varying amounts of fragmentation (one >25%, the rest lower). Two embryos were graded as good quality. She conceived twins on that cycle, and she is presently 34 weeks pregnant. Genetic testing on both fetuses showed normal results, and normal fetal anatomy was visible on ultrasound. She was 43 years old at the time of oocyte retrieval.

Discussion

It is possible that the failed fertilization of 2 oocytes in cycle 1 was merely by chance, since the average ICSI fertilization rate is 70-75%. However, it is also possible that the 6 oocytes from cycle 2 would also have failed to fertilize by simply performing ICSI without AOA.

Advanced age of the male or female partner could affect oocyte activation potential in a minority of cases. There is very little direct evidence that DOR or advanced age in the female partner leads to decreased fertilization rates, and advanced age of the male does not significantly affect fertilization either [32,35]. However, there are compelling arguments that older patients may benefit from artificial oocyte activation, not only to fertilize oocytes but also to increase embryo quality and development. Use of ICSI-AOA in patients >40 years old produced higher cleavage rates and significantly more top-quality embryos than use of ICSI alone [36]. This suggests that while fertilization steps may be initiated after oocyte activation, there may be factors, which are inadequate to create embryos with good implantation potential.

As discussed earlier, Ca++ oscillations trigger resumption of meiosis. Oocytes from females of advanced age may contain sufficient Ca++ stores for the initial release burst but may not be able to maintain the oscillations appropriately. Oocytes from a patient of advanced reproductive age commonly have meiotic errors, which may arise from a lack of ATP to power recombination and chromosomal segregation. Moreover, patients with previous unexplained fertilization failure tend to have lower oocyte mitochondrial DNA copy numbers than patients whose TFF was due to severe male factor [13]. This strongly indicates that lack of energy production in the oocyte correlates with defective fertilization.

The pattern and amplitude of Ca++ oscillations in both in vitro matured oocytes and vitrified / warmed oocytes differs from freshly retrieved MII oocytes. Vitrification itself depolarizes oocyte mitochondria, and vitrified oocytes show decreased response to calcium ionophore A23187 [36]. In vitro matured MIIs show a characteristic pattern of Ca++ peaks, often with significantly lower frequency than their in vivo matured counterparts [11]. This lower frequency pattern persists even after injection of recombinant hPLC-z to boost activation response [12]. Adding together the decreased number of mitochondria, impaired oscillation patterns, and correlation with fertilization failure, one possible conclusion is that oocytes from women of advanced age may need augmented oocyte activation to fertilize and thus could explain why a woman at a younger age could conceive naturally but now not only requires ICSI to fertilize the oocytes but the need also of AOA. The possibility could also be that the aging of sperm, despite the normal semen parameters could lead to inability to fertilize oocytes with advancing age of the male.

Inadequate Ca++ oscillations can result in cleavage anomalies or developmental arrest as well. Murugesu et al., found significantly decreased cleavage rates reported in his meta-analysis, 68.3% cleavage rate in the AOA group vs 48.7% in the non-AOA group, which supports a possible decline in embryo development resulting from inadequate oocyte activation processes [37]. In the same analysis, blastocyst formation was also significantly blunted: 51% in the AOA group vs 10.7% in the non-AOA group. Shafqat et al., mentions that embryos derived from Ca++-devoid fertilization have observably reduced inner cell mass at the blastocyst stage [1]. These numbers and observations strongly suggest that inadequate oocyte activation may show more pronounced effects as indicators further downstream when evaluated. While we cannot compare embryo development in the two cycles, due to total fertilization failure on cycle 1, deploying AOA-ICSI immediately after total fertilization failure may have saved this patient a third IVF attempt

by augmenting embryo development.

Not all male sperm defects are readily detected using only standard semen analysis. Males with mutations leading to abnormal PLC-z conformation do not necessarily have any other semen parameters affected [20]. Occasionally, however, observations from a previous IVF cycle may help clarify potential reasons for total fertilization failure. For example, had this woman used conventional insemination in her first cycle, evaluating the sperm attachment to the zona pellucida would have provided some treatment guidance. Failure of sperm to bind to the zona in appropriate numbers could indicate, among other things, that the male patient had developed antisperm antibodies, which can coat the sperm head and prevent normal binding and subsequent penetration of the oocyte [38,39]. ICSI, without AOA, overcomes sperm-zona binding issues quite successfully [38,39].

However, failed fertilization despite high numbers of sperm bound to the zona pellucida is predictive of subsequent poor fertilization with ICSI as well [39]. In this circumstance, ICSI with artificial oocyte activation is highly recommended for future treatment. Typically, it is also standard to proceed to ICSI with AOA in cases of failed fertilization with ICSI alone, as in the case described here, although the paucity of oocytes in the first cycle makes that decision less clear. Of note, the same oocyte stimulation protocol and maturation trigger was used in both cycle 1 and 2, so a change in protocol would not solely explain the fertilization seen in cycle 2 [40]. This fact supports the claim that ICSI with AOA played a key role in the successful outcome.

ICSI-AOA treatment has led to numerous successful pregnancies and deliveries as part of IVF treatment [16,18,19,37]. Just as important, the use of calcium ionophore does not increase the risk of fetal aneuploidy, preterm delivery or low birth weight [40-42]. Even somewhat extreme oocyte activation protocols do not cause a widespread increase in meiosis II errors. In one report, donated oocytes were exposed to 100um calcium ionophore for 40 minutes and were then analyzed via array-CGH (comparative genomic hybridization) and / or single-nucleotide polymorphism genotyping. Exposed oocytes showed a similar number of meiosis II errors as control oocytes [41]. As previously noted, the case report patient herein had oocytes exposed to 10um of calcimycin for 7 minutes; this was done specifically at 30 minutes post-ICSI since that is just prior to when the first Ca++ wave should occur in a normally activated oocyte. This is a tenfold lower calcium ionophore concentration as well as only ¼ the total exposure time, so presumably the error rate would be no higher than in the tested oocytes. All fetal testing on the patient discussed herein appears normal.

The present case clearly demonstrates that successful pregnancy is possible in a woman of advanced reproductive age, with DOR and a history of fertilization failure with ICSI, by employing artificial oocyte activation with calcium ionophore. This type of precedent is important to help both patient and physician decide, in similar circumstances, whether to try another IVF cycle using the partner's sperm and patient's oocytes despite a previous IVF cycle with failed fertilization. Furthermore, although the safety of calcium ionophore treatment in IVF has been reasonably established, it is important to show its effectiveness in older patients and those with DOR, not just younger patients with better prognoses.

One final note: the female patient described herein was also treated with dextroamphetamine sulfate therapy and will remain on it until delivery. Dextroamphetamine sulfate causes release of dopamine from sympathetic nerve fibers which decrease cellular permeability and thus reduces endometrial inflammation by inhibiting natural killer cell migration into the fetal placental environment [43,44]. This drug therapy has helped multiple other infertility patients, notably one who was 46.5 years old and (unlike the patient described herein) in complete menopause to achieve a live delivery. Using ethinyl estradiol to up-regulate FSH receptors, which led to follicular recruitment, oocyte release, natural intercourse, and progesterone support in the luteal phase, along with dextroamphetamine sulfate, the patient delivered a healthy full-term baby [45-47].

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