Artificial oocyte activation with calcium ionophore A23187 in intracytoplasmic sperm injection cycles using surgically retrieved spermatozoa

Edson Borges, Jr., M.D., Ph.D., a,b,c Daniela Paes de Almeida Ferreira Braga, M.S., a,b Tatiana Carvalho de Sousa Bonetti, M.S. b Assumpito Iaconelli, Jr., M.D., a and José Gonçalves Franco, Jr., M.D., Ph.D. d,e

a Fertility-Assisted Fertilization Center, São Paulo; b Sapientiae Institute, São Paulo; c Department of Gynecology and Obstetrics, Botucatu Medical School/UNESP Botucatu; d Centre for Human Reproduction, Ribeirão Preto; and e Post Graduation Program, Department of Gynecology and Obstetrics, Botucatu Medical School/UNESP Botucatu, São Paulo, Brazil

Objective: To evaluate the effect of artificial oocyte activation (AOA) on intracytoplasmic sperm injection (ICSI) cycles using surgically retrieved sperm.

Design: Laboratory study.

Setting: Fertility/assisted fertilization center.

Patient(s): Couples undergoing surgical sperm retrieval for ICSI (n = 204).

Intervention(s): Application of calcium ionophore A23187 for AOA.

Main Outcome Measure(s): Cycles were divided into experimental groups according to the origin of the sperm used for injection and the type of azoospermia: [1] testicular sperm aspiration in nonobstructive-azoospermic patients (TESA-NOA group, n = 58), [2] TESA in obstructive-azoospermic patients (TESA-OA group, n = 48), [3] and percutaneous epididymal sperm aspiration in obstructive-azoospermic patients (PESA-OA, n = 98). For each experimental group, cycles where AOA was applied (subgroup: activation) were compared with cycles in which AOA was not applied (subgroup: control). The fertilization, high-quality embryo, implantation, and pregnancy rates were compared among the subgroups.

Result(s): For patients undergoing TESA, AOA did not improve ICSI outcomes for either type of azoospermia. However, for cases in which the injected sperm were retrieved from the epididymis, a statistically significantly increased rate of high-quality embryos was observed with AOA.

Conclusion(s): Artificial oocyte activation may improve ICSI outcomes in azoospermic patients when epididymal, but not testicular spermatozoa, are injected. (Fertil Steril 2009;92:131–6. ©2009 by American Society for Reproductive Medicine.)

Key Words: ICSI, spermatozoa, calcium, testicle, epididymis

Azoospermia, the absence of sperm from the ejaculate, is present in about 15% of infertile men (1) and is classified as either obstructive or nonobstructive. Obstructive azoospermia (OA), with normal spermatogenesis, is characterized by an occlusion or partial absence of the reproductive tract, whereas nonobstructive azoospermia (NOA) is characterized by impaired spermatogenesis (2).

Since the first report of a birth after intracytoplasmic sperm injection (ICSI) (3), this technique has been widely used to treat severe male infertility. In addition to ejaculated sperm, testicular and epididymal sperm can be used for ICSI (4, 5), resulting in high levels of fertilization and good pregnancy rates (6, 7). Although the success rates of ICSI were thought to be independent of basic sperm parameters (8–10), recent reports have suggested that repeated failures after ICSI may be caused by the effect of sperm-derived factors on preimplantation embryo development. These sperm-derived factors are referred to as paternal effects (11, 12).

The effect of the sperm source on ICSI outcome has been widely discussed. We have previously discussed how, in azoospermic patients, embryo quality depends on the origin of the injected sperm (testicle or epididymis) (13). Furthermore, it has been observed that ejaculated and epididymal spermatozoa provide better quality embryos when compared with embryos derived from testicular sperm (13). Pasqualotto et al. (14) also found that testicular sperm injection resulted in lower fertilization and pregnancy rates when compared with
epididymal sperm injection. Moreover, high abortion rates were reported when using testicular sperm (14–16). These reports all suggest that male gamete immaturity may affect the developmental competence of the resulting embryos.

In addition to the source of the sperm, the type of the azoospermia seems to affect the success of ICSI. Reports comparing ICSI outcomes between patients with OA and NOA are not in agreement. Although some suggest no difference in outcome (17, 18), most reports show significantly lower fertilization and pregnancy rates in ICSI cycles using testicular sperm from NOA patients compared with patients who have normal spermatogenesis (14, 19, 20). The fertilization rate after ICSI with testicular spermatozoa from men with impaired spermatogenesis may be reduced because the spermatozoa are less mature than those from men who have normal spermatogenesis (19). Fertilization failure after ICSI may be due to the inability of a spermatozoa to trigger oocyte activation (21).

In mammalian oocytes, an intracellular calcium rise is the signal responsible for the resumption of meiosis and the beginning of embryo development, thus playing an important role during fertilization (22–24). It has been suggested that sperm provide a soluble factor that is released into the egg upon sperm–egg fusion (25). Sperm contain a specific phospholipase C isoform, PLC-ζ, that is present at a sufficient concentration to induce calcium spiking in the egg (26).

Many studies have demonstrated that artificial oocyte activation (AOA) with a calcium ionophore may increase the free intracellular calcium (27–35), thereby mimicking physiologic cell signaling mechanisms that result in oocyte activation (36).

Recently, Nasr-Esfahani et al. (37) reported that AOA with ionomycin can improve fertilization and cleavage rates in teratozoospermic patients; however, to date, there have been few studies using AOA in ICSI cycles with sperm from patients who have different types of azoospermia. We evaluated the effect of AOA with the calcium ionophore A23187 on ICSI cycles using sperm surgically retrieved from AO or NOA patients.

MATERIAL AND METHODS
Experimental Design
The present study was performed on 204 couples undergoing surgical sperm retrieval for ICSI cycles in a private assisted fertilization center, during the period of January 2006 to July 2007. Written informed consent to share the outcomes for research purposes was obtained from all of the couples. This study was approved by the Brazilian National Committee in Ethics and Research (n.593/2007).

In OA patients, sperm were surgically acquired using percutaneous epididymal sperm aspiration (PESA). If the initial procedure failed, testicular sperm aspiration (TESA) was performed. Because the preferred procedure for the OA patients is PESA, the PESA group was composed exclusively of OA patients. The TESA group was composed of both OA and NOA patients.

The cycles were divided into three experimental groups according to the origin of the injected sperm and the type of azoospermia: [1] sperm was retrieved from the testicle of NOA patients (TESA-NOA group, n = 58), [2] sperm was retrieved from the testicle of OA patients (TESA-OA group, n = 48), and [3] sperm was retrieved from the epididymis of OA patients (PESA-OA group, n = 98).

For each experimental group, cycles in which AOA was applied (subgroup: activation) were compared with cycles in which AOA was not applied (subgroup: control). The allocation of patients to either the AOA or the control subgroup was randomized. The number of cycles in each group was TESA-NOA control: 29 cycles and activation: 29 cycles; TESA-OA control: 24 cycles and activation: 24 cycles; and PESA-OA control: 49 cycles and activation: 49 cycles.

Fertilization rate, percentage of high-quality embryos on the third day of development, implantation rate, and pregnancy rate were compared among the subgroups. Implantation rate was defined as the number of total gestational sacs divided by the total number of embryos transferred. Clinical pregnancy was defined as the presence of a gestational sac visualized by ultrasound 4 to 6 weeks after embryo transfer.

TESA and PESA Procedures
After cord block anesthesia, TESA was performed using a 21-gauge butterfly needle that was longitudinally inserted into the superior testicle pole while avoiding the epididymis. Forward and backward movements and needle direction were changed slightly to ensure parenchymal disruption for needle aspiration.

Epididymal sperm aspiration, under local anesthesia, was performed using a 27-gauge needle inserted into the epididymis. Gentle, negative pressure was applied as epididymal fluid was aspirated.

For both PESA and TESA, aspirated material was collected into a conical tube and washed with a minimum volume of modified human tubal fluid medium (HEPES; Irvine Scientific, Santa Ana, CA) at 37°C. The recovered material was checked for the presence of spermatozoa and centrifuged at 300 × g for 8 minutes. The fraction was diluted or concentrated if necessary.

Controlled Ovarian Stimulation
Controlled ovarian stimulation was achieved with a long pituitary down-regulation using a gonadotropin-releasing hormone agonist (GnRH-a, Lupron Kit; Abbott S.A. Société Française des Laboratoires, Paris, France), followed by ovarian stimulation with recombinant FSH (Gonal-F; Serono, Geneva, Switzerland). Follicular dynamics were evaluated by ultrasound starting on day 4 of gonadotropin administration. When adequate follicular growth and serum estradiol level
were observed, recombinant human chorionic gonadotropin (hCG, Ovidrel; Serono) was administered to trigger ovulation. Thirty-five hours after recombinant hCG administration, oocytes were collected by transvaginal ultrasound ovum pick-up.

Preparation of Oocytes

After retrieval, oocytes were incubated in culture medium (G-1-V3-Plus; Vitrolife) and covered with mineral oil (Ovoil; Vitrolife) for 3 hours at 37°C and 6% CO₂. Cumulus cells were removed by a 30-second exposure to HEPES-buffered medium containing 80 IU/mL of hyaluronidase (Irvine Scientific), after which coronal cells were carefully removed using finely drawn glass Pasteur pipettes (Humagen Fertility Diagnostics, Charlottesville, VA). Denuded oocytes were then assessed for nuclear status. Oocytes showing the release of the first polar body were considered mature and were used for ICSI.

ICSI and Calcium Ionophore Treatment

For ICSI, individual oocytes were placed in 4-μL droplets of buffered medium (G-Mops-V3-Plus; Vitrolife). Sperm were placed in a central 4-μL droplet of polyvinylpyrroldone solution (PVP; Irvine Scientific) in a 50 × 40 mm glass dish culture (WillCo-dish, Vineland, NJ) covered with warm mineral oil (Ovoil; Vitrolife). Sperm injection was carried out on the heated stage (37°C) of an inverted microscope (Eclipse TE 300; Nikon, Tokyo, Japan) 38 hours after recombinant hCG trigger. After ICSI oocytes were incubated in culture medium containing 5 μM of calcium ionophore A23187 (4-bromo calcium ionophore A23187–Sigma B7272, EUA) for 30 minutes at 37°C and 6% CO₂, the oocytes were then washed and placed in culture medium (G-1-V3-Plus; Vitrolife) in the incubator under the same conditions.

Assessment of Fertilization, Embryo Quality Evaluation, and Embryo Transfer

Fertilization was assessed 18 hours after ICSI, and normal fertilization was characterized by two distinct pronuclei.

Embryo quality, on day 2, was evaluated 24 hours after fertilization and every 24 hours until the transfer day using an inverted microscope (Eclipse TE 300; Nikon). The following parameters were recorded: [1] number of blastomeres, [2] fragmentation percentage, [3] variation in blastomere symmetry, [4] presence of multinucleation, and [5] defects in zona pellucida and in cytoplasm.

Embryo transfer was performed on the third day of development. High-quality embryos were defined as those showing four cells on the second day or eight cells on the third day of development that had less than 10% fragmentation, symmetric blastomeres, no multinucleation, a colorless cytoplasm with moderate granulation and no inclusions, no perivitelline space granularity, and no zona pellucida dysmorphism.

For each couple, from one to four embryos were transferred, depending on the embryo quality and the age of the woman.

Statistical Analysis

Results were expressed as mean ± standard deviation (SD) for numeric variables, and proportions (%) were used for categorical variables. Mean values were compared by Student’s t-test, and proportions were compared by the chi-square or Fisher exact test, depending on the sample size. P<.05 was considered statistically significant (the 5% critical level). Data analysis was carried out using the Minitab statistical program (version 14; Minitab, Inc., State College PA).

RESULTS

In OA patients, all general characteristics of the activation and control subgroups were statistically equal when sperm

<p>| TABLE 1 | General characteristics of the activation and control subgroups for each sperm origin group. |
|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>n</th>
<th>Female age</th>
<th>Male age</th>
<th>Follicles</th>
<th>Oocytes</th>
<th>Transferred embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>TESA-OA</td>
<td>Activation</td>
<td>24</td>
<td>33.9 ± 4.91</td>
<td>41.3 ± 7.98</td>
<td>18.9 ± 9.59</td>
<td>14.6 ± 7.99</td>
<td>2.21 ± 1.12</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>32.6 ± 5.10</td>
<td>44.3 ± 8.16</td>
<td>19.8 ± 8.29</td>
<td>14.3 ± 6.51</td>
<td>2.73 ± 1.38</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>.408</td>
<td>.238</td>
<td>.483</td>
<td>.737</td>
<td>0.208</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PESA-OA</td>
<td>Activation</td>
<td>49</td>
<td>31.6 ± 4.82</td>
<td>43.8 ± 6.97</td>
<td>19.8 ± 12.1</td>
<td>12.6 ± 8.3</td>
<td>2.51 ± 1.31</td>
</tr>
<tr>
<td>Control</td>
<td>49</td>
<td>31.7 ± 5.0</td>
<td>46.0 ± 7.09</td>
<td>20.4 ± 12.0</td>
<td>14.6 ± 8.7</td>
<td>2.51 ± 1.20</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>.255</td>
<td>.206</td>
<td>.665</td>
<td>.303</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TESA-NOA</td>
<td>Activation</td>
<td>29</td>
<td>34.0 ± 3.76</td>
<td>37.0 ± 4.57</td>
<td>17.7 ± 10.4</td>
<td>13.2 ± 7.24</td>
<td>2.36 ± 1.13</td>
</tr>
<tr>
<td>Control</td>
<td>29</td>
<td>34.0 ± 4.34</td>
<td>40.4 ± 7.73</td>
<td>18.1 ± 10.1</td>
<td>13.6 ± 7.45</td>
<td>2.50 ± 1.33</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>1.000</td>
<td>.046&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.874</td>
<td>.812</td>
<td>.710</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Values expressed in average ± standard deviation. PESA-OA, percutaneous epididymal sperm aspiration in obstructive-azoospermic patients; TESA-NOA, testicular sperm aspiration in nonobstructive-azoospermic patients; TESA-OA, testicular sperm aspiration in obstructive-azoospermic patients.

<sup>a</sup>P<.05.
were retrieved from either testicle (TESA-OA) or epididymis (PESA-OA). In the TESA-NOA patients, the general characteristics of the activation and control subgroups were similar except for the age of the men (Table 1).

The subgroups were not statistically different for fertilization rate, percentage of high-quality embryos on the third day of development, implantation rate, or pregnancy rate when testicular sperm were retrieved from both types of azoospermic patients (TESA-NOA and TESA-OA groups; Table 2).

For the cases in which the injected sperm were retrieved from the epididymis of OA patients, we observed a statistically significant increase in high-quality embryo rates when AOA was performed. However, no statistically significant difference was observed in the rates of fertilization, implantation, or pregnancy among the subgroups (Table 2).

### DISCUSSION

Artificial oocyte activation can be induced by a variety of stimuli. Combining calcium ionophore treatment with ICSI has been effective in achieving higher rates of fertilization, implantation, and pregnancy (27–31, 33, 38–40).

In addition to calcium ionophore treatment, application of other chemical agents has been reported. Yanagida et al. (41) achieved fertilization, pregnancy, and childbirth with a combination of strontium and ICSI; however, the effects of strontium and calcium ionophore exposure on gametes and embryos is unknown.

Although possible risks associated with exposing human oocytes to chemical agents may still be uncovered, calcium ionophore treatment has been widely applied in human oocytes with no evidence of toxicity. In 1997, calcium ionophore treatment was used in a clinical trial that resulted in the delivery of a healthy infant (39). In 2001, pregnancy and delivery resulted from frozen-thawed embryos after a treatment with calcium ionophore (30). In addition, successful pregnancies and deliveries after calcium ionophore oocyte activation were reported for normozoospermic patients who had repeated failed fertilization (33) and had extremely low fertilization rates (29).

A recent case study reported a successful pregnancy and delivery after ICSI with a frozen-thawed nonviable testicular sperm and AOA with a calcium ionophore (42). In another study, oocytes that were injected with the testicular sperm of a globozoospermic patient and were subsequently exposed to calcium ionophore failed to become fertilized (43). These studies are both case reports in which only one cycle was described.

Our report is a unique study that describes the effects of AOA with calcium ionophore in ICSI cycles when oocytes were injected with testicular sperm retrieved from either obstructive or nonobstructive azoospermic patients or when oocytes were injected with epididymal sperm retrieved from obstructive azoospermic patients.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Subgroup</th>
<th>n</th>
<th>Fertilization</th>
<th>High-quality embryos</th>
<th>Implantation</th>
<th>Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>TESA-NOA</td>
<td>Activation</td>
<td>29</td>
<td>47.7</td>
<td>43.7</td>
<td>11.5</td>
<td>18.5</td>
</tr>
<tr>
<td>Control</td>
<td>29</td>
<td>44.0</td>
<td>.108</td>
<td>41.3</td>
<td>10.6</td>
<td>20.8</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td>.108</td>
<td>.819</td>
<td>.901</td>
<td>1.000</td>
</tr>
<tr>
<td>TESA-OA</td>
<td>Activation</td>
<td>24</td>
<td>55.0</td>
<td>35.6</td>
<td>21.6</td>
<td>39.1</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>65.2</td>
<td>.634</td>
<td>47.0</td>
<td>18.4</td>
<td>29.2</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td>.634</td>
<td>.233</td>
<td>.746</td>
<td>.471</td>
</tr>
<tr>
<td>PESA-OA</td>
<td>Activation</td>
<td>49</td>
<td>67.0</td>
<td>64.8</td>
<td>17.2</td>
<td>35.9</td>
</tr>
<tr>
<td>Control</td>
<td>49</td>
<td>65.8</td>
<td>.787</td>
<td>48.0</td>
<td>12.0</td>
<td>29.1</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td>.787</td>
<td>.045</td>
<td>.479</td>
<td>.381</td>
</tr>
</tbody>
</table>

Note: Values expressed as percentage (%). PESA-OA, percutaneous epididymal sperm aspiration in obstructive-azoospermic patients; TESA-NOA, testicular sperm aspiration in nonobstructive-azoospermic patients; TESA-OA, testicular sperm aspiration in obstructive-azoospermic patients.

reaction analysis has revealed that PLC-zeta expression occurs in spermatids but not in the earlier steps of spermatogenesis (26). This finding suggests that PLC-zeta is expressed in both testicular and epididymal sperm. Swann et al. (45) found that even though the sperm contains PLC-zeta it is inactive until it is introduced into the oocyte. Indeed, it was suggested that the activation of PLC-zeta is not a passive process but depends upon the interaction with factors present in the ooplasm (46).

The reason why AOA did not improve the outcome of ICSI cycles using testicular sperm may be explained by the male germ cell maturity status. One hypothesis is that the oocyte activation–sperm factor PLC-zeta may be at a different concentration or may have an impaired activation capacity in immature spermatozoa.

Fertilization and pregnancy rates achieved after testicular sperm injection (15) were lower than those achieved with sperm injection. This discrepancy raises the question of whether the sperm maturation in the epididymis is important for the spermatozoan competence. It has been reported that male germ cell maturation is not only responsible for the transformation of spermatozoon from an immotile, infertile state to a vigorously active cell (47), it also involves the fade of transient organelles (48). Although it has been reported that the PLC-zeta is expressed in the spermatid stage (26), whether it is able to be released and activated in immature or partially mature sperm has yet to be determined.

Although the increase of free intracellular calcium can be induced by extra physiologic stimulus (49–51), repetitive calcium oscillations after the initial intracellular surge are critical for successful oocyte activation in mammals. Nearly all parthenogenetic activation agents working via calcium increase fail to cause oscillations (45). These oscillations are the key event leading to fertilization and further embryonic development. We suggest that a testicular spermatozoon cannot maintain calcium oscillations in the ooplasm after the artificial stimulation of oocyte activation.

It has been demonstrated that AOA can promote a rise in intracellular calcium concentration, resulting in higher fertilization rates. In our present study, however, we observed that when epididymal sperm were injected further embryonic development beyond fertilization was also positively affected by AOA. Calcium oscillations may participate in long-term embryonic events that go beyond their role as the stimulus for meiotic resumption (52, 53).

It was reported that differences in the calcium signaling pattern can have effects not only on implantation and postimplantation development, but also on fetal morphology (53) and weight variation in offspring (54). The exact mechanism by which intracellular calcium influences embryonic development is not completely understood. Nevertheless, it has been hypothesized that the calcium oscillation pattern may partially act through the regulation of gene expression (53, 55).

Even though it was observed that the fertilization rate was increased when AOA was performed, especially when sperm were retrieved from OA patients, it was not a statistically significant difference; a greater number of patients may be necessary to show a statistically significant difference.

The improvement in the proportion of high-quality embryos observed in our present study does not guarantee improvements in implantation, pregnancy, or birth rates. Whether events after transfer may be affected by ionophore exposure in azoospermic patients was still not determined. However, our results have demonstrated for the first time that AOA might be useful in improving ICSI outcomes in azoospermic patients when epididymal, but not testicular spermatozoa, are injected, independent of the type of azoosperma.

REFERENCES


