# Artificial oocyte activation with calcium ionophore does not cause a widespread increase in chromosome segregation errors in the second meiotic division of the oocyte

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**Objective:** To study the effect of artificial oocyte activation (AOA) on chromosome segregation errors in the meiotic divisions.
**Design:** Prospective cohort study with historical control.
**Setting:** Private/academic IVF centers.
**Patient(s):** Fifty-six metaphase II oocytes were donated from 12 patients who had undergone IVF between June 2008 and May 2009.
**Intervention(s):** Oocytes were activated by 40 minutes' exposure to 100 $\mu$M calcium-ionophore. The activated oocyte was tubed and analyzed by array comparative genomic hybridization and/or single-nucleotide polymorphism genotyping and maternal haplotyping (meiomapping). A control sample of embryos derived from normally fertilized oocytes was included for comparison.
**Main Outcome Measure(s):** Incidence of chromosome segregation errors in artificially activated and normally fertilized oocytes in relation to pronuclear evaluation.
**Result(s):** Of 49 oocytes that survived the warming procedure, thirty-nine (79.6%) activated. Most activated normally, resulting in extrusion of the second polar body and formation of a single or no pronucleus (2PB1PN: 30 of 39, 76.9%; or 2PB0PN: 5 of 39, 12.8%). Twenty-seven of these were analyzed, and 16 (59.3%) were euploid, showing no effect of AOA on meiotic segregation. Single-nucleotide polymorphism analysis of normally activated oocytes confirmed normal segregation of maternal chromosomes. No difference in the proportion of meiosis II type errors was observed between artificially activated oocytes (28.6%; 95% confidence interval 3.7%–71.0%) compared with embryos obtained from normally fertilized oocytes (44.4%; 95% confidence interval 13.7%–78.8%). The abnormally activated oocytes, with $\geq$2PN (4 of 39, 10.3%) were diploid, indicating a failure to coordinate telophase of meiosis II with polar body extrusion.
**Conclusion(s):** From this preliminary dataset, there is no evidence that AOA causes a widespread increase in chromosome segregation errors in meiosis II. However, we recommend that it be applied selectively to patients with specific indications. (Fertil Steril® 2016;105:807–14. ©2016 by American Society for Reproductive Medicine.)
**Key Words:** Calcium ionophore, chromosome segregation, fertilization failure, meiosis, oocyte activation

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Received September 15, 2015; revised November 2, 2015; accepted November 9, 2015; published online December 1, 2015.
A.C. has nothing to disclose. C.S.O. has nothing to disclose. D.K.G. has nothing to disclose. F.M.U. has nothing to disclose. A.H.H. has nothing to disclose. L.R. has nothing to disclose.
A.C. and C.S.O. should be considered similar in author order.
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Fertility and Sterility® Vol. 105, No. 3, March 2016 0015-0282/$36.00
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http://dx.doi.org/10.1016/j.fertnstert.2015.11.017

Failure of fertilization resulting in few or no embryos for transfer continues to be a significant clinical challenge for a minority of patients undergoing IVF. The introduction of intracytoplasmic sperm injection (ICSI) in the early 1990s significantly improved the clinical outcome for patients with male factor infertility, particularly those with low sperm counts, who could not achieve normal levels of fertilization with conventional IVF (1). Typical fertilization rates with ICSI average 70% for most patients, including those with poor semen parameters or surgically retrieved sperm. However, a significant proportion of ICSI cycles still results in fertilization rates below 50% (2), with between 1% and 4% resulting in total failed fertilization (TFF) (3–5). Although ICSI is invasive, and operator-dependent factors may contribute to the proportion of oocytes not fertilizing normally, most commonly the failure of an oocyte to fertilize after sperm injection is failure of oocyte activation (6–8). In most mammals the mature ovulated oocyte is arrested in metaphase of the second meiotic division (meiosis II) until fertilization by a sperm. Sperm binding with the oolemma activates the oocyte, triggering a series of pulsatile increases in intracellular calcium concentration, which in turn results in the resumption and completion of meiosis II, extrusion of the second polar body (PB2), and the initiation of preimplantation development (9). Phospholipase C, zeta 1 (PLCζ), a sperm-specific phospholipase, is considered the trigger for the molecular pathway within the oocyte, resulting in the release of calcium stores from the endoplasmic reticulum (10). Recent studies have shown that fertilization failure after ICSI can be linked to sperm devoid of PLCζ or sperm with abnormal PLCζ function (11–13). It has also been demonstrated that oocyte factors as well as sperm factors are involved in failed fertilization after ICSI (14).

Artificially increasing intracellular calcium with a variety of stimuli, from a brief exposure to low concentrations of ethanol to calcium ionophore exposure to allow the influx of calcium ions from the medium, triggers oocyte activation in several mammalian species (15, 16). Artificial oocyte activation (AOA) can be induced by electrical stimulation (17, 18) and a variety of chemical substances. Most commonly, AOA is induced by chemical agents, including 6-dimethylaminopurine, strontium chloride, or calcium ionophores, such as ionomycin and calcimycin. Exposure to a medium containing a calcium ionophore is the most commonly used method for AOA in clinical trials.

In assisted conception, AOA with calcium ionophore has been used clinically in cases of failed fertilization after ICSI, resulting in completion of normal fertilization in a significant proportion of oocytes and live births after ET (19). Indeed, there is evidence to show that AOA can overcome both oocyte- and sperm-related failed fertilization (20).

However, information on the effect of AOA and its biosafety is limited to clinical follow-up of a small number of children conceived using the technique, which demonstrated that their early development is within the expected normal range (21). Additionally, because of the abnormal, sustained increase in intracellular calcium concentration, which may have effects on downstream molecular events, it has been argued that AOA should only be used in failed fertilization cases and not as a routine adjuvant to ICSI (22) or when a specific indication is present, such as globozoospermia (23) or PLCζ deficiency (11–13).

To address the limited information on biosafety, particularly in terms of potential genetic effects, here we have investigated the effect of AOA with calcium ionophore on the incidence of female meiotic errors resulting in abnormal chromosome copy number, or aneuploidy, in the activated oocytes. Chromosome copy number was analyzed by array comparative genomic hybridization (aCGH) and combined with genome-wide single-nucleotide polymorphism (SNP) genotyping of the oocyte donors and oocytes to identify the meiotic origin of any chromosome segregation errors, with a specific focus on errors occurring in the second meiotic division (meiosis II). Because all of the oocytes in the study had completed the first meiotic division (meiosis I), extruded the first polar body (PB1), and were arrested in metaphase of meiosis II, before activation, any effect of exposure to calcium ionophore should only affect the segregation of chromosomes at anaphase of meiosis II after resumption of meiosis.

## MATERIALS AND METHODS

### Patients and Ethical Approval

All oocytes for the study were obtained from 12 patients who had undergone IVF treatment at the Center for Reproductive Medicine GENERA in Rome between June 2008 and May 2009 (Table 1). According to Italian law at the time of the patient's IVF cycles, a maximum of three oocytes could be inseminated per patient, and any surplus mature oocytes were vitrified. Surplus vitrified oocytes were later recruited for the study after informed consent was obtained from the patients. Consent was also obtained from all donors to obtain buccal cell swabs for genotyping. The study was approved by the institutional review board of the Clinica Valle Giulia, where the oocytes were stored and processed for the study.

Cleavage-stage embryos derived from normally fertilized oocytes and analyzed with the same meiomapping method (24) were used for comparison with chromosome analysis of artificially activated oocytes. The limited number of oocytes available for activation and restrictions on creating embryos for research purposes in their country of origin prevented analysis of a normally fertilized control group using oocytes from the same donors.

### Oocyte Collection, Vitrification, and Warming

Ovarian hyperstimulation was achieved using long down-regulation agonist or standard antagonist protocols, and transvaginal oocyte collection was performed 35 hours after hCG administration. The vitrification and warming procedures were performed according to a published protocol (25), using commercially available vitrification and warming kits (Kitazato BioPharma). Vitrification was performed a maximum of 40 hours after hCG administration, and the oocytes were stored on Cryotop vitrification tools (Kitazato BioPharma) in liquid nitrogen.

TABLE 1

Details of cycles with vitrified oocytes.

| Patient | Etiology | Stimulation protocol | Maternal age at oocyte pickup/vitrification (y) | No. oocytes collected | No. oocytes vitrified | No. oocytes donated | Live birth from cohort? |
|---|---|---|---|---|---|---|---|
| 1 | Male factor | Agonist | 33.2 | 12 | 2 | 2 | Yes |
| 2 | Endometriosis | Agonist | 37.9 | 10 | 7 | 5 | Yes |
| 3 | Idiopathic | Agonist | 37.4 | 10 | 10 | 4 | No |
| 4 | Male factor | Antagonist | 40.6 | 12 | 6 | 5 | Yes |
| 5 | Male factor | Antagonist | 37.6 | 15 | 10 | 10 | Yes |
| 6 | Male factor and tubal | Agonist | 37.3 | 11 | 6 | 3 | Yes |
| 7 | Male factor | Agonist | 35.7 | 11 | 5 | 5 | No |
| 8 | Tubal | Agonist | 38.4 | 16 | 9 | 6 | Yes |
| 9 | Male factor | Agonist | 29.0 | 12 | 6 | 6 | Yes |
| 10 | Male factor | Agonist | 31.7 | 14 | 5 | 4 | Yes |
| 11 | Male factor | Agonist | 39.0 | 18 | 18 | 3 | No |
| 12 | Male factor | Agonist | 36.2 | 6 | 3 | 3 | Yes |
| Mean | | | 36.2 | 12.3 | 7.3 | 4.6 | |
| SD | | | 3.3 | 3.2 | 4.2 | 2.1 | |
| Range | | | 29.0–40.6 | 6–18 | 2–18 | 2–10 | |

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## Oocyte Culture and Activation

All oocyte culture was performed at 37°C in 6% $CO_2$ and 5% $O_2$. Individual oocytes were cultured separately, and culture drops and wells were numbered to allow traceability throughout the experiment. Immediately after warming, the surviving oocytes were moved to 35-$\mu$L microdrops of cleavage medium plus 10% human serum albumin under mineral oil (Sage; Cooper Surgical) and cultured for 2 hours before activation. Oocytes were activated by exposure to 100 $\mu$M calcium ionophore (A23187; Sigma-Aldrich) in cleavage plus 10% human serum albumin from a stock solution in dimethyl sulfoxide (Sigma-Aldrich) diluted 1:40. Oocytes were transferred to 35-$\mu$L drops of the activation medium under oil, for 40 minutes. The oocytes were then moved, after thorough washing, to separate wells of multiwell slides (Unisence Fertilitech) in cleavage medium under oil (medium as used directly after oocyte warming). The slides were placed in the time-lapse incubator (Embryoscope; Unisence Fertilitech) for assessment of PB2 extrusion and appearance of pronucleus (PN). Oocytes showing the extrusion of the second PB and one PN were considered as normally activated.

## Oocyte Isolation and Tubing

The zona pellucida was removed from activated oocytes, and the polar bodies were isolated by micromanipulation (Narishige) on an inverted microscope (Nikon) equipped with Hoffman Modulation contrast and a 37°C heated stage (Linkam Scientific Instruments), as previously described (26). Oocytes were secured by suction with the holding pipette (TPC), and a large aperture was made in the zona pellucida with a series of laser (Saturn laser; Research Instruments) pulses. The aspiration pipette (Zona drilling pipette; TPC) was then inserted through the opening and the polar bodies removed with gentle suction. The oocyte was then removed from the zona by both displacement and zona manipulation techniques using the aspiration pipette as detailed in Figure 1. Once free from the zona, the oocytes were washed and transferred to polymerase chain reaction (PCR) tubes, ensuring that the polar bodies did not contaminate the samples. Transfer of the oocytes to PCR tubes was performed using a plastic denuding pipette (COOK Medical) with a 130-$\mu$m lumen. Individually labeled PCR tubes (Cell Projects) were primed with 2 $\mu$L Dulbecco's phosphate-buffered saline (Gibco; Life Technologies) with 0.1% poly vinyl alcohol (Sigma-Aldrich). Individual oocytes were expelled into the Dulbecco's phosphate-buffered saline in approximately 1 $\mu$L of the medium containing the samples. The PCR tubes were then briefly centrifuged, snap frozen in liquid nitrogen, and stored at −20°C before whole-genome amplification.

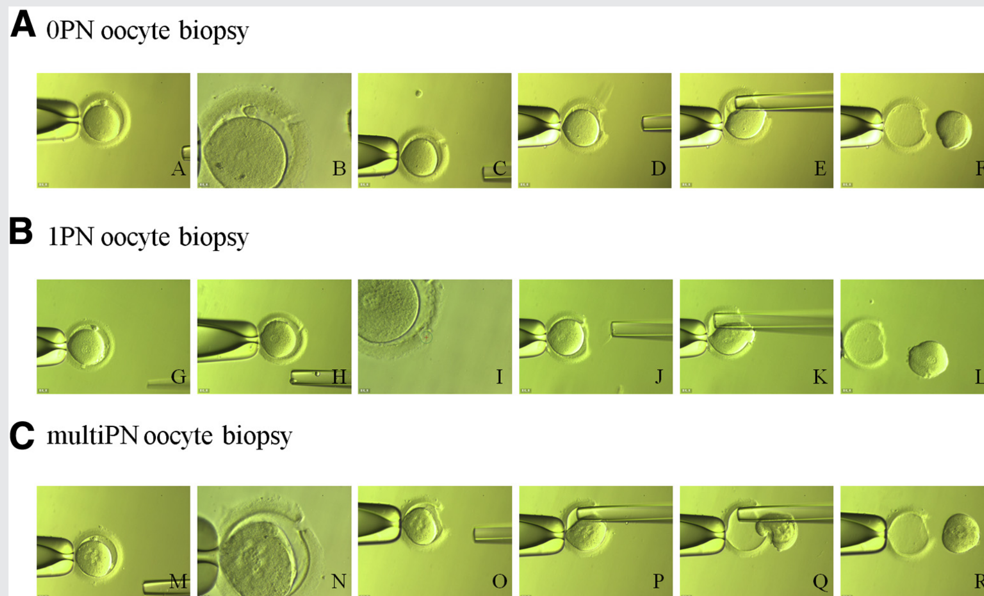
## Whole-genome Amplification and Genomic DNA Extraction

Deoxyribonucleic acid from all oocytes in the study were amplified by either multiple displacement amplification (MDA) (REPLI-g Single Cell Kit; Qiagen) or PCR library-based whole-genome amplification (WGA) (SurePlex; Illumina) according to the manufacturer's instructions, to obtain sufficient DNA for downstream analysis. Multiple displacement amplification was performed with a short, 2-hour incubation. Genomic DNA from all oocyte donors was obtained using buccal cell swabs (Isohelix; Cell Projects). Extraction of the genomic DNA from the buccal cells was performed using a proteinase K extraction kit to a final volume of 30 $\mu$L, following the manufacturer's instructions (Isohelix; Cell Projects).

## aCGH and SNP Genotyping

When feasible, both aCGH and SNP genotyping were performed on each sample. For aCGH analysis, 4-$\mu$L aliquots of WGA products from the oocytes were processed on 24Sure microarray slides (Illumina) according to the manufacturer's instructions. The data were imported and analyzed using

FIGURE 1




Isolation by displacement of activated oocytes showing either 0 (**A**), 1 (**B**), or multiple PN (**C**).

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dedicated software (BlueFuse Multi v 4.0; Illumina). Genomic DNA (400 ng) or 8 $\mu$L of WGA products from the oocyte samples were processed on SNP genotyping BeadChips for approximately 300,000 SNPs genome-wide (HumanCytoSNP-12 or HumanKaryomap-12; Illumina), according to the manufacturer's instructions. The genotype data were exported as a Micrsoft Excel file, using genotyping software (GenomeStudio; Illumina) for analysis.

## SNP Analysis

To detect aneuploidies of meiotic origin, each of the patients donating oocytes were genotyped, and informative maternal heterozygous SNP loci were phased by reference to either a presumed haploid sibling oocyte (or PB2 if no sibling oocyte was available). In cases in which the reference was itself aneuploid for a particular chromosome, a second reference was also used to confirm the status of the chromosome(s) involved. Mendelian analysis of the genotype of each of the activated oocytes at these informative SNP loci then allowed the identification of meiotic errors resulting in two chromatids instead of the normal single chromatid segregating to the oocyte (chromatid gain) by the presence of heterozygous regions. Furthermore, the distribution of these heterozygous regions allows the classification of these errors into [1] those that occur in the first meiotic division (meiosis I) and have chromatids from both homologous chromosomes, which result in heterozygosity in the pericentromeric and more distal regions of the chromosome arms; and [2] errors that occur in the second division (meiosis II) and have chromatids from the same homolog, which result in homozygosity in the pericentromeric region of the chromosome but are heterozygous in more distal regions. Finally, the absence of any informative maternal SNPs (chromatid loss) indicates the absence of a chromosome, and thus the meiotic origin of losses cannot be determined with this methodology. Therefore, chromatid loss in the oocyte could not be used for the study.

## Statistical Analysis

Continuous data and categorical variables are presented as mean and percentage frequency with standard deviations and 95% confidence interval (CI), respectively. Fisher's exact test was used to compare categorical variables, and $\alpha$ was set at 0.05.

# RESULTS

## Artificial Oocyte Activation

Fifty-six oocytes arrested at metaphase of meiosis II, which had been cryopreserved by vitrification from 12 patients with a mean ($\pm$SD) age of 36.2 $\pm$ 3.3 years, most of whom had pregnancies and live births after successful IVF treatment, mainly for male factor infertility, were donated for the study (Table 1). Forty-nine (88%; 95% CI 75.9%–94.8%) survived thawing, and 39 (79.6%; 95% CI 65.6%–89.8%) activated after exposure to calcium ionophore, as demonstrated by the formation of one or more PN and/or the extrusion of the PB2 (Supplemental Table 1, available online). Most of the activated oocytes extruded the PB2 and formed a single PN (2PB1PN: 30 of 39, 76.9%; 95% CI 76.9%–88.9%) or no visible PB (2PB0PN: 5 of 39, 12.8%; 95% CI 4.3%–27.4%), as expected. However, three (7.7%; 95% CI 1.6%–20.9%) formed two PNs (2PB2PN). Finally, one activated oocyte (2.6%; 95% CI 0%–13.5%) failed to extrude the PB2 and

formed a single normal-sized PN with several smaller pronuclei (1PB>2PN). For all activated oocytes, polar bodies were removed, and the oocyte cytoplasm was successfully isolated and tubed for chromosome analysis (Fig. 1).

## Chromosome Copy Number Analysis

Of the 39 activated oocytes, 31 (79.5%, 95% CI 63.5%–90.7%) successfully amplified and passed the quality control for chromosome analysis (Table 2). Chromosome copy number was analyzed in 31 activated oocytes (2PB1PN or 2PB0PN, n = 27; 2PB2PN, n = 3; 1PB>2PN, n = 1) by aCGH (n = 26) and/or SNP genotyping (n = 25) (Table 2). In total, 20 of 31 activated oocytes were analyzed by both aCGH and SNP genotyping, 6 of 31 were analyzed by aCGH only, and 5 of 31 were analyzed by SNP genotyping only (Table 2). Overall, 41.9% (13 of 31; 95% CI 24.5%–60.9%) of the activated oocytes had one or more chromosome copy number abnormalities (mean 2.0 per aneuploid oocyte; range, 1–7). In the 10 aneuploid activated oocytes analyzed by both aCGH and SNP genotyping, all 17 whole chromosome aneuploidies were detected by the two methods (Table 2). Among the 27 normally activated oocytes (2PB1PN), 16 (59.3%; 95% CI 38.8%–77.6%) were euploid, showing no effect of artificial activation on chromosome segregation in meiosis II. In relation to the number of pronuclei, 62.5% (15 of 24; 95% CI 40.6%–81.2%) of 2PB1PN and 33.3% (1 of 3; 95% CI 0.8%–90.57, not significant) of 2PB0PN activated oocytes were euploid. On a per-chromosome basis, 603 chromosomes segregated normally during meiosis II after activation in normally activated oocytes out of 621 chromosomes analyzed (97.1%; 95% CI 95.5%–98.3%), 94.2% (65 of 69) for 2PB0PN oocytes and 97.5% (538 of 552) for 2PB1PN oocytes.

## SNP Analysis of Meiotic Errors

Single-nucleotide polymorphism analysis of 24 activated oocytes, which extruded the PB2 and formed a single PN as expected (2PB1PN), or which failed to form a visible PN (2PB0PN), were all shown by SNP genotype analysis to have a haploid set of maternal chromosomes, with the exception of the aneuploid chromosomes. Of the seven chromosome gains identified by SNP genotype analysis, five had patterns of heterozygosity characteristic of meiosis I type errors (i.e., including the pericentromeric regions), and only two

**TABLE 2**

**Aneuploidies identified in normal and abnormal activated oocytes.**

| Patient | Oocyte ID | aCGH analysis | SNP genotyping analysis | No. of polar PB, PN | Amplification type |
|---|---|---|---|---|---|
| Normally activated (2PB1PN and 2PB0PN) | | | | | |
| 1 | 1.1 | −5, −10, +20 | −5, −10, +20 | 2PB, 1PN | SurePlex |
| | 1.2 | +11, −21 | +11, −21 | 2PB, 1PN | SurePlex |
| 2 | 2.1 | +22 | +22 | 2PB, 1PN | SurePlex |
| | 2.3 | +1 | +1[a] | 2PB, 1PN | SurePlex |
| | 2.5 | Euploid | Euploid | 2PB, 1PN | SurePlex |
| 4 | 4.1 | −4 | −4 | 2PB, 1PN | SurePlex |
| | 4.2 | Euploid | Euploid | 2PB, 1PN | SurePlex |
| | 4.3 | Euploid | Euploid | 2PB, 1PN | SurePlex |
| | 4.4 | Euploid | Euploid | 2PB, 1PN | SurePlex |
| 5 | 5.1 | Euploid | N/A | 2PB, 1PN | SurePlex |
| | 5.2 | −13 | N/A | 2PB, 1PN | SurePlex |
| | 5.3 | Euploid | N/A | 2PB, 1PN | SurePlex |
| 6 | 6.2 | Euploid | Euploid | 2PB, 1PN | MDA |
| | 6.3 | Euploid | Euploid | 2PB, 1PN | MDA |
| 7 | 7.3 | Euploid | Euploid | 2PB, 1PN | MDA |
| | 7.4 | −4 | −4 | 2PB, 1PN | MDA |
| 8 | 8.1 | −6, −18, +20 | −6, −18, +20 | 2PB, 1PN | MDA |
| | 8.2 | Euploid | Euploid | 2PB, 1PN | MDA |
| | 8.3 | Euploid | Euploid | 2PB, 1PN | MDA |
| 9 | 9.1 | N/A | Euploid | 2PB, 1PN | MDA |
| 10 | 10.1 | N/A | −17 | 2PB, 1PN | MDA |
| | 10.2 | N/A | Euploid | 2PB, 1PN | MDA |
| | 10.3 | N/A | Euploid | 2PB, 1PN | MDA |
| | 10.4 | N/A | Euploid | 2PB, 1PN | MDA |
| Normally activated 2PB0PN | | | | | |
| 2 | 2.2 | ++15 | +15 | 2PB, 0PN | SurePlex |
| 3 | 3.1 | Euploid | Euploid | 2PB, 0PN | SurePlex |
| 7 | 7.2 | −13, +20, −22 | −13, +20,[a] −22 | 2PB, 0PN | MDA |
| Abnormally activated | | | | | |
| 2 | 2.4 | −13 | −13 | 2PB, 2PN | SurePlex |
| 6 | 6.1 | Euploid | N/A | 1PB, >2PN | MDA |
| 7 | 7.1 | Euploid | N/A | 2PB, 2PN | MDA |
| 11 | 11.1 | +1, +4, +15, +16, +17, −18, −22 | N/A | 2PB, 2PN | SurePlex |

*Note:* aCGH = array comparative genomic hybridization; N/A = not available; PB = polar body; PN = pronucleus; SNP = single-nucleotide polymorphism.
[a] Aneuploidies occured following a meiosis two segregation error.

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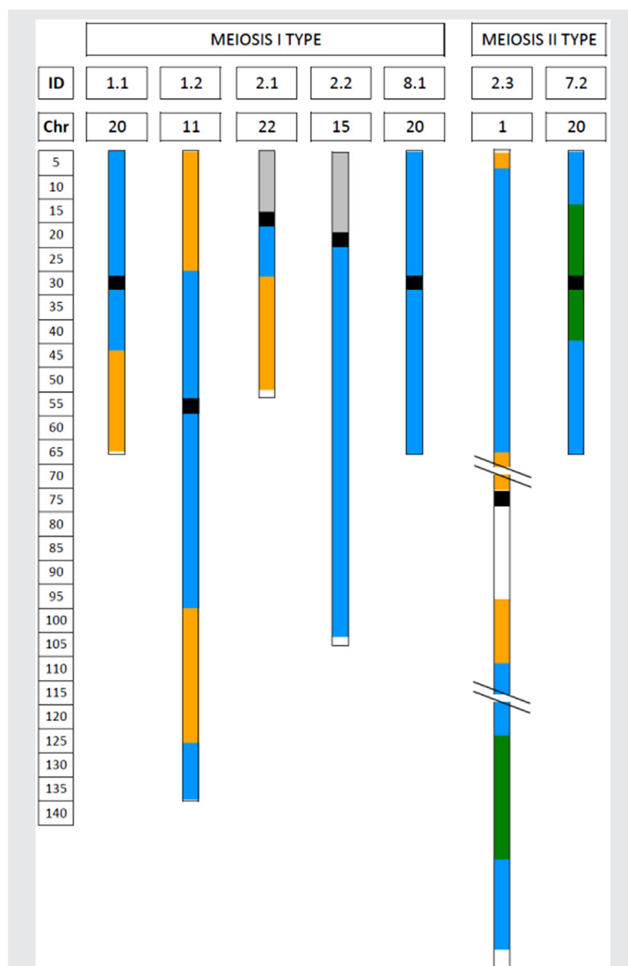
## FIGURE 2




Diagrammatic representation of all chromosome gains from oocytes demonstrating single or no PN formation with the extrusion of the PB2 after activation. Homozygous regions of the chromosomes are colored *yellow* or *green* (depending on the maternal haplotype present) and heterozygous regions in *blue* (both maternal haplotypes present). The centromeres are shown in *black*, and satellite DNA is colored *grey*. The scale bar to the left denotes the megabase pair (Mbp) position along the chromosomes. The gains present with either pericentromeric heterozygosity (*yellow* or *green* around the centromere) as MI errors or pericentromeric homozygosity (*blue* around the centromere) as MII errors.

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(28.6%; 95% CI 3.7%–71.0%) had patterns consistent with meiosis II type errors (Fig. 2, Table 2). No significant differences of the proportion of meiosis II type errors were observed when comparing these data from artificially activated oocytes with embryos obtained from normally fertilized oocytes (4 of 9, 44.4%; 95% CI 13.7%–78.8%; $P=.6$) from patients of similar female age (37.1 $\pm$ 2.3 years) and analyzed using the same meiomapping method (24).

Overall, these data showed a maximum estimated rate of chromosome errors in meiosis II of 1.9% per chromosome (12 of 621, 1.9%; 95% CI 1.0%–3.3%) and of 7.7% (2 of 26; 95% CI 0.9%–25.1%) on a per-oocyte basis in artificially and normally activated oocytes, respectively.

The remaining abnormally activated oocyte analyzed by SNP genotyping, which had extruded the PB2 and formed two PNs (2PB2PN), was shown to be diploid (digynic), with patterns of heterozygosity consistent with the presence of both sets of meiosis II chromosomes. The only exception was chromosome 13, which had only a single copy consistent with the loss observed with aCGH (Supplemental Fig. 1A). Similarly, the aCGH plot of another oocyte that formed two PNs (2PB2PN) (oocyte 11.1) and had two chromosome losses (chromosomes 18 and 21) was consistent with a single copy for these chromosomes, indicating a diploid (digynic) aneuploid oocyte (Supplemental Fig. 1B).

## DISCUSSION

Artificial activation of oocytes by exposure to calcium ionophore is being used increasingly to overcome low or failed fertilization after ICSI, and there are now several reports of pregnancies and healthy live births (21, 27, 28). In this preliminary study of potential genetic effects, exposure of oocytes arrested in meiosis II to calcium ionophore and chromosome copy number analysis of normally activated oocytes demonstrated the presence of aneuploidies of maternal meiotic origin (11 of 27, 40.7%). However, the incidence of aneuploidy was comparable to those in a small sample of normally fertilized embryos and in published studies of women of a similar age range (24, 29). The limited number of oocytes available for activation and restrictions on creating embryos for research purposes in their country of origin prevented analysis of a normally fertilized control group using oocytes from the same donors. Furthermore, analysis of informative heterozygous maternal SNP loci by meiomapping showed that five of the seven chromosome gains in the activated oocytes had patterns of heterozygosity characteristic of segregation errors in the first meiotic division (meiosis I) (i.e., including the pericentromeric region of the chromosomes), and only two had patterns consistent with meiosis II type segregation errors (Fig. 2, Table 2). This lower level of meiosis II errors compared with meiosis I errors is in line with studies on meiotic errors after IVF only (26). Our preliminary conclusion, therefore, is that AOA with calcium ionophore does not cause a widespread increase in chromosome segregation errors in meiosis II.

In contrast, SNP analysis of an activated oocyte, which had extruded the PB2 but then formed two PNs (2PB2PN), clearly demonstrated that all chromosomes had a pattern of heterozygosity consistent with the presence of both sets of meiosis II chromosomes and was therefore diploid (digynic). The exception was chromosome 13, which had no regions of heterozygosity, indicating the presence of a single chromosome and consistent with the log2 ratio observed by aCGH (Supplemental Fig. 1A). Notably, aCGH does not detect the overall ploidy of a cell because it normalizes copy number across the genome for comparison with individual chromosomes. Because this aCGH pattern of putative single loss (and single gain) from diploid copy number was observed in a second oocyte (Supplemental Fig. 1B), it is likely therefore that all activated oocytes in our data set with two or more

PNs were similarly digynic. This would suggest that the main risk after AOA with calcium ionophore is failure to coordinate telophase of meiosis II with extrusion of the PB2, resulting in retention of both chromosome sets in the oocyte (4 of 39, 10%). Thus, in clinical practice, these data suggest that a careful examination for PB2 extrusion and PN formation, possibly by time-lapse imaging, is essential to avoid the transfer of digynic triploid embryos after AOA. Similarly, after failed fertilization by ICSI and AOA, measures should be taken to avoid transfer of digynic parthenotes that appear morphologically identical to normally fertilized zygotes (2PB2PN) and may continue through cleavage. One possibility would be to analyze both polar bodies by meiomapping (24). However, this excludes analysis of any paternal and mitotic aneuploidies. More comprehensively, karyomapping or any other comprehensive chromosome screening (CCS) technology able to provide information also about the ploidy state of the cells could be used in trophectoderm biopsies at the blastocyst stage in a more effective way.

In a more general context, these data emphasize the importance of performing further evaluation of fertilized eggs after regular IVF and showing abnormal pronuclear patterns (including the presence of micro-pronuclei) with the use of new technologies that are able to give a clear picture of chromosome segregation errors during female meiosis and in embryos. Even if usually discarded from IVF cycles, 0PN or 1PN oocytes that progress to blastocyst have reproductive potential. Cytogenetic analysis of embryos from fertilized oocytes with one or no visible pronucleus (1PN or 0PN) has shown that a considerable proportion are diploid (30). Furthermore, normal diploid embryonic stem cell lines were successfully generated from 0PN or 1PN zygotes growing to the blastocysts stage (31, 32). Finally, the transfer of such embryos has resulted in healthy births (33, 34). Thus, the limited evidence suggests that 0PN or 1PN oocytes that go on to cleave and progress to blastocyst stage may be considered suitable for replacement if others are not available.

Our data on AOA also suggested a normal haploid chromosomal complement in activated oocytes when no visible pronucleus was identified. In our analysis a 2PB0PN accounted for 13% of the activated oocytes, a clinically significant proportion. Accordingly, the possibility to control for chromosome abnormalities with the use of accurate and reliable aneuploidy testing technologies at the blastocyst stage represents a practical and novel way to avoid discarding potentially viable embryos from IVF cycles simply because of an atypical pattern of pronuclei.

Although this preliminary evidence suggests that AOA is likely not to affect oocyte aneuploidy, we do not recommend it to be applied as routine practice in IVF to generally increase fertilization rates. More data are needed to corroborate these preliminary results on meiotic aneuploidy rates after AOA, and ideally a control group of normally fertilized oocytes is needed for a more powerful comparison with artificial activation. Furthermore, it should be highlighted that the oocytes used in this study were mostly from patients who had a successful pregnancy outcome using sibling oocytes from the same stimulation (Table 1). Finally, the protocol used for activation here involved prolonged exposure (40 minutes) to higher concentrations of calcium ionophore (100 $\mu$M) than are currently used clinically, to ensure a high level of activation (39 of 49, 80%). From a clinical perspective, we agree with the recent cautionary note suggesting that further validation is necessary for the clinical use of AOA (22). Until such time that the bio-safety of AOA is ascertained, its clinical application should be limited to patients with a known PLCζ deficiency (11–13), globozoospermia (23), or patients with a clinically relevant history of TFF after ICSI, even though the exact patient population with TFF that will benefit from such application needs still to be defined. Indeed, there is direct evidence from our data suggesting that the clinical application of AOA may not benefit all infertility patients. Although using our activation protocol we were able to achieve a consistently high rate of normal oocyte activation in the majority of our donors, one outlier (patient 9) had a low activation rate of 1 of 6 (17% activation; Supplemental Table 1). This supports previous reports demonstrating that AOA is not beneficial for all patients to maximize fertilization rates (2, 35).

In conclusion, it is of vital importance to extend this study to normal clinical AOA protocols to analyze the incidence and meiotic origin of aneuploidies in normally and abnormally activated oocytes and the resulting embryos with the use of suitable genetic technologies (36).

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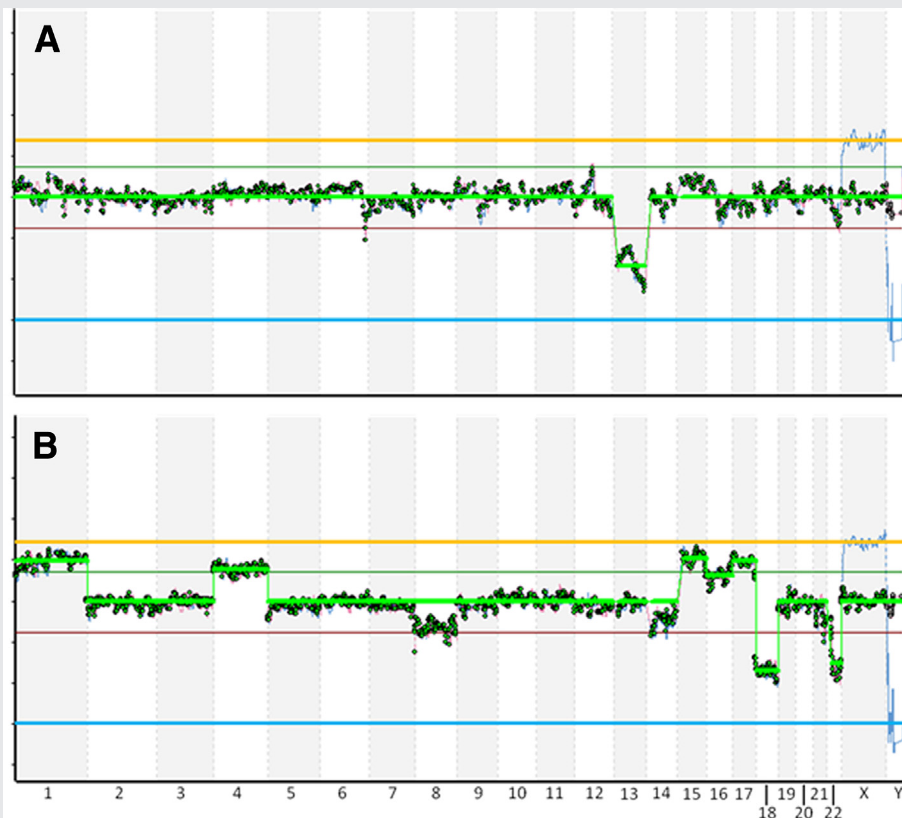
## SUPPLEMENTAL FIGURE 1




Array CGH plots of two activated diploid oocytes displaying two pronuclei, consistent with diploid state. This can be determined by the single chromatid loss (**A**) and multiple chromatid gains and losses (**B**). The Log2 separation ratio of X chromosome and Y chromosome when the diploid oocytes are compared with sex-mismatched male DNA are indicated by the *orange* and *blue* lines, respectively. (**A**) Separation for all probes on chromosome 13 does not reach the *blue* line, consistent with a single copy of the chromosome or chromatid loss from diploid state. (**B**) Separation of all gains and losses do not reach the *orange* and *blue* lines, respectively. The gains are consistent with three copies of the chromosome or chromatid gain from the diploid state. As in (**A**), the losses in (**B**) do not reach the *blue* line, consistent with chromatid losses from the diploid state.

*Capalbo. Aneuploidies after artificial activation. Fertil Steril 2016.*

## SUPPLEMENTAL TABLE 1

**Incidence of normal and abnormal patterns of PB extrusion and PN formation after artificial oocyte activation.**

| | | | | Normal activated | | Abnormal activated | |
|---|---|---|---|---|---|---|---|
| **Patient** | **No. oocytes thawed** | **No. oocytes survived** | **Total activated** | **2PB1PN** | **2PB0PN** | **1PB > 2PN** | **2PB2PN** |
| 1 | 2 | 2 | 2 | 2 | 0 | 0 | 0 |
| 2 | 5 | 5 | 5 | 3 | 1 | 0 | 1 |
| 3 | 4 | 4 | 2 | 1 | 1 | 0 | 0 |
| 4 | 5 | 4 | 4 | 4 | 0 | 0 | 0 |
| 5 | 10 | 5 | 3 | 3 | 0 | 0 | 0 |
| 6 | 3 | 3 | 3 | 2 | 0 | 1 | 0 |
| 7 | 5 | 5 | 5 | 2 | 2 | 0 | 1 |
| 8 | 6 | 6 | 5 | 5 | 0 | 0 | 0 |
| 9 | 6 | 6 | 1 | 1 | 0 | 0 | 0 |
| 10 | 4 | 4 | 4 | 4 | 0 | 0 | 0 |
| 11 | 3 | 2 | 2 | 0 | 1 | 0 | 1 |
| 12 | 3 | 3 | 3 | 3 | 0 | 0 | 0 |
| Total (%) | 56 | 49 (87.5) | 39 (80) | 30 (77) | 5 (13) | 1 (2.5) | 3 (7.5) |

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