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ORIGINAL ARTICLE *Embryology*

Artificial oocyte activation with $SrCl_2$ or calcimycin after ICSI improves clinical and embryological outcomes compared with ICSI alone: results of a randomized clinical trial

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STUDY QUESTION: Are pregnancy and birth rates affected by artificial oocyte activation (AOA) with $SrCl_2$ or calcimycin after ICSI for couples with male-factor infertility linked to abnormal sperm morphology or for couples with previous ICSI cycles of unexplained low fertilization or inadequate fertilization associated with impaired oocyte morphology?

SUMMARY ANSWER: AOA with either $SrCl_2$ or calcimycin can improve the rates of clinical pregnancy, ongoing pregnancy and live birth compared with ICSI alone, and the two agents have diverse effects for different subgroups of patients.

WHAT IS KNOWN ALREADY: ICSI is a successful treatment for infertility, but not in all individuals. AOA has potential to overcome inadequate fertilization in ICSI. Calcimycin and $SrCl_2$ are candidate agents for AOA, but their effectiveness remains to be compared.

STUDY DESIGN, SIZE, DURATION: This study was a randomized, open-label, three-arm, parallel-group, double-centre, superiority trial conducted between April 2015 and January 2016. The study evaluated the effects of AOA with calcimycin or $SrCl_2$ for clinical pregnancy rates after ICSI and included 343 couples divided into three groups.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Couples were included if they had two previous ICSI cycles of no or low fertilization (0–30%) with unknown causes or impaired oocyte morphology. Male-factor infertility cycles (frozen–thawed sperm, surgically retrieved sperm or ejaculates contained <10 millions spermatozoa/ml) undergoing their first ICSI attempt were also included if they had 100% abnormal sperm morphology (including globozoospermia and tapered-head). Couples were randomized to undergo ICSI with $SrCl_2$ AOA, ICSI with calcimycin AOA or ICSI alone, with clinical pregnancy as the primary endpoint. Effect sizes were summarized as absolute rate differences (ARDs) and odds ratios (ORs), with precision evaluated by 95% CIs.

MAIN RESULTS AND THE ROLE OF CHANCE: Both $SrCl_2$ and calcimycin AOA improved clinical pregnancy rates compared to ICSI alone (49, 42 and 27%; ARD 22, 95% CI: 9–33; $P = 0.0007$ and ARD 16, 95% CI: 3–27; $P = 0.014$). $SrCl_2$ and calcimycin AOA were also superior to ICSI alone on the rates of ongoing pregnancy (42, 36 and 23%; $P = 0.0019$ and $P = 0.023$) and live birth (40, 33 and 18%; $P = 0.0002$ and $P = 0.012$). Among couples with previous ICSI cycles of low fertilization, AOA with $SrCl_2$ (but not with calcimycin) was superior to ICSI alone for rates of clinical pregnancy (ARD 35 percentage points (pp), $P = 0.0007$), ongoing pregnancy (ARD 27 pp, $P = 0.009$) and live birth (ARD 37 pp, $P = 0.002$). Among couples affected by male-factor infertility, AOA with calcimycin (but not with $SrCl_2$) was superior to ICSI alone for rates of clinical pregnancy (ARD 22 pp, $P = 0.006$), ongoing pregnancy (ARD 19 pp, $P = 0.013$) and live birth (ARD 17 pp, $P = 0.02$).

LIMITATIONS, REASONS FOR CAUTION: This study was an open-label trial, and this design might have introduced bias, although randomization methods were used. The study did not include a longitudinal follow-up, so further evidence is required to demonstrate the safety of AOA.

WIDER IMPLICATIONS OF THE FINDINGS: The decision to use $SrCl_2$ or calcimycin for AOA after ICSI may depend on whether the activation failure originates in the oocyte or the sperm.

STUDY FUNDING/COMPETING INTEREST(S): The study received no funding and the authors have no conflicts of interest to declare.

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Key words: oocyte activation / calcium ionophore / calcium oscillation / failed fertilization / male factor infertility

Introduction

IVF and ICSI are effective treatments for infertility (Palermo *et al.*, 1992; Steptoe and Edwards, 1992; Kissin *et al.*, 2014). Successful fertilization requires oocyte activation, which depends on a proper interaction between the gametes (Yeste *et al.*, 2016). Activation failure results in poor fertilization rates (Flaherty *et al.*, 1998; Cheng *et al.*, 2011; Clift and Schuh, 2013).

Oocyte activation triggers a cascade of events including Ca^{2+} oscillation, leading to fertilization and embryo development (Yeste *et al.*, 2016). In animal models, the duration, amplitude and frequency of Ca^{2+} oscillation affect the normal oocyte activation and embryo development (Sfontouris *et al.*, 2015). The precise mechanisms underlying oocyte activation remain elusive. A likely mechanism is that the phospholipase C zeta (PLCζ) of a fertilizing spermatozoon produces inositol-1,4,5-trisphosphate within the oocyte which interacts with receptors in the endoplasmic reticulum, causing Ca^{2+} oscillation (Ridgway *et al.*, 1977; Steinhardt *et al.*, 1977; Miyazaki *et al.*, 1992, 1993; Wassarman *et al.*, 2001; Ducibella *et al.*, 2002; Saunders *et al.*, 2002; Horner and Wolfner, 2008; Kashir *et al.*, 2010; Amdani *et al.*, 2013; Nomikos *et al.*, 2013; Escoffier *et al.*, 2014). Reports suggest that abnormal sperm morphology reduces the rates of fertilization and implantation (De Vos *et al.*, 2003; Lu *et al.*, 2012; Greco *et al.*, 2013). The oocyte also contributes to the activation events via identified and unidentified factors (Tesarik *et al.*, 2002; Eldar-Geva *et al.*, 2003; Heindryckx *et al.*, 2005, 2008; Combelles *et al.*, 2010; Yelumalai *et al.*, 2015).

Artificial oocyte activation (AOA) can repair a defective activation (Yeste *et al.*, 2016), improving ICSI outcomes (Montag *et al.*, 2012). Mechanical, electrical and chemical activation serve as AOA methods (Vanden Meerschaut *et al.*, 2012; 2014). Chemical AOA is the prominent, and it induces either a single, prolonged wave or pulsatile waves of intracytoplasmic Ca^{2+} (Yeste *et al.*, 2016; Murugesu *et al.*, 2017). Calcimycin, ionomycin, puromycin and 6-dimethylaminopurine increase membrane permeability to extracellular Ca^{2+}, inducing a single, prolonged Ca^{2+} wave (Rybouchkin *et al.*, 1997; Ebner *et al.*, 2015; Yeste *et al.*, 2016). Strontium chloride ($SrCl_2$), phorbol esters or anhydrous alcohol release the Ca^{2+} from the endoplasmic reticulum in pulsatile waves (Kline and Kline, 1992; Kishikawa *et al.*, 1999; Kim *et al.*, 2014; Yeste *et al.*, 2016).

AOA with calcimycin or $SrCl_2$ can improve outcomes for ICSI cycles of impaired fertilization (Yanagida *et al.*, 2006; Chen *et al.*, 2010; Kim *et al.*, 2014; Murugesu *et al.*, 2017). Variable studies including retrospective designed analyses, historical comparisons or relatively underpowered studies have synthesized this evidence. Most of the studies have only examined calcimycin AOA, although $SrCl_2$ has shown potential for AOA (Kim *et al.*, 2014; Yeste *et al.*, 2016). Recently, Lu *et al.* (2018) showed that $SrCl_2$ AOA cannot provoke calcium oscillation or parthenogenic division for human oocytes. However, Lu *et al.* used *in vitro* matured oocytes or oocytes characterized by smooth endoplasmic reticulum. In addition, there has been no comparative randomized clinical trial (RCT) to evaluate the relative effectiveness of calcimycin and $SrCl_2$. Whether the sperm or the oocyte is most responsible for the activation defects remains unidentifiable from a clinical perspective (Murugesu *et al.*, 2017).

To address these questions, we compared $SrCl_2$ and calcimycin AOA with each other and with ICSI alone in this prospective RCT, identifying the rate of clinical pregnancy as a primary endpoint. To determine whether the sperm or the oocyte is most responsible, infertile couples included were those undergoing ICSI cycles for previous low fertilization (<30%) but normal semen parameters or first ICSI cycles for male-factor infertility with abnormal sperm morphology.

Materials and Methods

Trial design

The study adopted a randomized, open-label, three-arm, parallel-group, double-centre, superiority trial design (NCT02424214, registered at www.ClinicalTrials.gov). Two private IVF facilities in Egypt (Ibnsina IVF Centre, Suhag and Banoon IVF Centre, Asyut) conducted the trial. The trial included a screening period of 18 months (from the time of getting the consent to the start of assignment of participants). Research instructors disclosed the details to eligible couples. From January to April 2015, research instructors recalled participants for randomization and enrolment. The first couple was recruited on 30 April 2015, and the last oocyte retrieval was performed on 17 January 2016. Participants provided written informed consent. The ethics committees of the centres approved the study (Number 09; 02/2013). Independent experts and safety committees oversaw this study.

Participants

Women were 18–40 years of age with a BMI of <31 kg/m^2. Inclusion criteria were deliberately broad to include couples with two previous ICSI cycles of no or low fertilization (0–30%) or with male-factor infertility

undergoing their first ICSI cycle. We classified previous cycles of low fertilization as 'unexplained low fertilization' or as 'low fertilization associated with impaired oocyte morphology', but within normal sperm limits. Impaired morphology of oocytes, according to the Istanbul Consensus, was non-conformity with the following definition: a spherical structure in a uniform zona pellucida with a uniform translucent cytoplasm free of inclusions and with a size-proper polar body (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). The male-factor infertility cycles (frozen–thawed sperm, surgically retrieved sperm or ejaculates containing <10 million spermatozoa/ml) undergoing their first ICSI attempt had 100% abnormal sperm morphology (including globozoospermia and tapered-head). Couples had primary infertility with no previous completed pregnancy or abortion. Exclusion criteria were couples with endometriosis, thin endometrial lining or pinhead sperm defects.

Randomization and masking

Using an online randomization tool (http://www.openepi.com/Random/Random.htm), independent data managers assigned participants to $SrCl_2$ AOA, calcimycin AOA or ICSI alone with a 1:1:1 allocation ratio. Block randomization was performed to ensure equal representation of couples in the study groups. Each allocation was sealed in a sequentially numbered, opaque envelope. On 27 April 2015, independent instructors recorded each envelope number in each woman's case-record file. On Day 21 of the cycle preceding ICSI, research instructors opened the envelope and assigned the participant to the relevant group. Immediately, the instructors transferred the allocation result to the laboratory team, keeping gynaecologists and participants unaware of the result until the embryo transfer.

Stimulation protocol, embryo transfer and luteal-phase support

Pituitary downregulation was attainable by subcutaneous GnRH-a (Decapeptyl 0.1 mg, Ferring, Saint-Prex, Switzerland) from Day 21 of the cycle preceding ICSI. On Day 2 of the ICSI cycle, women received 225–300 IU of rFSH (Gonal-F, Merck Serono) and hMG injections (Menogon, Ferring) with a 2:1 ratio, with the dose adjusted according to the response. When three follicles measured 18 mm diameter, final maturation was induced by 10 000 IU human chorionic gonadotropin (hCG, Choriomon, IBSA, Lugano, Switzerland). Oocytes were collected 37 h later and the metaphase II (MII) were inseminated by ICSI (Palermo *et al.*, 1992). On Day 5, specified clinicians followed a standard protocol, transferring one to two blastocysts for each woman. For luteal phase support, women began intramuscular progesterone (100-mg, Prontogest, IBSA) from the day after oocyte retrieval to the 12th week of gestation, unless the biochemical pregnancy test was negative.

Sperm preparation, oocyte retrieval, denudation and ICSI

Fresh, frozen–thawed and surgically retrieved sperm were prepared at room temperature by density-gradient centrifugation (PureSperm, Nidacon, Mölndal, Sweden) (Björndahl *et al.*, 2010). Samples were then washed and incubated in AllGrade Wash medium (LifeGlobal, Guilford, CT, USA). Follicles were aspirated into, and handled in, Global HEPES medium (LifeGlobal) at 37°C, using a tube warmer. With chemical and mechanical tools (80 IU hyaluronidase, LifeGlobal; Denudation Pipette, Vitrolife, Göteborg, Sweden), oocytes were denuded at 39 h after hCG. Immediately, ICSI was performed at 37°C, in Global HEPES (LifeGlobal).

Culture protocol and embryo scoring

In the two centres and across the study, laboratory variables were controlled using: only Minc-1000 incubators (Cook, USA); Global Total medium (LifeGlobal); 37 ± 0.1°C culture temperature verified by a thermocouple (Chino, Tokyo, Japan); 7.25 ± 0.02 pH validated weekly by a blood gas analyser (Epocal, Ottawa, Canada); group culture of three oocytes; 15 µl medium droplet in micro-droplet dish (Vitrolife) overlaid by 5 ml oil (NidOil, Nidacon); continuous culture from Day 0 through Day 5/6, without medium renewal; premixed gas (7.5% CO_2, 5% O_2 and 87.5% N_2); and the same batches of disposables. Fertilization check and embryo grading were performed on Days 1, 3 and 5 of culture (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Interventions

Immediately after ICSI, AOA was performed and comprised incubation for 1 h in 10 mM $SrCl_2$ (Sigma, St Louis, MO, USA) diluted in Global Total medium or incubation for 20 min in a commercial calcimycin (Cult-active Ca-Ionophore; Gynemed, Lensahn, Germany). AOA droplets were 200 µl each in 60-mm dishes (353652, BD Falcon, USA) overlaid with 10 ml oil (NidOil) and were equilibrated overnight at 37°C and in an atmosphere of 7.5% CO_2, 5% O_2, and 87.5% N_2 (pH 7.25 ± 0.02).

Study outcomes

The primary endpoint was clinical pregnancy rate (positive heartbeat on ultrasonography at ≥4 weeks after ET). Secondary endpoints included the rates of (i) fertilization (fertilized oocytes with two pronuclei per MII oocyte injected); (ii) high-quality embryos on Day 3 (seven or eight blastomeres of equal-size and <10% fragmentation by volume); (iii) formed blastocyst on Day 5/6 from fertilized oocytes; (iv) high-quality blastocysts (blastocysts ≥3.1.1 grade, per fertilized oocyte); (v) biochemical pregnancy (positive b-hCG at ≥14 days following ET); (vi) ongoing pregnancy (pregnancy following week 12 of gestation); (vii) multiple pregnancy (≥two foetuses with a heartbeat); (viii) implantation (sacs with a heartbeat); (ix) miscarriage (pregnancy loss before gestational week 12); and (x) live birth (viable neonate at ≥30 weeks of gestation).

Statistical analysis

Previous cycles of low fertilization for women younger than 40 years of age showed a 16% clinical pregnancy rate (Murugesu *et al.*, 2017). This trial was powered to detect a 14% rise in pregnancy rate (16–30%) with 80% power at 5% significance level, requiring 342 participants (114 per arm) to undergo $SrCl_2$ AOA, calcimycin AOA or ICSI alone and reach the primary endpoint. An alternative was to enrol up to 450 women, to allow for any significant dropout. The independent data and safety committees performed an interim analysis at 343 participants, advising that no more enrolment is needed. Data were analysed using binomial logistic regression with log link, one-way ANOVA, Fisher's exact and Chi-square tests where appropriate. Between-clinician and between-embryologist differences were controlled by Fleiss' Kappa analysis (McHugh, 2012). Results are reported as an intention-to-treat and per indication analyses and are presented as an absolute rate difference (ARD), odds ratio (OR) with 95% CI) or means with SD where appropriate. $P < 0.05$ was regarded as statistically significant. All analyses were performed using SPSS (version 22, IBM. Armonk, NY, USA).

Results

Between 27 April 2015 and 17 January 2016, we recruited 343 participants ($SrCl_2$ AOA = 115, calcimycin AOA = 113 and ICSI alone = 115)

after excluding 49 (Fig. 1). Patient and cycle characteristics were similar in the three groups (Table I).

Overall (Table II), the clinical pregnancy rate was significantly higher with $SrCl_2$ and calcimycin AOA than with ICSI alone (49, 42 and 27%; ARD 22, 95% CI: 9–33 and ARD 16, 95% CI: 3–27). $SrCl_2$ and calcimycin AOA also significantly increased the biochemical pregnancy rate compared with ICSI alone. The ongoing pregnancy rate was considerably higher with $SrCl_2$ and calcimycin AOA than with ICSI alone (42, 36 and 23%; ARD 19, 95% CI: 7–30 and ARD 14, 95% CI: 2–25). The implantation rate was significantly also higher with $SrCl_2$ and calcimycin AOA than with ICSI alone (32, 28 and 18%; ARD 14, 95% CI: 5–23 and ARD 10, 95% CI: 2–19). These results culminated in significantly higher rates of live birth for $SrCl_2$ and calcimycin AOA than for ICSI alone (40, 33 and 18%; ARD 22, 95% CI: 10–33 for $SrCl_2$ and ARD 14, 95% CI: 3–25). The rates of cancelled transfer, chemical pregnancy, miscarriage and multiple pregnancy were similar between the groups. Both $SrCl_2$ and calcimycin AOA resulted in similar rates of ongoing pregnancy, implantation and live birth (Supplementary Table SV).

Among couples with previous low fertilization, $SrCl_2$ AOA significantly improved the clinical pregnancy rate compared with ICSI alone (ARD 35, 95% CI: 15–52; Supplementary Table SI) or calcimycin AOA (ARD 30, 95% CI: 10–47; Supplementary Table SV), whereas calcimycin AOA and ICSI alone were comparable (ARD 5, 95% CI:−14 to 24; Supplementary Table SI). $SrCl_2$ AOA was superior to ICSI alone regarding the rates of biochemical pregnancy, ongoing pregnancy and implantation, resulting in a higher live birth rate (ARD 37, 95% CI: 16–53; Supplementary Table SI). $SrCl_2$ AOA was also superior to calcimycin AOA, resulting in higher rates of ongoing pregnancy and live birth (ARD 22, 95% CI: 2–40 and ARD 26, 95% CI: 6–44; Supplementary Table SV). Only the implantation rate was significantly higher with calcimycin AOA than with ICSI alone (Supplementary Table SI).

Among couples of male-factor infertility with abnormal sperm, calcimycin AOA significantly improved the clinical pregnancy rate compared with ICSI alone (ARD 22, 95% CI: 6–36; Supplementary Table SII), whereas $SrCl_2$ AOA and ICSI alone were comparable (ARD 12, 95% CI:−3 to 26). Calcimycin AOA was superior to ICSI alone regarding the rates of biochemical pregnancy, clinical pregnancy

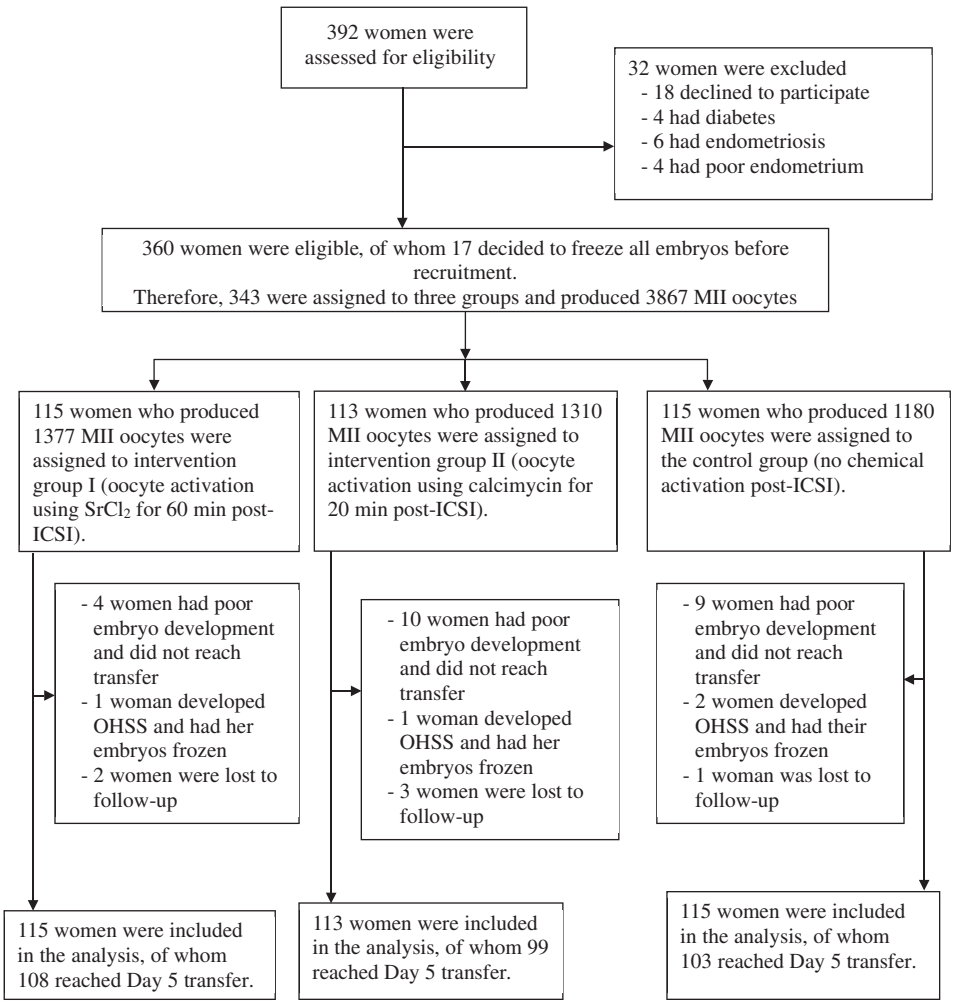

Figure 1 Study flow diagram.

Table I Baseline characteristics by trial group.

	Artificial oocyte activation (AOA) groups		
	$SrCl_2$ AOA ($n = 115$)	Calcimycin AOA ($n = 113$)	Control (no AOA) ($n = 115$)
Age (years): mean ± SD	31 ± 5.1	32.4 ± 5.2	32 ± 5.2
BMI (kg/m^2): mean ± SD	28.4 ± 2.7	28.4 ± 2.7	28.3 ± 2.7
Duration of infertility (years): mean ± SD	6 ± 2.7	6 ± 2.5	6 ± 2.2
Basal FSH (IU/L): mean ± SD	6 ± 1.2	6 ± 1.3	6 ± 2
Antral follicle count: mean ± SD	15.0 ± 2.2	14.0 ± 2.1	14.3 ± 2.3
Total FSH/hMG: mean ± SD	2245 ± 244	2294 ± 195	2264 ± 227
Impaired-fertilization cycles:			
Previous fertilization failure: *n* (%)	7 (6)	5 (4)	7 (6)
Previous low fertilization (<30%) with average oocyte quality: *n* (%)	14 (12)	13 (12)	12 (11)
Previous low fertilization (<30%) associated with impaired oocyte quality: *n* (%)	26 (23)	27 (23)	24 (21)
Male-factor infertility cycles:			
Frozen–thawed sperm of affected-morphology samples: *n* (%)	22 (19)	24 (21)	23 (20)
Surgically retrieved sperm of affected-morphology samples: *n* (%)	19 (17)	21 (19)	22 (19)
Globozoospermia: *n* (%)	4 (3)	3 (3)	5 (4)
100% tapered-head sperm: *n* (%)	23 (20)	20 (18)	22 (19)
Number of oocytes collected: mean ± SD	14 ± 9.4	13 ± 9.1	12 ± 8.0
Number of mature oocytes: mean ± SD	13 ± 6.5	12 ± 8.0	11 ± 6.2
Number of embryos transferred: mean ± SD	1.7 ± 0.5	1.6 ± 0.5	1.6 ± 0.5

Comparison of the differences between the three groups was performed by one-way ANOVA and the chi-square test where appropriate.

and ongoing pregnancy, implantation, live birth and live-birth implantation, whereas $SrCl_2$ AOA and ICSI alone were comparable, as were $SrCl_2$ and calcimycin AOA were comparable (Supplementary Tables SII and SV).

$SrCl_2$ AOA resulted in significantly greater rates of fertilization, top-quality embryo and compaction on Day 3, blastocyst development, high-quality blastocysts and cryopreservation compared with ICSI alone (all $P < 0.0001$; Table III). Calcimycin AOA also resulted in significantly higher rates of fertilization, blastocyst formation and cryopreservation compared with ICSI alone ($P = 0.003$, $P = 0.026$ and $P < 0.0001$; Table III). The embryological outcomes for the subgroup analyses are shown in Supplementary Tables SIII and SIV.

Discussion

To our knowledge, this RCT is the first to compare $SrCl_2$ and calcimycin AOA after ICSI to ICSI alone for cycles of previous low fertilization or male-factor infertility. Overall, this trial demonstrated significantly higher rates of clinical pregnancy and live birth for either $SrCl_2$ or calcimycin AOA after ICSI compared with ICSI alone, whereas $SrCl_2$ and calcimycin AOA were comparable. Our findings concur with a recent meta-analysis (Murugesu *et al.*, 2017).

Among couples with previous ICSI cycles of low fertilization, $SrCl_2$ AOA was more effective than ICSI alone, whereas calcimycin AOA was comparable to ICSI alone. In this subgroup, neither sperm entry nor ICSI appears sufficient to initiate the oocyte activation. This is likely to relate the activation defects to the oocyte (Miyara *et al.*, 2003; Ebner *et al.*, 2006; Xing *et al.*, 2011; Kilani and Chapman, 2014). Reports suggest that ICSI with $SrCl_2$ AOA appears to stimulate pulsatile waves of Ca^{2+} oscillation from the endoplasmic reticulum during fertilization and further development (Kline and Kline, 1992; Kishikawa *et al.*, 1999; Kim *et al.*, 2014; Yeste *et al.*, 2016). Although $SrCl_2$ AOA did not show signs of Ca^{2+} oscillation or parthenogenetic division for *in-vitro* matured oocytes (Lu *et al.*, 2018), this situation may differ from the MII oocytes undergoing ICSI with AOA. Therefore, given its potential to improve ICSI outcomes, further studies are required to identify whether $SrCl_2$ AOA activates the oocyte by Ca^{2+} oscillation or other mechanisms.

For calcimycin AOA and previous ICSI cycles of low fertilization, our findings showed that calcimycin AOA and ICSI alone were comparable. Our results can be explained as calcimycin might be less effective than $SrCl_2$ because it induces a single Ca^{2+} wave that is not converted into oscillation. Although our observations contradict a recent meta-analysis (Murugesu *et al.*, 2017) yet concur with an earlier one (Sfontouris *et al.*, 2015), the conclusions from the meta-analyses seem to be not substantial due to the heterogenous designs of their studies. Therefore, calcimycin AOA appears to be not beneficial for ICSI cycles with previous impaired fertilization, but normal sperm parameters.

Among couples with male-factor infertility, calcimycin AOA was superior to $SrCl_2$ AOA or ICSI alone. It is likely that when the problem results from male-factor infertility, Ca^{2+} oscillation is functional in the oocyte, but it has insufficient stimulation by the ICSI alone with affected sperm (Vanden Meerschaut *et al.*, 2013; Ferrer-Buitrago *et al.*, 2018). Calcimycin AOA provides additional Ca^{2+} influx from the

Table II Clinical Outcomes in the trial groups by intention-to-treat analysis.

	Artificial oocyte activation (AOA) groups (%)			Absolute rate difference, percentage points (95% CI)		Odds ratio (95% CI)	
	$SrCl_2$ AOA ($n = 115$)	Calcimycin AOA ($n = 113$)	Control (no AOA) ($n = 115$)	$SrCl_2$ to control	Calcimycin to control	$SrCl_2$ to control	Calcimycin to control
Cancelled transfer rate	7/115 (6)	14/113 (12)	12/115 (10)	−4.3 (−12 to 3)	2 (−6.5 to 11)	0.56 (0.21–1.47) $P = 0.23$	1.21 (0.54–2.75) $P = 0.64$
Biochemical pregnancy rate	62/115 (54)	61/113 (54)	39/115 (34)	20 (7–32)	20 (7–32)	2.28 (1.34–3.88) $P = 0.002$	2.29 (1.34–3.90) $P = 0.002$
Clinical pregnancy rate	56/115 (49)	48/113 (42)	31/115 (27)	22 (9–33)	16 (3–27)	2.57 (1.48–4.46) $P = 0.0007$	2.0 (1.15–3.49) $P = 0.014$
Chemical pregnancy rate	6/115 (5)	13/113 (11)	8/115 (7)	−1.7 (−8 to 5)	5 (−3 to 13)	0.74 (0.25–2.19) $P = 0.58$	1.74 (0.69–4.37) $P = 0.24$
Ongoing pregnancy rate	48/115 (42)	41/113 (36)	26/115 (23)	19 (7–30)	14 (2–25)	2.45 (1.38–4.35) $P = 0.0019$	1.95 (1.10–3.49) $P = 0.023$
Miscarriage rate	14/115 (12)	20/113 (18)	13/115 (11)	0.9 (−8 to 9.4)	6.4 (−2.9 to 15.7)	1.09 (0.49–2.43) $P = 0.84$	1.69 (0.79–3.58) $P = 0.17$
Multiple pregnancy rate	5/115 (4)	4/113 (4)	2/115 (2)	2.6 (−2.4 to 8.2)	1.8 (−3.1 to 7.2)	2.57 (0.49–13.5) $P = 0.45$	2.07 (0.37–11.6) $P = 0.44$
Implantation rate	61/190 (32)	52/184 (28)	33/182 (18)	14 (5–23)	10 (2–19)	2.14 (1.31–3.47) $P = 0.0019$	1.78 (1.08–2.92) $P = 0.022$
Live birth rate	46/115 (40)	37/113 (33)	21/115 (18)	22 (10–33)	14 (3–25)	2.98 (1.63–5.45) $P = 0.0002$	2.18 (1.18–4.03) $P = 0.012$
Live-birth-implantation rate	46/190 (24)	37/184 (20)	21/182 (12)	13 (5–20)	9 (1–16)	2.45 (1.39–4.30) $P = 0.0015$	1.93 (1.08–3.45) $P = 0.025$

The logistic regression analysis verified that there was no association between the primary outcome and the confounding variables including each centre's cycle characteristics, except for $SrCl_2$ AOA and Calcimycin AOA. Chemical pregnancy indicates a positive pregnancy test with no gestational sac identified 15 days after the test.

surrounding medium, boosting the initiation of Ca^{2+} oscillation (Yeste *et al.*, 2016). In this situation, $SrCl_2$ may have less effect because the defect is not with the Ca^{2+} oscillation machinery itself, just the initiation of oscillation. Our observations concur with a recent meta-analysis (Murugesu *et al.*, 2017) as it also included cycles with affected sperm function. Therefore, calcimycin AOA appears promising for ICSI cycles with abnormal sperm morphology.

Regarding embryo development, AOA with $SrCl_2$ or calcimycin gave significantly better results than conventional ICSI in the overall and subgroup analyses. The effect sizes for $SrCl_2$ were larger than for calcimycin. Both AOA protocols were superior to ICSI alone for the blastocyst cryopreservation rate, suggesting that, besides the clinical improvements observed, AOA might improve the cumulative pregnancy rate. The improvements in embryo development could have resulted from restoration of Ca^{2+} oscillation to physiological or supraphysiological status. However, the mechanisms by which this restoration might have occurred are beyond this study, and whether possible supraphysiological Ca^{2+} oscillation might have epigenetic effects remains a subject for further investigation.

Previous studies (Murugesu *et al.*, 2017) have shown that the clinical pregnancy rate is 16% for ICSI cycles with previous low fertilization. This rate appears relatively lower than that we observed in our facilities and we reported for this group. In our study, the rigorous control for the laboratory and clinical confounders, the relatively young age of women included (~32), and the short time of conduct seemed to be reflected in the observed outcomes in the three groups.

To limit the sample size required, the margin of superiority was set at 14%, which is a relatively high. This margin was considered because although calcimycin and $SrCl_2$ appear safe, this safety has not been proved unequivocally (Yeste *et al.*, 2016).

Our study has several strengths. It represents, to our knowledge, the first RCT with an appropriate sample size to compare $SrCl_2$ to calcimycin for AOA. Although clinical pregnancy was the primary outcome, ongoing pregnancy and live birth rates were also assessed. Our

Table III Embryological outcomes in the trial groups.

	Artificial oocyte activation (AOA) groups (%)			Absolute rate difference, percentage points (95% CI)		Odds ratio (95% CI)	
	$SrCl_2$ AOA (n = 115)	Calcimycin AOA (n = 113)	Control (no AOA) (n = 115)	$SrCl_2$ to control	Calcimycin to control	$SrCl_2$ to control	Calcimycin to control
Maturation rate: MII oocytes/collected oocytes	1377/1581 (87)	1310/1483 (88)	1180/1329 (89)	−1.7 (−4.1 to 0.7)	−0.45 (−2.8 to 1.9)	0.85 (0.68–1.07) $P = 0.16$	0.96 (0.76–1.21) $P = 0.71$
Fertilization rate: 2PN oocytes/injected MII oocytes	1168/1377 (85)	874/1310 (67)	720/1180 (61)	24 (20–27)	5.7 (1.9–9.5)	3.57 (2.96–4.31) $P < 0.0001$	1.28 (1.09–1.51) $P = 0.003$
Cleavage rate: cleaved embryos/fertilized oocytes	1134/1168 (97)	854/874 (98)	702/720 (98)	−0.4 (−1.9 to 1.2)	0.2 (−1.3 to 1.8)	0.86 (0.48–1.53) $P = 0.597$	1.09 (0.5–72.09) $P = 0.78$
Top-quality Day 3 embryos/fertilized oocytes	1021/1168 (87)	513/874 (59)	413/720 (57)	30 (26–34)	1.3 (3.5–6.0)	5.16 (4.11–6.48) $P < 0.0001$	1.06 (0.87–1.29) $P = 0.59$
Compaction rate: compacted Day 3 embryos/fertilized oocytes	518/1168 (44)	212/874 (24)	189/720 (26)	18 (14–22)	−2 (−6.3 to 2.3)	2.24 (1.83–2.74) $P < 0.0001$	0.89 (0.72–1.13) $P = 0.36$
Blastocyst formation rate: blastocysts/fertilized oocytes	722/1168 (62)	447/874 (51)	328/720 (46)	17 (12–21)	5.6 (0.7–10.5)	1.93 (1.60–2.34) $P < 0.0001$	1.25 (1.03–1.52) $P = 0.026$
High-quality blastocysts/fertilized oocytes	475/1168 (41)	232/874 (27)	195/720 (27)	14 (9–18)	−0.5 (−0.5 to 0.4)	1.85 (1.51–2.26) $P < 0.0001$	0.97 (0.78–1.22) $P = 0.806$
Cryopreservation rate: vitrified blastocysts/fertilized oocytes	518/1168 (44)	251/874 (29)	133/720 (18)	26 (22–30)	6.1 (6–14)	3.52 (2.82–4.39) $P < 0.0001$	1.7 (1.40–2.26) $P < 0.0001$

2PN, two pronuclei; MII, metaphase II.

findings provide insight into the relative suitability of $SrCl_2$ and calcimycin for AOA for particular causes of infertility, which can improve the outcomes for subgroups of patients who experience poor outcomes of ICSI.

Our study also has several limitations. The open-label trial design might have introduced bias, although randomization, at least partly, addressed this possibility. The study did not include a longitudinal follow-up period, so further evidence regarding the safety of AOA is still required. The study included couples with different indications for ICSI, rather than focusing on a single subgroup, but post hoc analyses were performed to determine the particular effects of AOA in these subgroups.

In conclusion, in our study population, ICSI followed by AOA with $SrCl_2$ or calcimycin was associated with significantly better embryological and clinical outcomes than ICSI alone. Calcimycin AOA appears effective in overcoming defects related to abnormal sperm morphology, whereas $SrCl_2$ AOA appears to improve outcomes for ICSI cycles characterized by previous impaired fertilization. In consideration of the current lack of knowledge regarding the long-term safety and transgenerational effects of AOA, we do not recommend this treatment for the general ICSI population. Instead, we recommend that more studies are performed, to further elucidate the mechanisms that underlie our findings, in particular those related to $SrCl_2$ AOA. We also recommend that a multicentre trial is conducted to identify the cumulative live birth rate and to assess the health of the offspring as primary outcomes. Until these studies have been conducted, we recommend that AOA should be used cautiously, and the advantages and disadvantages regarding its use should be weighed in each case.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

M.F. created the concept and design of the study, was the primary investigator and wrote the article. M.E. participated in the investigation as well as the collection, analysis and interpretation of the data. All other authors participated in critically reviewing the concept upon which the study was conducted and participated in the investigation. All authors critically revised the manuscript and gave their final approval of the version for publication.

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Conflict of interest

The authors have no conflicts of interest to declare.

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