Ionophore application for artificial oocyte activation and its potential effect on morphokinetics: a sibling oocyte study

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Abstract

Purpose To evaluate whether ionophore application at the oocyte stage changes the morphokinetics of the associated embryos in cases of artificial oocyte activation.

Methods In a prospective sibling oocyte approach, 78 ICSI patients with suspected fertilization problems had half of their MII-oocytes treated with a ready-to-use ionophore (calcimycin) immediately following ICSI (study group). Untreated ICSI eggs served as the control group. Primary analyses focused on morphokinetic behavior and the presence of irregular cleavages. The rates of fertilization, utilization, pregnancy, and live birth rate were also evaluated.

Results Ionophore-treated oocytes showed a significantly earlier formation of pronuclei (t2PNa) and a better synchronized third cell cycle (s3) \( (P < .05) \). The rate of irregular cleavage was unaffected \( (P > .05) \). Ionophore treatment significantly improved the overall rates of fertilization \( (P < .01) \) and blastocyst utilization \( (P < .05) \).

Conclusion Ionophore application does not negatively affect cleavage timing nor is it associated with irregular cleavage.

Keywords Artificial oocyte activation · Calcimycin · Ionophore · Morphokinetics · Time-lapse imaging

Introduction

One of the new technologies in the field of medically assisted reproduction from the last decade [1] is time-lapse technology (TLT). Since its development, TLT has shifted from an observational approach to a tool involved in the selection and prediction of embryo growth. To date, there are no randomized controlled trials supporting the clinical relevance of TLT [2], although this lack of evidence has not remained unchallenged [3, 4]. However, regardless of the potential causative impact on reproductive outcome, TLT has several advantages that would justify its use.

First, time-lapse incubators guarantee the safest and most stable embryo culture environment available by simply avoiding extensive exposure outside the incubator [5]. Second, continued embryo monitoring that allows for a 24-h surveillance of the oocytes, zygotes, and embryos facilitates the detection or further investigation of previously unknown phenomena in preimplantation development [6–8]. The good practice recommendation paper for the use of TLT by the ESHRE working group on time-lapse technology [9] further highlighted that TLT allows for a more flexible and better management of a laboratory workload.

Finally, TLT is an important tool within the framework of existing quality management systems. In fact, it may be used to train embryologists and assess intra- and inter-operator variability with respect to the annotation of morphokinetic parameters [10–12]. More importantly, TLT can also be used to identify and monitor in vitro stress that may arise from suboptimal culture conditions. In particular, a large number of consumables or new culture media for testing could interfere with anticipated cleavage timings [13, 14]. Although subtle changes may have no clinical consequences [14, 15], larger deviations in morphokinetic behavior may reflect severe physiological problems [16].

In other words, in the course of the validation of a new process in an IVF laboratory [17], TLT could be an effective tool to support the capability and safety of the same. One such process [18–20] is assisted or artificial oocyte activation (AOA). To date, AOA has been successfully used to
artificially induce intracellular calcium (potentially stimulating activation and mitosis) in cases of fertilization failure, fertilization problems (< 30%), and severe male factor infertility, and in cases with impaired embryo development.

In this context, information on the potential effects of AOA, for example application of an ionophore on cleavage behavior, is scarce and primarily based on preliminary data from posters [21, 22] or oral presentations [23]. Recently, Martínez et al. [24] were the first to evaluate the association between AOA and morphokinetics in donor oocyte cycles of patients with a known mutation in the PLCζ1 gene in great detail.

This study aimed to identify any potential influence of a ready-to-use ionophore (calcimycin) on cleavage timings and synchronicity. In order to find an optimally matched control group, a sibling oocyte approach was chosen, with half of the eggs being treated with AOA and the other half not treated.

**Material and methods**

**Patients**

In the 3-year study period (2017–2019), a total of 78 patients were included in this prospective observational study with retrospective analysis of morphokinetic data (ethical votum #1115/2021). All patients provided informed consent and accepted that for approximately half of their mature gametes, oocyte activation was artificially supported by calcimycin, which is a known Ca²⁺-ionophore. This additional support was offered due to a history of borderline fertilization (n = 47) defined as < 50% [25] or suspected severe male factor infertility (n = 31) [26]. The patients were not charged for this AOA technique because of the study conditions. Every patient was only included once since the purpose of the split ionophore cycle was diagnostic. Based on the results of the present sibling oocyte approach, a subsequent cycle would either be without ionophore application or with all eggs receiving AOA.

The female mean age was 32.9 ± 4.1 years, and the patients suffered from endometriosis (9.0%), PCOS (6.4%), or bilateral tubal factor (1.3%). Apart from four cases of unexplained infertility (5.1%), all couples had an additional male factor (94.9%). Most of the stimulations were performed according to an antagonist protocol (n = 52, 69.2%), whereas the remaining cycles were treated with a long protocol. Ovulation induction was always performed with hCG when the lead follicle(s) reached a diameter of 18–20 mm and serum estradiol level was adequate (approximately 150 pg/ml E2 follicle seen on ultrasound). Subsequently, ovarian puncture was performed transvaginally using an ultrasound 36 h after ovulation induction.

### ICSI, AOA, and embryo culture

Cumulus-oocyte complexes (COCs) were collected from GM501 Wash (Gynemed, Lensahn, Germany). After a 2–3 h resting period in a big box incubator (Labo C201, Labotect, Göttingen, Germany), COCs were carefully denuded (combining enzymatic and mechanical steps). Oocytes that were identified as mature (MII) were injected using Microtech pipettes (Gynemed) and a Luigs and Neumann micromanipulator (Ratingen, Germany). Untreated control oocytes were immediately placed into a special time-lapse culture dish (CultureCoin®, Esco Medical Technologies) for use in the time-lapse system Miri TL (Esco Medical Technologies, 6.5% CO₂, 5% O₂). The other half of the MII-oocytes were treated with a ready-to-use ionophore compound (GM508 CultActive, Gynemed) within 10 min after ICSI, as published earlier [26, 27]. Since GM508 CultActive is a bicarbonate-buffered product, it requires preincubation in a CO₂ atmosphere (4 h according to the supplier). This process is usually performed in a 4-well dish in which one well contains a droplet of GM508 CultActive (30–50 µl in our hands) and three wells contain washing medium (GM501 Wash). All agents must be covered with mineral oil. It is recommended to agitate the bottle with GM508 CultActive before use in order to avoid precipitation.

After 15 min of incubation in GM508 CultActive and a thorough washing process, the oocytes subjected to AOA were transferred to the same CultureCoin. Notably, the ionophore used in the ready-to-use product was calcimycin, also known as A23187.

Although video sequences were automatically generated, static morphological assessments were performed at fixed time points [28]. It should be noted that on day 1 (circa 22 h post ICSI), the videos were screened for the extrusion of the second polar body and the presence of two pronuclei so that fertilization was never missed. This led to the scenario in which the number of fertilizations equals the number of cleaved embryos. Good-quality embryos on days 2–3 showed a stage-appropriate cell number, with less than 10% of fragments showing no signs of multinucleation. It should be clarified that it was not screened for multinucleated cells and, thus, the fact that there were multinucleated cells at time points [29] other than those suggested by Alpha and ESHRE cannot be overlooked [28]. Any “evidence of compaction that involves virtually the entire embryo volume” on day 4 was considered to be a proper development and reflected good quality [28]. Blastocyst quality was scored on day 4 [30] or day 5 according to usual criteria such as grade of expansion and quality of the two cell lineages, namely trophectoderm (TE) and inner cell mass (ICM).

Whenever possible, blastocysts were transferred, and this was performed in the majority of cases (n = 60,
samples were screened for rapid cleavage (t3–t2 < 5 h), tri-nomenclature from Ciray et al. [13] was used. In detail, the et al. [13] were automatically calculated using the Miri
synchronicity of embryonic cell cycles) according to Ciray
initiation of a certain process (tSB).

A distinct separation of cells (e.g., t2PB, t2-t8) or by the
determined any longer (t2PNf) or the first frame that shows either
in [13], these events are marked either by the first frame
2PNf, as well as later cleavage events (t2-t8, tSC, tSB, tB),
parameters were annotated by a single embryologist (T. E.)
missing any cellular events of interest. Morphokinetic
frequency was chosen (5 min, seven focal planes) to avoid
non-AOA) were synchronized. The shortest possible image
time of microinjection [13] so that all videos (AOA and
Apartment annotation

Notably, the starting time of the videos was set at the mid-
time of microinjection [13] so that all videos (AOA and
and non-AOA) were synchronized. The shortest possible image
frequency was chosen (5 min, seven focal planes) to avoid
missing any cellular events of interest. Morphokinetic
parameters were annotated by a single embryologist (T. E.)
according to previously published criteria [13]. In detail,
early morphokinetic parameters such as t2PB, t2PNa, and
2PNf, as well as later cleavage events (t2-t8, tSC, tSB, tB),
were taken into consideration. According to the definition
in [13], these events are marked either by the first frame
in which a certain morphological feature cannot be visual-
ized any longer (t2PNf) or the first frame that shows either
distinct separation of cells (e.g., t2PB, t2-t8) or by the
initiation of a certain process (tSB).

In addition, dynamic cell cycle variables (duration and
synchronicity of embryonic cell cycles) according to Ciray et al. [13] were automatically calculated using the Miri
TL software (s2, s3). Special care was taken to identify the
embryos with irregular cleavages. In this study, the nomenclature from Ciray et al. [13] was used. In detail, the
samples were screened for rapid cleavage (t3–t2 < 5 h), tri-
chotomous mitoses (e.g., t3–t2 = 0), cell fusions (reduction
of blastomere number at any stage), and the presence of
planar embryos [31]. Slight modifications were applied for
trichotomous cleavage events, as the trichotomous mitoses
were further subdivided according to the embryonic cell
cycle. In other words, we not only focused on the zygote
stage but also on the 2- and 4-cell embryo stages. Finally,
irregular chaotic division (ICD) was characterized by the
inability to divide clearly into separate blastomeres. Desai et al. [32] found that in “such embryos, the process
of cleaving can be prolonged, eventually resulting in an
embryo with irregular blastomeres and large cellular
fragments.”

Statistics

Seventeen days after intrauterine transfer, the blood concentration of hCG was measured (biochemical pregnancy).
Clinical pregnancy was determined by visualization of at
least one gestational sac with positive heart activity a month
after embryo transfer. A subclinical pregnancy showed no
fetal heartbeat. The implantation rate was defined by ultra-
sound visualization of a gestational sac per transferred
embryo (subclinical pregnancies were included). Neonatal
and perinatal outcomes were either obtained from an in-
house registry or were requested from peripheral maternity
clinics. Additional information was received from our neo-
natal intensive care unit (in the case of preterm delivery) and
from the pediatric unit (in the case of minor malformations).

Using the Kolmogorov–Smirnov test, all variables were
tested for a normal distribution. According to the outcome
of the distribution, either a paired sample Wilcoxon sign
rank test (no normal distribution) or a t-test for paired sam-

The primary endpoint of the study was the comparison
of the exact timing of morphokinetic events in study and
control oocytes leading up to full blastocyst formation (tB).
The secondary endpoints included the presence of irregular
cleavages and the rates of fertilization and blastulation. In
addition, the focus was on utilization rate, clinical pregnancy
rate, and live birth rate.

It is very likely that developmental arrest of cleavage
stage embryos may be adumbrated by an insidious cleav-
age delay at earlier phases. In order to account for this con-
founding factor, statistical analysis was not only done for the
entirety of embryos but also for the subgroup of embryos
that entered the blastocyst stage (and in which imminent
growth delay or arrest was not a major issue). Bootstrap-
ning method (2000 repeats) was used to calculate 95%
CIs. However, in all cases, statistical significance was set
at P < 0.05.

Results

A total of 939 COCs were collected from 78 patients under-
going ICSI. After the denudation process, 747 oocytes
(79.6%) were found to be mature (mean ± SD: 9.6 ± 4.3
MII oocytes), i.e., at metaphase II. These MII-eggs were
then injected, which led to 551 zygotes (73.8%). Minor per-
centages were found to be degenerated after ICSI (1.7%),
parthenogenetically activated (1PN, 1.7%), or showed 3PN
(2.3%). Table 1 indicates that the overall fertilization rate was significantly higher ($P < 0.01$, Wilcoxon test) in the oocytes that had been treated with ionophore (78.2%) than in the untreated controls (69.3%). Interestingly, ionophore application was not related to parthenogenetic activation or degeneration after ICSI in the present study.

Of all day 2 embryos, 40.5% were of good quality, and a similar rate was observed for day 3 (38.3%). The next day, 50.3% (241/479) showed signs of compaction. Finally, on day 5, blastulation was observed in 305 embryos (63.7%), 247 (81.0%) of which were of good quality so that they could be transferred or vitrified. Embryo/blastoyst formation quality was not affected by ionophore application (Table 1). On average, a 6% higher blastocyst formation rate in the ionophore-treated cohort did not reach the level of significance. ($P = 0.063$, Wilcoxon test).

The only cleavage timing that was significantly affected ($P = 0.002$) by the ionophore application was the appearance of the two pronuclei (t2PNa), while the remaining cleavage timings showed no correlation with AOA (Fig. 1, Table 2). The developmental benefit was still observable if only those oocytes were considered for calculation, which made it to the blastocyst stage ($P = 0.02$). Notably, the synchronicity of the four blastomere divisions within embryonic cell cycle 3 (referred to as s3) was significantly shortened in the ionophore group ($P = 0.036$). Again, analyzing only those embryos that made it to day 5 did not change the results ($P = 0.048$). Table 3 highlights that the application of an ionophore was not associated with the occurrence of irregular cleavages.

Including those four patients with mixed embryo transfers, 83 embryos were transferred to 78 patients. The overall implantation rate was 50.6% (42/83). Positive hCG levels were measured in 42 out of 78 (53.8%) patients. Pregnancies were only achieved in patients who had either embryos from the study group ($n = 45$) or from the control group ($n = 29$) transferred (Table 4). However, one extrauterine pregnancy (2.4%) and seven pregnancy losses (16.7%) reduced the clinical pregnancy rate to 43.6%. One late pregnancy loss due to an infection in gestation week 16 (ionophore group) was observed. Consequently, the overall live birth rate was 42.3% (33/78).

### Table 1

| Untreated oocytes | Ready-to-use ionophore |
|------------------|------------------------|
| N                | 371                    | 376                    |
| Degenerated      | 7 (1.9)                | 6 (1.6)                |
| 0PN             | 93 (25.1)              | 60 (16.0)              |
| 1PN             | 8 (2.1)                | 5 (1.3)                |
| 2PN             | 257 (69.3)             | 294 (78.2)             |
| 3PN             | 6 (1.6)                | 11 (2.9)               |
| Good quality embryos d2 | 102 (39.7)       | 121 (41.1)             |
| Good quality embryos d3 | 96 (37.4)            | 115 (39.1)             |
| Embryos d5 culture | 230                    | 249                    |
| Day 4 compaction | 111/230 (48.3)          | 130/249 (52.2)         |
| Blastocyst formation | 139 (60.4)           | 166 (66.7)             |
| Utilization rate | 111 (48.2)             | 136 (54.6)             |

$^{a,b}P < .01$

$^{c}P < .05$

Values in parentheses are percentages
Since two twin (monochorionic-diamniotic) pregnancies (one in each group) were achieved (4.8%), 35 babies were born. There was one minor malformation per group, for example, a bilateral preauricular adnexa in the untreated group and a small femoral hemangioma in the ionophore counterpart. According to Table 4, there was no effect of the ionophore treatment on pregnancy outcome since the observed 10% higher live birth rate did not reach the level of significance. The same holds true for neonatal outcomes, which was the same for both groups.

**Discussion**

Although AOA is a technique necessary for some patients to succeed in fertilization, embryo transfer, and pregnancy [33, 34], it is considered an add-on intervention [35]. However, since AOA techniques may overcome physiological problems in a rather “unphysiological” mode, caution should be taken and overuse should be avoided [18, 19].

In principle, AOA embryologists can choose from modified ICSI techniques [36], electrical [37, 38], or chemical activation approaches [39] to artificially activate oocytes or to stimulate mitosis [40, 41].

Many efforts have been made to emphasize that AOA, as it is applied today, can be considered a safe technique. In
detail, gene expression of ICSI plus AOA was found to be closer to that in conventional IVF than with ICSI alone [42]. More reassuring evidence comes from an Italian group [43] that did not find an increased rate of chromosome segregation errors in meiosis II after exposure to 100 μM A23187 (which approximately corresponds to the tenfold concentration that is used in routine AOA). Others [44] reported that AOA did not increase the rate of aneuploid blastocysts compared to conventional ICSI. These results are in line with the present finding that irregular cleavages, potentially associated with aneuploidy [45], were not increased in ionophore-treated oocytes compared to their untreated counterparts.

More importantly, neurological development (cognition, language, motor skills) was not notably impaired in children aged 3–10 years [46]. Similarly, no increased rates of congenital birth defects were reported using AOA with calcimycin [47] or ionomycin [48]. The same holds true for the ready-to-use ionophore used in the present study since more than 130 healthy live births have been published [26, 27, 40, 41, 49, 50]. Although there is growing evidence supporting the clinical relevance of ionophores and their application in IVF laboratories, it must be clarified that AOA should not be considered a routine technique. Rather, it is an (still experimental) add-on technique that should only be used in case of a proper indication.

Annotation of morphokinetic parameters could increase the knowledge on safety aspects of this procedure. However, all AOA techniques that use data on morphokinetics are only available for chemical activation using ionophores. Preliminary published data are in disagreement regarding the potential effect of iono- and calcimycin on annotated morphokinetic parameters. Montag et al. [23] found that artificially activated oocytes (with ready-to-use calcimycin) and the associated embryos did not differ in terms of morphokinetics (t2PB to tB), while others [21, 22] did see such an association. In particular, t2PN [22], as well as tPNf, t3, and t5 [21], was found to be accelerated once ionomycin was applied for AOA.

Notably, the three preliminary studies mentioned above [21–23] analyzed only a limited number of AOA patients each (63 cycles in total). This flaw makes the only available original study on AOA and time-lapse imaging of particular interest. These authors [24] highlighted that the effect of ionomycin on the morphokinetics of (donated) oocytes and the associated embryos was minimal and limited to very early events such as t2PB and t3. They speculated that ionomycin produces a quicker increase in intracellular Ca²⁺ compared to sole injection of sperm alone. This temporal advance would then lead to an earlier inactivation of the maturation promoting factor, an earlier zink spark, earlier meiotic resumption, and, consequently, an earlier extrusion of the second polar body (t2PB). This hypothesis of our Spanish colleagues would further confirm that the first Ca²⁺ peak is responsible for the oocyte response and drives further downstream effects, for example, CaMKII modulation [51].

To the best of our knowledge, this is the first study to analyze the potential association between ionophore usage and morphokinetics. In line with Martínez et al. [24], an accelerated series of events at very early stages of oocyte activation/fertilization was observed. In particular, in the present study, the shortening of the latency of the Ca²⁺ signal was reflected by an earlier t2PNa in the ionophore group. In contrast to the Spanish study [24], no further morphokinetic parameters up to tB were affected. More interestingly, this study suggests a more synchronized cleavage pattern in embryonic cell cycle 3 (referred to as s3), in which the individual blastomeres of the 4-cell embryo cleaved in an orchestrated manner until the 8-cell stage was reached. A shorter s3 is considered a positive biomarker and was found to be predictive of blastocyst formation, blastocyst quality, and implantation [52–55].

These contradictory findings may have been caused by the different ionophore types used. Nikiforaki et al. [56] observed a higher amplitude of the initial Ca²⁺ peak with ionomycin than with ready-to-use A23187, although the latency of the peak did not show obvious differences.

Alternatively, different study designs may have played a role. All the time-lapse literature cited above [21–24] used controls that were not cultured under identical (although comparable) culture conditions. We believe that a sibling oocyte approach could circumvent these limitations.

There were also differences in patient cohorts. Martínez et al. [24] exclusively focused on a patient with a poor prognosis and a known history of complete fertilization failure due to a mutation in the male PLCζ gene, while our patient group was composed of couples with suspected fertilization problems and, as such, represents a cohort known to have a better prognosis. No patients with complete fertilization failure were included in the present work because, in such a case, splitting of oocytes is obsolete and all gametes would have been treated with GM508 CultActive to minimize the risk of transfer cancelation. In other words, there is a chance that the initial Ca²⁺ peak arises faster in patients with a substantial male factor, such as PLCζ gene mutations, while other patients with less severe impairments have a slightly longer latency of the calcium signal. The idea of “individualized” artificial oocyte activation, meaning that different medical indications might require different AOA approaches, is not new [49].

However, in light of the minimal changes in morphokinetics due to ionophores [24] and bearing in mind that a faster development at earlier stages (even if it would be conserved up to the blastocyst stage) is a rather positive sign [30], the present annotated dataset does support a relative innocuousness of ionophore treatment and, specifically, the
ready-to-use ionophore GM508 CultActive [57, 58]. These results can assist in further evaluating the safety issues of AOA.

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Declarations

Competing interests T. E. reports honoraria from Cooper Surgical, Esco, Ferring, and Gynemed outside the submitted work. All other co-authors have no conflicts of interest to declare.

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