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ASSISTED REPRODUCTION TECHNOLOGIES

Obstetric and neonatal outcome following ICSI with assisted oocyte activation by calcium ionophore treatment

Ileana Mateizel[1] · Greta Verheyen[1] · Hilde Van de Velde[1] · Herman Tournaye[1] · Florence Belva[2]

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Abstract

Purpose Calcium ionophore treatment is being used in assisted reproductive technology (ART) for cases with previous low fertilization rate or total absence of fertilization after insemination by intracytoplasmic sperm injection or when a specific indication such as globozoospermia is present. As this technique is more invasive and differs from the physiological process of fertilization, a thorough investigation of the health of the children born following this procedure is required. We intent to report the medical outcome of all children conceived following calcium ionophore treatment in our IVF center.

Methods One-armed descriptive study is performed to report the obstetrical and neonatal outcome of children born after using calcium ionophore treatment during the intracytoplasmic sperm injection procedure in our center.

Results A number of 237 cycles were included in this study, with 74 pregnancies reported, from which 47 children (31 singletons and 16 twin children) were born. No major malformations were detected in singletons. In twins, three children were diagnosed with major malformations. Minor malformations were present in seven singletons and in one twin.

Conclusions In conclusion, our results regarding birth characteristics and congenital malformations are within the expected range but, although reassuring, should be interpreted with caution due to the small number of children included.

Keywords Assisted oocyte activation · Calcium ionophore · Malformations · Neonatal outcome · Obstetrical outcome

Introduction

The introduction of intracytoplasmic sperm injection (ICSI) into clinical practice has allowed fertilization in couples with severe male factor infertility. However, ICSI does not completely prevent poor or total fertilization failure even, in case of normal sperm parameters [1]. For these cases, or when a specific indication such as globozoospermia is present, treatment with calcium ionophore during ICSI is offered in order to improve fertilization. This procedure increases the intracellular Ca^{2+} concentration and consequently artificially activates the oocytes [2]. Indeed, human fertilization is the result of a cascade of events: following the penetration of the sperm into the oocyte, intracellular Ca^{2+} oscillations are generated that will consequently regulate different events leading to oocyte fertilization and embryo development [3].

Although the use of the calcium ionophore treatment after ICSI has been shown to improve fertilization as well as overall pregnancy and live birth rates [4], the aberrant intracellular Ca^{2+} rise without subsequent oscillations differs from the physiological situation where periodic Ca^{2+} oscillations are generated [5] which is, among other issues, a reason for concern [6]. Indeed, it has been reported in animal models that a reduction or increase in the number of oscillations may have a long-term effect on both gene expression and development to term [7].

Until now, the few studies on the outcome of children born after the use of calcium ionophore report neonatal and neurodevelopmental outcomes within the normal range [8, 9]. Miller et al. [10] reported six birth defects in 62 (9.6%) children born after oocyte activation with ionophore A23187 (calcimycin), but this number was not statistically different from the 6.1% (26/426) children after ICSI without oocyte activation in their study. Reassuringly, Capalbo et al. [11]

✉ Ileana Mateizel
Ileana.Mateizel@uzbrussel.be

[1] Centre for Reproductive Medicine, Vrije Universiteit Brussel (VUB), Universitair Ziekenhuis Brussel (UZ Brussel), Laarbeeklaan 101, 1090 Brussels, Belgium

[2] Centre for Medical Genetics, Vrije Universiteit Brussel (VUB), Universitair Ziekenhuis Brussel (UZ Brussel), Laarbeeklaan 101, 1090 Brussels, Belgium

reported no widespread increase in chromosome segregation errors in the second meiotic division following the use of calcium ionophore for the activation of human oocytes.

However, these studies are based on questionnaire data or on small sample sizes, as only a small number of children were conceived using this technique. Moreover, differences in treatment protocol, in indications, and in choice of the ionophore (ionomycin or calcimycin) impede direct comparisons.

Here, we report the medical outcome in terms of obstetric and neonatal characteristics and congenital malformations of all children conceived following calcium ionophore treatment in our IVF center.

Materials and methods

Study population and study design

All cycles requiring assisted oocyte activation (AOA) with calcium ionophore treatment between 2004 and 2015 were included in this study. For some cycles, the treatment was applied to all the oocytes, while in other cycles, the sibling oocytes were treated or not with ionophore. In total, 237 initiated cycles resulted in 176 cycles with a fresh embryo transfer (ET). In 62 cycles, supernumerary embryos were frozen resulting eventually in 55 cycles with a frozen ET. Of the 231 cycles with ET (including 176 fresh and 55 frozen ET), 204 cycles were performed only with embryos originating from oocytes that had been exposed to calcium ionophore treatment. The remaining 27 cycles are not included since they had either mixed transfer with embryos originating from both treated and non-treated oocytes ($n = 10$), or transfer with embryos originating only from non-treated oocytes ($n = 17$). Two out of the 204 cycles included in the study were lost to follow-up, and finally 74 cycles with positive hCG were achieved.

All children born from these cycles were considered for analysis in this single-center observational study. The study is part of the existing follow-up program of the Centre for Medical Genetics in collaboration with the Centre for Reproductive Medicine from the UZ Brussel. As standard procedure, before starting an ART treatment, the couples were asked to participate in a prospective clinical follow-up study that focusses on the health of the offspring born after ART. This program includes the completion of standardized questionnaires (parents/gynecologist/pediatrician) after birth in addition to a thorough clinical examination performed by trained pediatricians at the Centre for Medical Genetics at the age of 2–3 months of age for families living in Belgium. Pre- or postnatal karyotyping in the child was recommended to the parents.

Identical definitions and classifications of congenital malformations have been used in all children conceived in our center since the set-up of a follow-up program [12]. Briefly, major malformations were defined as anomalies that generally cause functional impairment or require surgical correction. The remaining malformations were considered minor. The total major malformation rate was calculated as (affected live births + affected stillborns + induced abortions for major anomalies) divided by (live births + stillbirths).

No additional informed consent was required. The study was approved by the Ethical Committee of the UZ Brussel (B.U.N. 143201732431).

Assisted oocyte activation with calcium ionophore

Two calcium ionophores were used for the group of patients requiring AOA: ionomycin or calcimycin (A23187). The ionomycin treatment was performed as previously described by Heindryckx et al. [13] but with a shorter time of ionomycin exposure. Briefly, during ICSI, a small amount of 0.1 mol/L $CaCl_2$ was injected with the sperm, followed by 30 min incubation at 37 °C in 6% CO_2, 5% O_2, and 89% N_2. Consequently, the oocytes were incubated for 7 min with 10 μmol/L ionomycin (MP Biomedical, Brussels, Belgium) and carefully washed in cleavage culture medium. A second incubation with ionomycin for 7 min was applied 30 min later, followed by extensive washing and further culture in cleavage medium at 37 °C in 6% CO_2, 5% O_2, and 89% N_2.

In case of calcimycin (A23187) treatment, after ICSI, the oocytes were simply incubated for 15 min in the ready-to-use A23187 solution (CultActive; Gynmed, Lensahn, Germany) according to the manufacturer's instructions. After extensive washing, the oocytes were cultured in cleavage medium at 37 °C in 6% CO_2, 5% O_2, and 89% N_2. The A23187 ionophore was used in only few cases ($n = 17$), in cycles where sibling oocytes were treated with ionomycin.

Statistical analysis

Descriptive statistics including mean and standard deviations (SD) were calculated for all patient characteristics.

One-sample *T* test was performed to compare the neonatal data (birthweight and length), and Fisher's exact test was used to compare the total major malformation rate of children born after ionophore treatment with results in children conceived by ICSI with the use of ejaculated sperm [14].

Results

Evolution of pregnancies and number of children

In total, 74 pregnancies with positive hCG were achieved after transfer of fresh ($n = 55$) or frozen ($n = 19$) embryo(s) originating from ICSI with calcium ionophore treatment: two following A23187 treatment, five after mixed ET from cycles

where sibling oocytes were treated either with ionomycin or with A23187, and 67 following ionomycin treatment.

Of these 74 pregnancies, 9 (12.2%) were biochemical, 12 (16.2%) ended in a spontaneous abortion (< 12 weeks), and 14 (18.9%) pregnancies were lost to follow-up. There were no late spontaneous abortions, no extra-uterine pregnancies, and no terminations of pregnancies. Finally, 39 pregnancies (34 after fresh and 5 after frozen ET) were obtained in 35 patients and resulted in 47 children: 31 singletons and 16 twins. Regarding the 31 singletons, 29 were born following ionomycin treatment and 2 following A23187 treatment. Regarding the twins, 12 were a consequence of ET following ionomycin treatment and 4 following a mixed ET in cycles where sibling oocytes were treated either with ionomycin or with A2317.

Parental characteristics and indications for calcium ionophore treatment

In the majority of the cycles which resulted in live born children (29/39; 74%), ICSI was performed because of poor sperm quality. In 2 cycles, ICSI was performed because of female factor infertility and in 1 cycle due to a combination of both male and female infertility factors. In 4 cycles, PGD was performed. Three couples suffered from idiopathic infertility.

The indications for the use of calcium ionophore treatment were as follows: low ($n = 14$) or no ($n = 16$) fertilization in previous cycles, globozoospermia ($n = 6$), small acrosome ($n = 2$), and poor embryo development ($n = 1$).

Except for one case in which fresh testicular semen was used, the ICSI cycles were performed with ejaculated sperm (37 with fresh and 1 with frozen sperm).

From the 35 patients included in this study, 29 had previous cycles without AOA (17 with 1 cycle and 12 with at least 2 cycles), from which five deliveries were obtained. In all five cases, poor or no fertilization was obtained in at least 1 cycle before calcium ionophore treatment was indicated.

Genetic counseling and karyotype analysis were performed in 21 out of the 35 couples. All tests showed normal karyotypes, except for one father that had Klinefelter syndrome.

Obstetric outcome

Mean maternal age was 32.3 ± 3.9 years and mean maternal BMI was 24.6 ± 5.0 kg/m^2.

Complications in singleton pregnancies were as follows: pregnancy-induced hypertension ($n = 1$), preterm labor/PROM ($n = 2$), early hemorrhage (< 20 weeks) ($n = 1$), abruptio placentae ($n = 1$), and gestational diabetes ($n = 4$). Complications in twin pregnancies were as follows: preterm labor/PROM ($n = 2$) and early hemorrhage (< 20 weeks) ($n = 1$).

Table 1 Neonatal characteristics and congenital malformations in singletons and twins born after ionophore treatment

	Singletons ($N = 31$)	Twins ($N = 16$)
Neonatal characteristics		
Liveborns	31	16
Stillborns	0	0
Gender ratio: male/female	18/13	9/7
Birth weight (g)	3290.8 ± 445.6	2241.5 ± 440.2
Birth weight < 2500 g	1 (3.2%)	13 (81.2%)
Length (cm)	50.3 ± 2.6	45.5 ± 3.4
Head circumference (cm)	34.1 ± 1.2	N.A.
Gestational age (weeks)	39.2 ± 1.5	35.9 ± 1.7
Preterm 32–37 weeks	2	10
Preterm < 32 weeks	0	0
Mode of delivery		
Vaginal	21	6
Cesarean section (%)	8	10
Other	2	0
Apgar score < 7 at 5 min	1	1[#]
Early neonatal death	0	1
Late neonatal death	0	0
Infant death	0	0
Perinatal death	0	1
De novo karyotype anomaly (pre- or postnatally) detected	0/10	0/6
Congenital malformations		
Major malformations	0	3
Unilateral hip and knee dislocation treated by surgery		1
Anus imperforatus		1
Severe renal dysplasia and pulmonal hypoplasia		1[#]
Minor malformations°	7	1
Metatarsus adductus	1	
Overriding toes	1	
Supernumerary nipple	1	
Hydronephrosis without treatment	1	
Epicanthal folds	1	
Hypertelorism	1	
Foramen ovale	1	
Large hemangioma (> 12 cm)		1

Mean (SD) or *n* (%)

[#] Child died shortly after birth due to major congenital malformations (normal karyotype)

°A child can have more than one minor malformation

Neonatal outcome

Birth parameters and congenital malformations of the singletons and twins are presented in Table 1. Birth characteristics were based on questionnaire data and were available for all

children. In five children, examination at 2–3 months of age could not be performed in our center because they were living abroad, but extensive written information regarding their health provided by their pediatrician was available. From ten children, only written information provided by parents or pediatricians was available at follow-up. From three children, no information was available at follow-up.

All children were born alive, but one of the twins died shortly after birth due to congenital malformations.

Mean birth weight was 3290.8 g ± 445.6 for singletons and 2241.5 g ± 440.2 for twins, while the mean length was 50.3 cm ± 2.6 and 45.5 cm ± 3.4, respectively.

When compared to historical control data from our center regarding children born after ICSI using ejaculated sperm [14], no statistically significant difference was observed for both birth weight and length in singletons (mean 3224 g ± 582 and 49.6 cm ± 3.1; $P = 0.4$ and $P = 0.1$ respectively) and twins (mean 2394 g ± 522 and 46.2 cm ± 3.3; $P = 0.2$ and $P = 0.5$, respectively).

Mean gestational age was 39.2 weeks ± 1.5 for singletons and 35.9 weeks ± 1.7 for twins. Karyotype analysis was performed in ten singletons and in six twins; no anomalies were found.

No major malformations were detected in singletons. In twins, three children were diagnosed with major malformations: one member of a twin with a unilateral hip and knee dislocation treated by surgery, one member of a twin with an imperforate anus, and one member of a twin died shortly after birth due to severe renal dysplasia and pulmonal hypoplasia (normal karyotype). Minor malformations were present in seven singletons and in one twin.

In the present sample, total major malformation rate was 6.3% (3/47) which was comparable to 4.1% ($P = 0.4$) as described previously in a cohort of children born after ICSI using ejaculated sperm in our IVF center [14].

Discussion

The present report describes the obstetrical and neonatal outcome of children born after AOA with calcium ionophore treatment performed in our IVF center, and represents, to our knowledge, the largest series reported to date on this subject. Although the findings are reassuring for singletons and twins regarding birth characteristics and congenital malformations, these results should be interpreted cautiously given the small sample size.

The main strength of this single-center study is that the majority of the results were based on a clinical examination performed by trained pediatricians. Families living abroad were obviously not invited for clinical visit in our center, but parents were encouraged to provide medical reports of their children which resulted in full written data of five children. Furthermore, only in ten (21%) children, we had to rely on questionnaire data only.

The fact that a quarter of the pregnancies was lost to follow-up is unfortunately inevitable since our IVF center is one of the few that offer this treatment and patients return to their original place of residence. Nevertheless, many efforts are made to retrieve information on the course of the pregnancy. Although beyond the scope of this report, the pregnancy rate after AOA (per embryo transfer) was 36.2% which is in line with the reported 36.9% in a recently published meta-analysis including 1521 cycles spread over 14 studies [4]. Our reported miscarriage rate of 16.2% following AOA is in line with results from other groups: 15.1% [8] and 16.9% [10].

As AOA represents an invasive non-physiological technique [15], follow-up of the children is crucial. However, several pitfalls are encountered when describing the health of children born after use of calcium ionophore treatment. Firstly, due to its specific indication, the number of patients that may benefit from this technique is rather small, leading to a low number of children born after transfer of an embryo obtained after oocyte activation. While we described the medical outcome of 47 children conceived at our center, only two other reports on medical follow-up of children born after AOA are available in literature, both from the same group [8, 9]. Obviously, the described number of children should be much higher in order to draw firm conclusions regarding the safety of AOA for the offspring, particularly regarding congenital malformations.

Secondly, the variation in oocyte activation protocols impede proper comparison with other centers. Most papers [10, 16–19] reported data based on the use of ionophore A23187 to activate the oocytes, using a straightforward one-step incubation protocol, however, with different A23187 concentrations (5 μmol/L, 10 μmol/L, or unspecified) and/or time of exposure (30, 15, and 10 min). Overall birth defect rate in singletons and twins has been reported as high as 9.6% after use of A23187 [10]. However, the ionomycin treatment implies the injection of $CaCl_2$ into the oocyte during ICSI followed by two incubation steps with ionomycin, and is more invasive for the oocyte than the single incubation with A23187. We have used both ionophores in our study, and the total congenital malformation rate in our study is 6.3% (3/47), with all malformations reported in children born after ionomycin treatment. In the study of Vanden Meerschaut et al. [8], who used only ionomycin treatment, no birth defects were reported at delivery, whereas at toddler age, birth defects were reported in 14.3% (3/21) of the children following assessment. Nevertheless, in all studies, the numbers are small and should be interpreted with caution.

Thirdly, the variation in indications and in patient selection for AOA makes it very difficult to compare outcomes, not only between centers but also between populations. In our series, both fresh and frozen embryo transfers are included,

as well as embryos biopsied for pre-implantation genetic diagnosis. Moreover, the use of non-ejaculated sperm was required in some cycles. This wide variety in cycle and patient characteristics hampers the comparison with an appropriate control group. Therefore, we compared our current findings of birth weight, height, and malformation rate with our previously reported findings in children born after ICSI with ejaculated sperm in our IVF center [12]. The fact that the changes in culture media and freezing protocols over time were simultaneously adopted in all treatment cycles (control and AOA population) in our center is another asset. Reassuringly, birth characteristics and total major malformation rate were comparable between the present findings and our control data.

Ideally, the best study group should be composed of siblings born to the same parents from cycles with or without ionophore treatment, which could reveal adverse effects linked to the invasive technique of oocyte activation itself. In our series, five couples have both a child after ICSI and after ICSI with calcium ionophore treatment. Obviously, this number of children will always be limited, and confounders (e.g., effect of parity or type of embryo transfer: frozen rather than fresh embryo transfer) should also be taken into account when interpreting results.

Another plausible study group could be the children born from embryos originating from sibling oocytes, treated or not with calcium ionophonore, that were transferred and lead to pregnancy in different cycles.

Although we present rather reassuring findings, possible adverse epigenetic effects following oocyte activation may only be revealed in future generations [15]. Considering that Ca^{2+} oscillations around fertilization coincide with the active and rapid demethylation of the paternal genome followed by a passive DNA demethylation of maternal genome, it is plausible that an altered Ca^{2+} signal, as was reported following calcium ionophore treatment [20], may be associated with modifications in genomic imprinting.

In conclusion, although keeping in mind that this is an one-armed descriptive study with limited sample size, neonatal outcome of singletons and twins born after calcium ionophore treatment seems to be reassuring so far. Nevertheless, we join others [8, 15] in their concern of using calcium ionophore in other than rare cases of fertilization failure or very low fertilization rate after ICSI (< 20%). Our prudent attitude towards the use of calcium ionophore is indeed reflected in the rather low number of AOA cycles applied during the past 11 years. Although AOA with calcium ionophore has been reported to improve pregnancy rates, no information on the long-term health of the offspring is yet available. As male infertility is often the case in patients treated with AOA, long-term follow-up is mandatory, particularly in view of the recent findings regarding low semen quality in young adult ICSI offspring born to fathers with impaired spermatogenesis [21].

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Compliance with ethical standards

No additional informed consent was required. The study was approved by the Ethical Committee of the UZ Brussel (B.U.N. 143201732431).

Conflict of interest The authors declare that they have no conflict of interest.

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