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Live birth after artificial oocyte activation using a ready-to-use ionophore: a prospective multicentre study

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Abstract Artificial oocyte activation has been proposed as a suitable means to overcome the problem of failed or impaired fertilization after intracytoplasmic sperm injection (ICSI). In a multicentre setting artificial oocyte activation was applied to 101 patients

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who were diagnosed with fertilization abnormalities (e.g. less than 50% fertilized oocytes) in a previous conventional ICSI cycle. Female gametes were activated for 15 min immediately after ICSI using a ready-to-use Ca^{2+} -ionophore solution (A23187). Fertilization, pregnancy and live birth rates were compared with the preceding cycle without activation. The fertilization rate of 48% in the study cycles was significantly higher compared with the 25% in the control cycles ($P < 0.001$). Further splitting of the historical control group into failed (0%), low (1–30%) and moderate fertilization rate (31–50%) showed that all groups significantly benefitted ($P < 0.001$) in the ionophore cycle. Fewer patients had their embryo transfer cancelled compared with their previous treatments (1/101 versus 15/101). In total, 99% of the patients had an improved outcome with A23187 application resulting in a 28% live birth rate (35 babies). These data suggest that artificial oocyte activation using a ready-to-use compound is an efficient method. 

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Introduction

The introduction and implementation of intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992) into the field of assisted reproduction has been a major benefit for the treatment of male infertility. This breakthrough has allowed fertilization even in couples with severe male infertility because depositing a single sperm directly into the cytoplasm bypasses all anatomical structures. It has been stated that the origin of the sperm has no negative effect on ICSI outcome (Bukulmez et al., 2001) regardless of whether the male gamete was obtained by masturbation or by invasive techniques such as microsurgical epididymal sperm aspiration or testicular sperm extraction (Devroey et al., 1994; Silber et al., 1994).

It is obvious that the success rate for using spermatozoa from different sources depends on the sperm count and motility of the male gametes (Palermo et al., 1996). On average, ICSI guarantees constantly high fertilization rates of up to 70–80% (Montag et al., 2012). Today, complete fertilization failure after ICSI is indeed a rare event (Liu et al., 1995), but does happen even in the presence of a presumptively normal spermatozoon. Moreover, low or moderate fertilization (<30%) can be observed in repeated ICSI cycles for some patients (Montag et al., 2012). The presence of such fertilization problems after ICSI raises the question of the underlying cause, especially the role of the male gamete for achieving successful oocyte fertilization. In this process, it is especially activation of the oocyte by phospholipase C zeta (PLC zeta), a sperm borne factor, which is of utmost importance (Saunders et al., 2002; Swann et al., 2004). This physiological agent causes the production of inositol-triphosphate (IP3) in the ooplasm, which then binds to its receptors at the endoplasmic reticulum (Kashir et al., 2010). Calcium is then released from these stores in an oscillatory mode. Sperm-induced Ca^{2+} oscillations stimulate mitochondrial respiration and, in turn, the resulting adenosine triphosphate production required to maintain sperm-triggered calcium waves.

Recently, a close relationship between an absence of, or reduction in, PLCzeta content and failed oocyte activation and fertilization was demonstrated in humans (Heytens et al., 2009; Yoon et al., 2008). Intriguingly, a deficiency in intracellular calcium can be compensated by approaches that aim for an artificial calcium entrance or release.

Modified ICSI techniques have successfully been applied to obtain fertilization (Ebner et al., 2004; Tesarik et al., 2002). Electrical activation is another alternative, although not frequently applied (Baltaci et al., 2010; Mansour et al., 2009). More commonly, artificial oocyte activation is induced by a

variety of chemical agents, such as 6-dimethylaminopurine, strontium chloride or calcium ionophores, such as ionomycin and calcimycin (A23187) to name but a few (Heindryckx et al., 2008; Kashir et al., 2010; Rybouchkin et al., 1997). Although the potential of calcium ionophores to support oocyte activation and achieve acceptable fertilization rates has been tested in mice (Heytens et al., 2008, 2010), and has been emphasized since the early years of ICSI in humans (Tesarik et al., 1995), larger studies on a patient cohort with ICSI fertilization problems have not been conducted. Montag et al. (2012) were the first to retrospectively analyse their oocyte activation patients and to propose that the benefit of artificial oocyte activation is dependent on the fertilization rate of the previous treatment cycle. The cut-off value for fertilization rate in the preceding cycle that distinguished between an increase in fertilization rate in the presence of A23187 and no improvement was 30% (Montag et al., 2012).

In this context, the present multicentre trial was set up to test prospectively the above mentioned retrospective data (Montag et al., 2012), but on a different population, having no severe male factor. In addition, a larger number of patients was treated with a ready-to-use compound instead of a home-made product.

Material and methods

This almost 2-year prospective multicentre analysis was conducted at six IVF clinics in Germany ($n = 5$) and Austria ($n = 1$). All patients involved (female age: 33.9 ± 4.1 years, male age: 37.3 ± 5.8 years) gave written consent, and ethical approval was granted (reference number 4-J-09 from the 4 June 2009). Splitting of oocytes (with and without A23187) was avoided as previously suggested (Ebner et al., 2012; Montag et al., 2011), as all patients included had a history of fertilization failure or problems. To guarantee the prospective approach, all cycles ($n = 101$) were reported to the director of the study (MM) on the day of oocyte collection. Each patient was only included once.

Patients

The inclusion criteria comprised a history of ICSI fertilization problems (e.g. <50% fertilization rate) as well as the presence of at least three cumulus-oocyte-complexes in order to exclude the role of chance in terms of failed fertilization. Severe male factor indication (Ebner et al., 2012) was

Table 1 Comparison of study and historical control groups. The latter was subdivided according to the previous fertilization rate (Montag et al., 2012).

	Control group			Study group
	0%	1–30%	31–50%	
Cycles (n)	15	52	34	101
COC (n)	93	759	375	1088
MII oocytes (n)	79	735	373	884
Two pronuclei ^a	0 (0)	148 (20.1)	147 (39.4)	422 (47.7)
Cultured to blastocyst (n)	0	19	14	86
Blastulation rate ^a	0	4 (21.1)	3 (21.4)	49 (57.0)

COC = cumulus–oocyte–complex; MII = metaphase II.

^a $P < 0.001$ (control versus study group); calculation of blastocyst formation rate was only possible in one centre (F) responsible for one-quarter of the cases.

excluded from the study. Ovarian stimulation and oocyte retrieval followed standard protocols using either long protocol with a GnRH-agonist or short version with antagonist application. Ovulation was triggered by administering human chorionic gonadotrophin (HCG), and ovum retrieval was scheduled 36 h later (Ebner et al., 2012; Montag et al., 2012).

ICSI, culture, embryo transfer and pregnancy

All interventions, including sperm collection and preparation, oocyte retrieval, ICSI, embryo culture and transfer were carried out according to the standard protocol of the individual centre (Ebner et al., 2012; Montag et al., 2012). In center F, the only Austrian clinic involved, all oocytes were cultured after insemination and pronuclei check to the blastocyst stage, and one to two blastocysts were chosen for transfer based on morphology. In the German centres, however, the presence of pronuclei was checked 16–20 h after ICSI. Two to three two-pronuclear oocytes were chosen for further culture and transfer and surplus two pronuclei oocytes were cryopreserved. Embryo transfer was carried out on day 2–3 according to the policy of the centre. Outcome, however, was not affected by national regulations as live birth rate between centre F (8/25) and centres A–E (20/76) were comparable. The mean number of embryos and blastocysts transferred was 1.85 (± 0.5). Quality was assessed according to previously published guidelines (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

A first beta-HCG test was carried out 14 days after ovum retrieval, and, if positive, the test was repeated 2 days later. The presence and number of fetal sacs was confirmed by ultrasound scan 2 weeks later, and fetal heart beat was investigated another 14 days later. The implantation rate was calculated by the number of fetal sacs divided by the number of embryos transferred. Pregnancy was defined as rising beta-HCG. Only pregnancies with a proven implantation were classified as clinical pregnancies. Pregnant patients were asked to submit relevant data after the birth of the child. In those rare cases in which the said birth report did not work properly, obstetric and neonatal data were actively requested from the maternity hospital in charge. Spontaneous abortion was defined as the spontaneous loss of a pregnancy before 24 weeks of gestation.

Artificial oocyte activation

Oocyte activation was carried out as described (Montag et al., 2011), with some slight modifications. Dishes for activation were prepared with a 50- μ l droplet of a ready-to-use A23187 solution (CultActive, Gynemed, Lensahn, Germany) and several droplets of standard culture medium for washing after activation, and were covered by mineral oil. Freshly prepared dishes were incubated in CO₂ atmosphere for at least 2–4 h before use. Immediately after ICSI, oocytes were incubated for 15 min in the activation solution, then thoroughly washed in culture medium and incubated under standard conditions in a humidified atmosphere. Except for the activation procedure, the different participating centres used their standard culture scheme with no further modifications.

Statistics

As in our other ionophore studies (Montag et al., 2011; Ebner et al., 2012), the previous cycles without artificial activation served as control groups in order to work out the positive action of the ionophore. For statistical analysis of the effect of an ionophore treatment on fertilization rate, a repeated measures analysis of variance, including the between-subject factor 'centre' was performed using the Statistical Package for Social Sciences SPSS version 22 (IBM Corp., USA). Post-hoc Scheffé corrected tests were carried out for the three major centres (A, D, F). Furthermore, Wilcoxon rank sum test or McNemar tests were applied whenever appropriate as indicated in the text. $P < 0.05$ was considered significant.

Results

As highlighted in Table 1, oocyte collection in the present 101 cycles resulted in 1088 cumulus–oocyte complexes. Of these complexes, 81.3% contained metaphase II oocytes usable for ICSI. The average number of mature eggs per patient was 8.8 \pm 4.3. Of these, 422 (47.7%) showed the presence of two pronuclei the following day. In addition, 373 zygotes had no signs of fertilization (42.2%), 36 revealed one or three pronuclei (4.1%) and another 53 (6.0%) degenerated after ICSI.

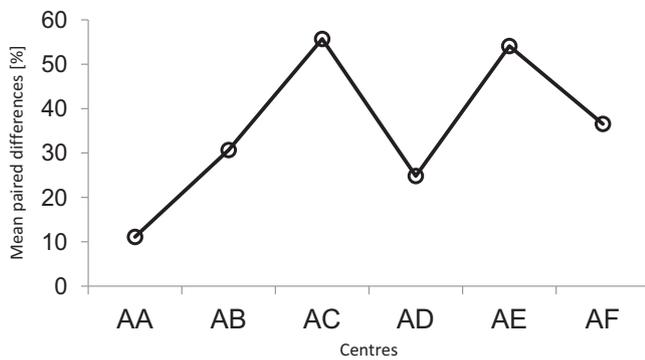


Figure 1 Mean paired differences between fertilization rates between the study cycle and the control cycle.

A repeated measure analysis of variance revealed a significant difference between the fertilization rates of study and control cycles ($P < 0.001$). Moreover, the between-subject factor 'centre' as well as the interaction factor 'treatment x centre' revealed significant differences ($P = 0.042$ and $P = 0.022$, respectively). Post-hoc tests on the three major centres showed a significant difference between centre D and F ($P = 0.036$). For statistical reasons, it was decided to compare only the three centres responsible for larger number of cycles. The three remaining clinics had limited numbers of cycles treated ($n = 6$, $n = 6$, and $n = 2$). The mean paired differences by centres are shown in **Figure 1**.

Further splitting of the historical control group as suggested by Montag et al. (2012) into failed (0%), low (1–30%) and moderate fertilization rate (31–50%) revealed that the distribution of all groups (**Table 1**) changed significantly after the artificial oocyte activation (Wilcoxon rank sum test, $P < 0.001$). More detailed analysis showed that this significant increase was observable in all major contributing centres ($P = 0.032$ for centre A; $P < 0.0001$ for centres D and F). Overall, the maximum likelihood estimate for the probability of an improvement in fertilization rate by artificial activation using the present calcium ionophore was 83% (79% centre A, 81% centre D, and 90% centre F).

All zygotes kept in culture at the German centres as well as 98.6% of the zygotes cultured in the Austrian centre cleaved on day 2. Embryo quality was not statistically significantly different compared with present and previous treatments. For the above-mentioned reason, calculation of blastocyst formation rate was only possible in one centre, which contributed one-quarter of the cases ($n = 25$). The blastulation rate based on those 17 patients whose embryos were cultured to day 5 was 57%.

In general, significantly fewer patients had their embryo transfer cancelled compared with their previous treatments (1/101 versus 15/101; McNemar's test: $P < 0.0001$). Overall, 100 patients had an embryo or blastocyst transfer that resulted in a 35% clinical pregnancy rate. In total, seven (20%) spontaneous abortions were observed, which occurred in the 5th, 8th, 9th ($\times 3$), 11th and 19th week of gestation. The live birth rate was 28% as indicated in **Table 2**. Because of legal situation in Germany, the multiple pregnancy rate was almost 30%, but no triplets were conceived. The male–female ratio in singleton pregnancies was 2.4 and in twin pregnancies 1.25. All children were born healthy except one male newborn (from

Table 2 Treatment outcome in the study group with a ready-to-use ionophore (rate in per cent).

Started cycle	101
Cycles with at least one fertilization	100
Pregnancy	37 (37)
Ectopic pregnancy	2
Clinical pregnancy	35 (35)
Multiple pregnancy	10/35 (28.6)
Vanishing twins	3
Spontaneous abortion	7 (20.0)
Implantation rate	47/185 (25.4)
Live birth per started cycle	28 (27.7)
Children born from singleton pregnancy	18
Children born from twin pregnancy	17
Malformation	1 (2.9)

Table 3 Neonatal outcome of children born after artificial oocyte activation.

	Singleton pregnancy	Twin pregnancy
Children	18	17
Delivery		
Median (week)	39	37
Range (week)	32–42	30–41
Length (cm)		
Median (cm)	50	48
Range (cm)	45–54	39–54
Weight (g)		
Median (g)	3180	2440
Range (g)	2510–4040	1475–3890
Malformation ^a	1	0

^aAnal atresia.

a singleton pregnancy) who was diagnosed with anal atresia. Therefore, malformation rate was not increased compared with a regular ICSI cohort (Ludwig and Katalinic, 2002) in this poor-prognosis cohort and stayed below 3%.

In **Table 3**, birth weight and other neonatal data are shown to be related to the order of the pregnancy (i.e. singleton or twin). A comparison with prior 'control' cycles to establish whether ionophore treatment had an effect on neonatal data was not possible, as only a single pregnancy resulting in live birth had been reported from the preceding control cycles.

Discussion

Much of what is known about biochemical processes during artificial oocyte activation comes from animal research. Vanden Meerschaut et al. (2013) pointed out that the percentage of oocytes showing calcium rises as well as the number of transients per oocyte was significantly lower in a mouse having sperm with globozoospermic phenotype compared with the wild-type. Application of electrical pulses, strontium chloride, or ionomycin all led to higher fertilization rates. Post-implantation development did not differ significantly between the three activation groups, but pregnancy rate was favoured by use of Sr^{2+} and electrical pulses. Interestingly, the

birth weight of female pups at certain time points was significantly lower in the strontium cohort compared with wild-type mice (Vanden Meerschaut et al., 2013). In mice, gene expression patterns were found to be affected after chemical oocyte activation with strontium (Rogers et al., 2006). Whether oocyte activation may cause epigenetic modifications in a similar way as assumed for in-vitro culture or culture media (Dumoulin et al., 2010) needs further investigation.

In contrast to strontium chloride (Kline and Kline, 1992; Zhang et al., 2005), calcium ionophores (e.g. CultActive) do not result in physiological calcium oscillations, rather a single Ca^{2+} transient is caused, which helps to pass a critical threshold of calcium availability (Heytens et al., 2008; Kline and Kline, 1992; Rinaudo et al., 1997). Evidence that the above-mentioned physiological oscillations in mouse oocytes can be carried over to the human is still needed, as Rogers et al. (2004) failed to observe calcium transients when they incubated human oocytes in Sr^{2+} -containing medium.

In humans, as in most mammals, PLC zeta was identified to be one of the most likely causes of failed oocyte activation and fertilization (Saunders et al., 2002; Swann et al., 2004), although other intrinsic factors may be involved as well and, de facto, fertilization failure or problems can be either oocyte- or sperm-related (Swain and Pool, 2008; Tesarik et al., 2002).

The actual potential of spermatozoa from a male with proven fertilization problems to activate a human oocyte can be estimated by injecting such sperm (or a sperm extract) into mouse eggs, which is known as the so-called mouse oocyte activation test (Heindryckx et al., 2005; Vanden Meerschaut et al., 2012). When this test was applied in patients with severe male factor, a high rate of oocyte activation failure was reported (Heytens et al., 2009; Kyono et al., 2008). Therefore, it is likely that fertilization rates below 50% in repeated ICSI cycles in the same patient will be caused by impaired oocyte activation probably owing to a sperm deficiency.

Routinely, artificial oocyte activation with various chemical stimuli is used to overcome the problem of failed or very low fertilization. Such agents have been applied successfully to patients with borderline normozoospermia who otherwise had no underlying disease (Heindryckx et al., 2005, 2008; Kyono et al., 2008; Yanagida et al., 2006). It is beyond doubt that calcium ionophores, such as ionomycin or calcimycin (A23187), are the most popular artificial activating substances for human oocytes, especially when dealing with severe teratozoospermia, such as globozoospermia at the worst (Ahmady and Michael, 2007; Ebner et al., 2012; Moaz et al., 2006; Nasr-Esfahani et al., 2008; Taylor et al., 2010; Tejera et al., 2008).

It is evident, however, that calcium ionophore treatment cannot be the solution for all activation failures (Check et al., 2007). Indeed, Vanden Meerschaut et al. (2012) suggested that assisted oocyte activation is not beneficial for all patients with a suspected activation deficiency. This conclusion is supported by both our retrospective (Montag et al., 2012) and the present analysis, which show that success of ionophore treatment is related to the fertilization rate in a previous cycle. In detail, it could be confirmed, that the best effect is obtained if A23187 is used in patients with a history of less than 30% fertilization in a previous ICSI cycle. In contrast to the retrospective approach, however, a ready-to-use ionophore also significantly improved the fertilization rate in patients with a moderate fertilization rate (31–50%) in the preceding cycle. This supports data from Belgium (Vanden Meerschaut

et al., 2012) showing successful treatment of patients suffering from oocyte-related activation problems causing total fertilization failure. In cases of known low fertilization, Vanden Meerschaut et al. (2012) recommend working with split sibling oocytes (with and without ionophore), which may facilitate 'distinguishing between a molecular oocyte-related activation deficiency and a previous technical or other biological failure'.

Despite the significant increase in fertilization rates, the present values do not reach the range (70–77% reported by others [Heindryckx et al., 2005, 2008; Vanden Meerschaut et al., 2012]). This may be due to differences in the patient cohort (Montag et al., 2012; Vanden Meerschaut et al., 2014a), but, more likely, disparities in type, concentration, exposure time, mode of application of the ionophore, or both, are responsible for this inconsistency. In particular, the latter parameter is all but standardized, as highlighted in the elegant review of Vanden Meerschaut et al. (2014a). A wide variety of application modes is listed in this review reaching from single ionophore (ionomycin or calcimycin) exposure for 10 min to twofold exposure for an overall 20 min. The most invasive approach, and obviously the most effective one in terms of fertilization, is a combination of ICSI in which the spermatozoon is injected along with a 0.1 mol/L CaCl_2 solution. This process is then followed by a double exposure to ionomycin, 10 and 30 min apart from ICSI (Heindryckx et al., 2008; Vanden Meerschaut et al., 2012). By adapting this Belgian protocol (Heindryckx et al., 2008; Vanden Meerschaut et al., 2012), by repeating the 15-min ready-to-use ionophore incubation period after a break of 30 min (unpublished data), the efficiency of CultActive can be increased.

Regardless of the invasiveness of the activation technique, it seems that either a ready-to-use Ca^{2+} -ionophore or a home-mixed solution results in apparently healthy offspring (Ebner et al., 2012; Heindryckx et al., 2005; Montag et al., 2012; Vanden Meerschaut et al., 2014b). Even more interestingly, the developmental outcome of children aged 3–10 years and born after artificial oocyte activation was within expected ranges (Vanden Meerschaut et al., 2014b). The malformation presented here, however, is associated with maternal age and sperm source rather than with ionophore treatment. Morphokinetic analysis of embryos derived from artificially activated oocytes (using a ready-to-use ionophore) was developmentally comparable to embryos stemming from conventional ICSI (Montag et al., 2013). Furthermore, Borges et al. (2009) stressed that A23187 application is not a significant determinant of the likelihood of spontaneous abortion when using ejaculated, epididymal, or testicular sperm.

To the best of our knowledge, this is the first report of a major malformation (anal atresia) after artificial oocyte activation using the aforementioned ionophore calcimycin. It is acknowledged that assisted reproductive technologies *per se* might increase the risk of anal atresia compared with those infants conceived *in vivo* (Källén et al., 2010). It has been observed that anal atresia may also happen in conventional IVF or ICSI without the use of artificial oocyte activation (Chen et al., 2013; Kanasugi et al., 2013). Numbers of live births registered after ionophore treatment are still too low to allow proper calculation of the actual risk of anal atresia in a cohort of ionophore treated patients. To date, there is no evidence that the incidence of this gastrointestinal defect in

ionophore treated patients differs from that of babies conceived after other assisted reproductive technologies (e.g. 0.14% in ICSI using non-ejaculated sperms) (Belva et al., 2011).

One question still remains to be answered. What to do if ionophore treatment does not result in normal fertilization? If one uses a less invasive approach (i.e. ready-to-use ionophore CultActive in this study), one option would be to switch to a two-fold exposure strategy or to combine different activating stimuli (Nakagawa et al., 2001; Nakasaka et al., 2000). On the other hand, if the methodology used is already at the upper end of the manipulation scale (Heindryckx et al., 2008; Vanden Meerschaut et al., 2012), there is not much left to try. Theoretically, evidence shows that recombinant PLC zeta might make it possible to overcome the unwanted scenario of failed fertilization (Nomikos et al., 2013; Yoon et al., 2012). Although this would result in more physiological calcium oscillations, the dosage of PLC zeta to inject is uncertain.

Either way, artificial oocyte activation still needs to be considered as experimental (Montag et al., 2012; Vanden Meerschaut et al., 2014a) and its application requires thorough consultation with the patient and should only be done if the correct indications are present.

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