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MINI-REVIEW

Assisted oocyte activation following ICSI fertilization failure

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Frauke Vanden Meerschaut was born in Belgium in 1984 and concluded her medical studies at the University of Ghent, Faculty of Health Sciences and Medicine in 2009. Since then, she has joined the research team of the Centre for Reproductive Medicine at the Ghent University Hospital. She is interested in different aspects of reproductive medicine. She recently finalized her PhD on assisted oocyte activation and started working full time as an gynaecology obstetrics trainee.

Abstract The capacity of intracytoplasmic sperm injection (ICSI) to permit almost any type of spermatozoa to fertilize oocytes has made it the most successful treatment for male factor infertility. Despite its high success rates, fertilization failure following ICSI still occurs in 1—3% of couples. Assisted oocyte activation (AOA) is being increasingly applied in human assisted reproduction to restore fertilization and pregnancy rates in couples with a history of ICSI fertilization failure. However, controversy still exists mainly because the artificial activating agents do not mimic precisely the initial physiological processes of mammalian oocyte activation, which has led to safety concerns. This review addresses the mechanism of human oocyte activation and the relatively rare phenomenon of fertilization failure after ICSI. Next, it describes the current diagnostic approaches and focuses on the application, efficiency and safety of AOA in human assisted reproduction.

KEYWORDS: assisted oocyte activation, diagnosis, failed fertilization, ICSI, male infertility, safety

Introduction

During intracytoplasmic sperm injection (ICSI), a single spermatozoon is injected directly into the cytoplasm of a mature oocyte via micromanipulation. This is an effective method for assisting reproduction in men with suboptimal semen parameters, typically oligoasthenoteratozoospermia (OAT) or in couples who experience zero or low fertilization rates after conventional IVF. The first pregnancies following ICSI were reported in the early 1990s (Palermo et al., 1992). With the advent of ICSI, almost any type of spermatozoa can now fertilize oocytes, making it the most successful treatment of male factor infertility.

The indications to perform ICSI have been broadened widely in the past decade. Men suffering from obstructive and nonobstructive azoospermia in whom spermatozoa are

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surgically retrieved from the epididymis (microsurgical epididymal sperm aspiration) or testis (testicular sperm extraction) can now be treated by ICSI. Other indications for ICSI are cycles in which preimplantation genetic diagnosis is performed and HIV-discordant couples to minimize the exposure of the oocyte to multiple spermatozoa. Fertilization rates of up to 80% and clinical pregnancy rates of up to 45% are observed following ICSI. The latter success rates have reduced the need for donor sperm insemination or adoption in couples with severe male factor infertility (Palermo et al., 2009).

Despite the high success rate of ICSI, total fertilization failure still occurs in 1–3% of all ICSI cycles and can recur in subsequent cycles, even when a sufficient number of oocytes and motile spermatozoa are available (Esfandiari et al., 2005; Flaherty et al., 1998). Failure of oocyte activation is considered to be the main cause of fertilization failure following conventional ICSI (Flaherty et al., 1998; Liu et al., 1995; Rawe et al., 2000). Less common causes include failed sperm head decondensation, premature sperm chromatin condensation, oocyte spindle defects and sperm aster defects (Swain and Pool, 2008). Furthermore, fertilization failure can be due to technical problems (e.g. incorrect sperm injection), a limited availability of mature or morphologically normal oocytes, a lack of motile spermatozoa and severe forms of teratozoospermia, such as globozoospermia (Dam et al., 2007; Yanagida, 2004).

Several reports show that the majority of couples suffering from ICSI failure benefit from the application of ICSI combined with assisted oocyte activation (AOA) (Heindryckx et al., 2005, 2008; Montag et al., 2012; Nasr-Esfahani et al., 2010; Vanden Meerschaut et al., 2012). Until adequate scientific evidence regarding the safety and efficacy of ICSI–AOA is available, it should not be considered an established treatment and should be applied with caution. This review tackles the different clinical aspects of fertilization failure following ICSI and the use of AOA in assisted reproduction clinics.

The mammalian oocyte activation mechanism

Oocyte activation is a series of events that converts a metaphase-II-arrested oocyte into a fertilized egg ready to begin embryogenesis. In most mammals, oocyte activation is a spatial–temporal regulated process triggered by the sperm entry. Firstly, the zona pellucida undergoes physical and chemical changes by the extrusion of the cortical granules from the oocyte to prevent polyspermy and to protect and support the embryo. The oocyte has to be released from the metaphase-II arrest to allow the completion of the cell cycle and the formation of the haploid female pronucleus capable of combining with the sperm-derived pronucleus. Also, maternal mRNA and proteins undergo dynamic changes and post-translational modifications. Finally, cytoskeletal rearrangements have to occur to support the zygote's growth and embryo development (Horner and Wolfner, 2008).

There is now a general consensus that the master key to initiate all the cytological changes in fertilized oocytes is a series of intracellular calcium rises that start shortly following spermatozoon–oocyte fusion (Miyazaki and Ito, 2006; Ramadan et al., 2012) (**Figure 1**). In vertebrates, the intracellular calcium rise is largely due to the release of calcium from intracellular stores within the endoplasmic reticulum (ER; Wakai and Fissore, 2013). Intracellular calcium oscillations upon fertilization continue from a few minutes to about several hours, depending mainly on the species considered (Ducibella and Fissore, 2008). Progressive decline and eventual termination of calcium oscillations typically occur when pronuclei are formed (Day et al., 2000; Jones, 2005). In IVF the initial calcium increase starts within a few minutes of spermatozoal fusion, whereas following ICSI this trigger is provoked immediately during injection of the spermatozoa by the artificial calcium influx from the surrounding injection medium (Tesarik et al., 2000). Changes in calcium concentration are translated into cellular responses through the activation of calcium-sensitive downstream pathways which activates the early events of oocyte activation. Despite increasing knowledge about signal transduction mechanisms, the nature of the functional linkage between the frequency, number, amplitude and duration of the calcium signals and the kinetics of oocyte activation is still a matter of debate. By activating mouse eggs with experimentally controlled and precisely defined calcium transients, Ducibella et al. (2002) demonstrated that each of the early events of mammalian oocyte activation are initiated by a different number of calcium transients, while their completion requires a greater number of calcium transients than for their initiation. The same group of researchers subjected freshly ovulated mouse eggs to a series of repetitive calcium influxes of various patterns modulated

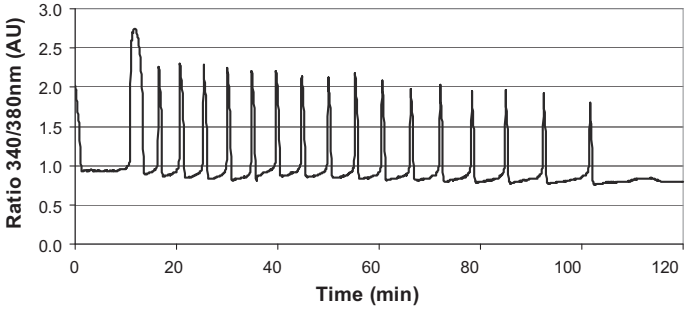

Figure 1 Calcium oscillating pattern recorded following mouse ICSI. The first calcium rise, partially visible at time point 0, is provoked by the ICSI procedure itself. A second large calcium spike is seen within 10–15 min following ICSI and this is followed by a series of calcium oscillations. AU = arbitrary units (recorded by F Vanden Meerschaut, Physiology Group, Department of Basic Medical Sciences, Ghent University).

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by a noninvasive membrane electropermeabilization method. Using a combination of two suboptimal treatments, they showed that mouse eggs can 'sum' the effects of these various patterns of intracellular calcium transients during the period of egg activation (Tóth et al., 2006). Thus, very distinct calcium signals might lead to cell cycle resumption. The exact pattern of calcium oscillations might also have an impact on future downstream events following oocyte activation long after the calcium oscillations have ceased. It was shown that modifying the calcium pattern can influence pre- and post-implantation development in the rabbit and mouse (Ozil, 1990; Ozil and Huneau, 2001; Ozil et al., 2005, 2006). Moreover, inhibiting or stimulating the natural pattern of calcium signalling of inseminated eggs was shown to have long-term effects on both gene expression and development to term, although the development to the blastocyst stage was not altered (Ozil et al., 2006).

Although recently conflicting results have been reported (Aarabi et al., 2012), substantial evidence suggests that the spermatozoa releases an oocyte-activating protein, phospholipase C zeta (PLCζ), into the oocyte's cytosol, which triggers calcium oscillations via an inositol-1,4,5-triphosphate (IP_3)-mediated pathway (Saunders et al., 2002). PLCζ is considered the physiological 'sperm factor' responsible for triggering intracellular calcium release and therefore oocyte activation. PLCζ hydrolyses internal phopholipid phosphatidylinositol bisphosphate stores into diacylglycerol and IP_3, which then binds to its receptor on the ER, causing calcium release into the oocyte's cytosol (Yu et al., 2011).

Given the main cause of fertilization failure following ICSI is failure of oocyte activation and the proposed role of PLCζ in oocyte activation, it is possible that abnormal forms or aberrant function of PLCζ may be the underlying cause of certain types of male factor infertility and oocyte activation failure. Recent studies have shown that the spermatozoa of some men suffering from fertilization failure are either totally unable to trigger robust calcium oscillations upon injection into mouse oocytes or induce calcium oscillations of reduced frequency and amplitude compared with those from fertile men (Vanden Meerschaut et al., 2013a; Yoon et al., 2008). Furthermore, immunofluorescence and immunoblot analysis revealed that infertile patients showed abnormal PLCζ expression (Heytens et al., 2009; Kashir et al., 2012).

Successful fertilization also depends on the inherent quality of the oocyte, which depends on successful oocyte maturation. Cytoplasmic as well as nuclear maturation are crucial steps for oocytes to obtain the ability to respond properly to the sperm PLCζ signal at the moment of fertilization (Swain and Pool, 2008). The ability to generate calcium oscillations requires several cytoplasmic changes: reorganization of the ER, an increase in the number of IP_3 receptors, changes in the biochemical properties of the receptors (sensitivity to IP_3), an increase in the concentration of calcium ions stored in ER and redistribution of calcium-binding ER proteins (Ajduk et al., 2008; Goud et al., 1999, 2002; Vanderheyden et al., 2009). Studies on unfertilized oocytes in IVF/ICSI cycles have revealed the presence of abnormal spindle and interphase microtubules, indicating that deficiencies in ooplasmic and nuclear components may be a cause of failed fertilization (Combelles et al., 2010; Kovacic and Vlaisavljevic, 2000; Rawe et al., 2000).

Diagnostic tools following ICSI fertilization failure

Following ICSI fertilization failure, the question remains whether the fertilization failure should be attributed to a sperm- or an oocyte-related fertilization deficiency. This is crucial information both for the physician and the patient while discussing the prognosis, future treatment options and the transmission risk when a genetic cause is involved. Therefore, reliable diagnostic tests should be available to each couple suffering from ICSI fertilization failure.

The mouse oocyte activation test (MOAT), a heterologous ICSI model, evaluates the capacity of human spermatozoa to activate mouse oocytes (Rybouchkin et al., 1996). This diagnostic test is usually offered to patients following total fertilization failure or very low fertilization following ICSI. MOAT compares the activation percentage of mouse oocytes after heterologous ICSI using spermatozoa from the patient versus control spermatozoa with proven activation capacity (positive control). Injection of culture medium serves as negative control (**Figure 2**). MOAT enables the classification of patients with failed or low fertilization after ICSI into three patient groups with a mouse oocyte activation percentage: (i) 20% or less (i.e. the upper limit of the negative control), who are classified as the low-activation group (MOAT group 1); (ii) 21–84%, who are classified as the intermediate-activation group (MOAT group 2); and (iii) 85% or more (i.e. the lower limit of the positive control), who are classified as the high-activation group (MOAT group 3) (Heindryckx et al., 2008). In MOAT group 1, patients a sperm-related deficiency is likely to be at the origin of previous fertilization failure, while in MOAT group 3 patients, a sperm-related deficiency can be refuted in most cases. For MOAT group 2 patients, MOAT reveals a reduced activation capacity of the patient's spermatozoa and the MOAT result is called inconclusive; hence, both spermatozoa and oocyte deficiencies might have contributed to the previous ICSI fertilization failure in those couples. In order to enhance the sensitivity (diagnostic and prognostic information) of the heterologous ICSI model, this study group incorporated the analysis of the calcium oscillation pattern. For all of the patients with low and most of the patients with high MOAT activation capacity (MOAT groups 1 and 3, respectively), the calcium oscillatory pattern analysis confirmed the MOAT result. For patients with a former inconclusive (intermediate) MOAT activation capacity result (MOAT group 2), no or strongly dissimilar calcium oscillatory patterns were seen, with significantly lower amplitude and frequency compared with control spermatozoa. A new threshold value can now assist in confirming or refuting, on a single-patient basis, the probability of a sperm-borne activation defect (Vanden Meerschaut et al., 2013a). This value, being the product of the amplitude and frequency, was based on the analysis of the calcium oscillatory patterns in mouse oocytes provoked by the spermatozoa from 26 MOAT patients and four control patients. The calcium pattern analysis was shown to be especially interesting for attributing MOAT group 2 patients to a sperm- or oocyte-related activation deficiency. Three other studies have also examined the calcium oscillatory patterns in mouse oocytes after the injection of human spermatozoa

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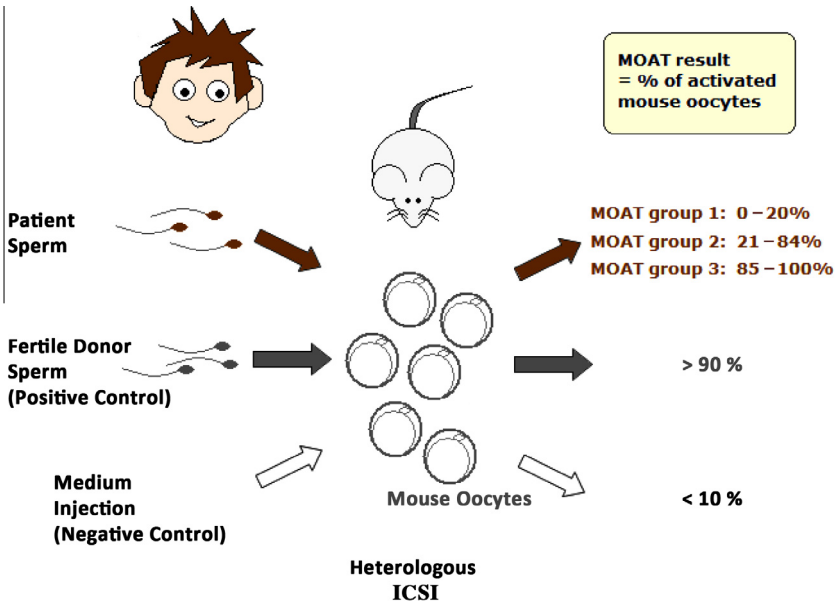

Figure 2 Schematic overview of the mouse oocyte activation test (MOAT).

but they were only on a limited number of patients (Heytens et al., 2009; Morozumi et al., 2006; Yoon et al., 2008).

When interpreting MOAT or calcium oscillatory patterns following heterologous ICSI, one should bear in mind that human PLCζ exhibits a greater potency compared with mouse PLCζ (Cox et al., 2002; Ito et al., 2008). Therefore, the activation rate in mouse oocytes cannot be extrapolated strictly to human oocytes. In support of this, it has been reported that when two spermatozoa, instead of one, were injected in a mouse oocyte, the activation rate of those oocytes after ICSI was almost doubled. The latter might indicate that the spermatozoa of some patients carry a quantitative oocyte activation defect (Yanagida et al., 1999).

Other groups have also employed heterologous ICSI of human spermatozoa into mouse oocytes as a diagnostic tool in patients with a history of fertilization failure following ICSI or for other obvious morphological indications, such as globozoospermia. Araki et al. (2004) investigated the nuclei of human spermatozoa that failed to fertilize human oocytes after ICSI. The rate of two pronuclei formation was 0% (0/46) by the infertile spermatozoa and 81.5% (66/81) by fertile ones ($P < 0.01$). Following ICSI, the oocytes were fixed, stained and subjected to chromosomal analysis. Sperm chromosomes in nonactivated oocytes were present as premature chromosome condensation. The results of this study confirmed in its cohort of patients with fertilization failure following ICSI that infertile spermatozoa had a low potential to spontaneously activate mouse oocytes. Another report described heterologous ICSI testing to distinguish between sperm-borne and oocyte-borne oocyte activation failure before modified ICSI treatment in a series of six patients (Tesarik et al., 2002). As well as mouse oocytes, also bovine, hamster and rabbit oocyte have been used to assess the function of the human spermatozoa (Goud et al., 1998; Nakamura et al., 2001, 2002; Terada et al., 2004). Injection of human spermatozoa into bovine eggs in particular can be used to assess human sperm centrosomal function (Nakamura et al., 2001, 2002), which is considered to be necessary for the normal human fertilization process. In contrast, mouse and hamster eggs cannot be used to assess the human sperm centrosomal function because the centrosome is maternally derived in rodent species (Hewitson et al., 1997; Schatten et al., 1985). Hamster oocytes have also been used for karyotyping human spermatozoa with poor fertilizing capacity (Goud et al., 1998).

Assisted oocyte activation and artificial activating agents

In general, artificial activation of human oocytes is not easily accomplished and it has been mainly investigated in the scope of overcoming oocyte activation failure in human assisted reproduction clinics (Ramadan et al., 2012; Rybouchkin et al., 1997). Artificial activation can also be used to obtain parthenogenetic mammalian embryos that could serve as a model to study the biochemical and morphological events during early embryogenesis (Paffoni et al., 2008). Additionally, artificial activation is one of the crucial steps following somatic cell nuclear transfer (Campbell et al., 2005).

A variety of artificial activating methods is used in human assisted reproduction treatment, including physical, mechanical or chemical stimuli, which provoke one or more calcium rises in the oocyte cytoplasm. The most popular physical stimulus is electrical activation, which induces calcium influx via the formation of pores in the plasma membrane (Egashira et al., 2009; Yanagida et al., 1999). Yanagida et al. (2008). The electric field generated by a direct current voltage causes charged proteins in the lipid bilayer of the cell membrane to move, thereby forming pores in the membrane. Extracellular calcium in the culture medium flows into the oocyte through these pores, transiently elevating the interior calcium concentration and activating the oocyte. Calcium concentration increases

immediately after the application of the stimulus and then gradually decreases and returns to the original concentration without subsequent oscillation.

Mechanical AOA in the human can be achieved by disrupting the plasma membrane and performing vigorous cytoplasmic aspiration during a modified ICSI procedure (Ebner et al., 2004; Tesarik et al., 2002). Vigorous aspiration of the oocyte cytoplasm may increase the oocyte calcium load at the time of injection and thus lead to a higher fertilization rate. Also, it may establish a closer contact of the injected sperm head with intracellular calcium stores of the oocyte (Tesarik and Sousa, 1995).

Calcium ionophores, such as ionomycin or calcimycin (A23187), are the most commonly used artificial activating agents in human assisted reproduction (Nasr-Esfahani et al., 2010; Yanagida et al., 2008). An ionophore is a lipid-soluble molecule, usually synthesized by microorganisms, which transport ions across the lipid bilayer of the cell membrane. Calcium ionophores help to activate the oocyte by increasing the calcium permeability of the cell membrane, thereby allowing extracellular calcium to flow into the cell. Additionally, calcium is released from its intracellular stores, and it has been shown in *Xenopus* that ionomycin mainly acts on the same intracellular calcium stores as IP_3 (Yoshida and Plant, 1992). This treatment causes a single transient increase in calcium ion concentration, but no subsequent calcium oscillations (Swann and Ozil, 1994; Tesarik and Testart, 1994). Rybouchkin et al. (1997) described the combination of mechanical and chemical stimuli by the injection of calcium directly into the oocyte cytoplasm during ICSI followed by ionomycin treatment. This ionomycin-based human AOA protocol consists of the injection of a spermatozoon into the oocyte together with a small amount of 0.1 mol/l $CaCl_2$ (corresponding to the diameter of the oocyte) using conventional ICSI. Thirty minutes following ICSI, the injected oocyte is exposed to 10 μmol/l ionomycin for 10 min, and then again after another 30 min. We have also demonstrated by prolonged calcium pattern analysis on human in-vitro matured oocytes that were donated for research that this AOA protocol is not able to provoke repeated calcium oscillations in human oocytes when sperm from globozoospermic patients is injected (**Figure 3**).

Another chemical artificial activating agent which has been described in human assisted reproduction treatment is strontium chloride (Kyono et al., 2008; Yanagida et al., 2006). In mouse models, strontium chloride treatment has been shown to cause oocyte activation accompanied with calcium oscillations and is very efficient in both mouse parthenogenesis and somatic cell nuclear transfer (Bos-Mikich et al., 1995; Ma et al., 2005). However, the efficiency of strontium chloride as an activating agent for human oocytes is still under debate. No calcium oscillations are observed in human oocytes following strontium chloride exposure (Rogers et al., 2004). The exact mechanism by which strontium chloride induces calcium oscillations in an oocyte remains unclear. Zhang et al. (2005) demonstrated that calcium oscillations induced by strontium chloride were mediated through the IP_3 receptor and required PLC activation and the synergistic action of IP_3. Strontium chloride is thought to move into the oocyte down the concentration gradient, causing calcium to be released from the endoplasmic reticulum.

Efficiency of assisted oocyte activation in humans

Several studies on the application of AOA to overcome fertilization failure or low fertilization after conventional ICSI have been published (Ebner et al., 2012; Heindryckx et al., 2008; Montag et al., 2012; Vanden Meerschaut et al., 2012). Rewarding fertilization rates are usually obtained and pregnancies have been achieved in many couples who previously faced fertilization failure (Kashir et al., 2010). Unfortunately, due to the heterogeneity and the low number of the patients included and due to the different activating agents used, it is difficult to compare different reports. The reported fertilization and pregnancy rates are highly variable. The most plausible reason for this is the differences in patient baseline characteristics between different reports. Also, in most reports, no diagnostic testing (e.g. by heterologous ICSI) was performed beforehand. The difference in efficiency of the used activating agents might also be an explanation for the diverging results when comparing different reports on the clinical use of AOA.

The largest reports on the use AOA in human assisted reproduction treatment describe the use of calcium ionophores as activating agent (**Table 1**). Remarkably, the AOA protocols used in the different reports diverge in the ionophore concentration used (5–10 μmol/l or unknown), the duration of ionophore exposure (10–30 min), the moment of ionophore exposure following ICSI (immediately or 30 min after ICSI) and the number of ionophore exposures (once or twice). Unfortunately, a direct comparison, ideally set up as a randomized controlled trial, has not been performed yet in terms of their efficiency. However, such a study would almost be impossible, since AOA is a rarely required assisted reproduction treatment.

Recently, a ready-to-use calcimycin solution (GM508 Cult-Active; Gynemed, Germany) was put on the market for clinical use in human assisted reproduction treatment as a method for AOA to overcome fertilization failure after ICSI (Ebner et al., 2012). Studies in rat liver mitochondria and starfish oocytes revealed that ionomycin is more potent and more specific compared with calcimycin (Kauffman et al., 1980; Vasilev et al., 2012). The effect of different activation stimuli on activation and developmental potential in the human warrants further investigation.

In this study centre, AOA is performed using the combination of calcium chloride injection into the oocyte at the moment of ICSI and a 2-fold ionomycin exposure afterwards, 30 min apart (Heindryckx et al., 2005, 2008). Fertilization rates after this particular AOA protocol are restored to a normal level in most patients (75% average fertilization rate) and successful pregnancies have been established in many patients (33% average pregnancy rate). Pregnancy rates tend to be higher in MOAT groups 1 and 2 (low and medium activation rates) compared with group 3 (high activation rate; 34 and 43% versus 17%, respectively; Heindryckx et al., 2008). The latter might be explained by the hypothesis that MOAT group 3 suffers from an oocyte-borne activation deficiency as no sperm-borne deficiency was revealed by MOAT (high activation percentage of mouse oocytes). It has been shown that certain oocyte deficiencies, such as spindle abnormalities, might lead to lower activation and

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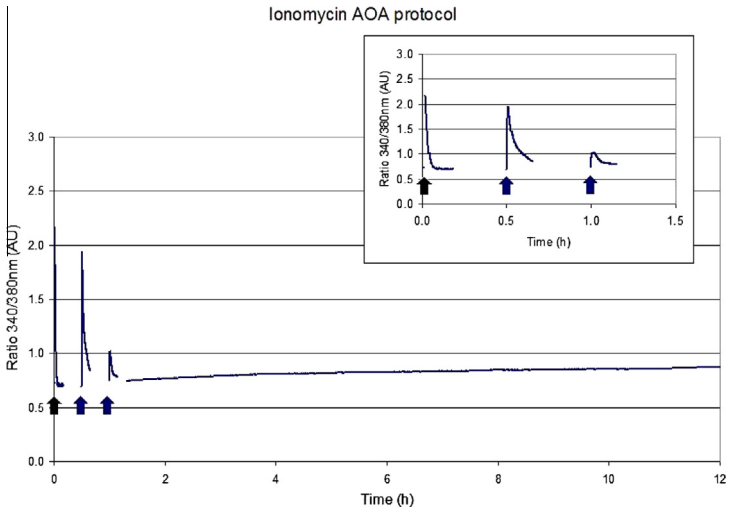

Figure 3 Calcium response in in-vitro matured human oocytes during assisted oocyte activation (AOA) using ionomycin, as described by Rybouchkin et al. (1997). The first calcium rise is provoked by the ICSI procedure combined with calcium chloride injection (black arrow). The second and third calcium rises are provoked by the first and second ionomycin exposures, respectively (blue arrows). The insert represents the first 1.5 h of calcium measuring. AU = arbitrary units (recorded by F Vanden Meerschaut, Physiology Group, Department of Basic Medical Sciences, Ghent University). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pregnancy rates (Combelles et al., 2010). ICSI–AOA is not beneficial for all patients with a suspected oocyte-related activation deficiency (MOAT group 3) and fertilization history should be taken into account when choosing the appropriate diagnostic and therapeutic approach (Vanden Meerschaut et al., 2012). When subgroups of MOAT group 1 were examined in more detail, it became clear that pregnancy rates were very high in the globozoospermic patients and patients with moderate OAT (42–67%); only the group of patients with extreme OAT showed lower pregnancy rates after AOA (9%; Heindryckx et al., 2008).

Safety of assisted oocyte activation in humans

Until adequate scientific evidence is provided regarding its safety, ICSI combined with AOA cannot yet be considered an established treatment. The artificially induced calcium rises do not mimic precisely the physiologically sperm-induced calcium oscillations and little is known yet about the possible adverse effects of ionophores on post-implantation embryo development. Therefore, concerns exist about the use of a calcium ionophore as an oocyte-activating agent in human assisted reproduction.

The influence of ionomycin exposure during the zygote stage on pre- and post-implantation development has been evaluated in the mouse. Zygotes treated with ionomycin showed normal pre- and post-implantation development, and normally developing and fertile pups originated from ionomycin-treated zygotes (Heytens et al., 2008). Another report showed no adverse effects following the application of ionomycin, electrical pulses or strontium chloride in an activation-deficient mouse model; healthy, normally developing and fertile pups were born (Vanden Meerschaut et al., 2013b). Nevertheless, since these results were obtained from animal models, results should be extrapolated with caution to a subfertile human population (Harper et al., 2012). Even though the exposure to the calcium ionophore for the purpose of AOA is short and limited to the zygote stage, knowledge is lacking regarding the potential adverse effects of ionomycin in children born following AOA. Recently, the current study centre performed a follow-up study on 21 children aged 3–10 years born following AOA (Vanden Meerschaut et al., 2014). The overall results of this case series of children born following ICSI combined with AOA showed that the obstetric, neonatal, neurodevelopmental and behavioural outcomes of children born following AOA are reassuring. No serious adverse effects were observed; however, the size of this study was too small to reach a definite conclusion. Therefore, AOA should still not yet be considered as routine practice.

Takisawa et al. (2011a,b) described a group of 22 babies born following AOA. Of these babies, 10 were born following A23187 exposure and 12 following strontium chloride treatment. Growth and health of these babies at birth were not different between both AOA protocols. In a similar communication, the weight and height of 21 AOA children was assessed up to the age of 6 (Sato et al., 2011). For the singletons, the physical growth was within the 10–90% percentile. In the latter report, it was not mentioned whether the children were born following calcium ionophore or strontium treatment.

Future perspectives

Although extensive research has been performed concerning the mammalian oocyte activation process, the role of both the spermatozoon and the oocyte and the reasons for fertilization failure following ICSI, major blind spots remain present in the current knowledge. One of the major concerns should be the lack of knowledge regarding the exact mechanisms of action of currently used artificial activating agents as well as the lack of robust diagnostic tools

Table 1 Overview of the calcium ionophore AOA protocols used in human assisted reproduction treatment.

Reference	*AOA protocol*	*Cases*	*Fertilization rate (%)*		
			Conventional ICSI	*AOA*	*P-value*
Moaz et al. (2006)	Twofold exposure to 10 μmol/l ionomycin for 10 min at 1 h and 1.5 h following ICSI	Abnormal sperm morphology			
		Amorphous heads (n = 18)	36.7	82.7	0.0008
		Tapered heads (n = 23)	39.3	81.7	0.005
		Bent necks (n = 15)	49.4	48.2	NS
Heindryckx et al. (2008)	Injection of 0.1 mol/l $CaCl_2$ together with spermatozoa during ICSI, followed 30 min later by a 2-fold exposure to 10 μmol/l ionomycin for 10 min, 30 min apart	Previously failed or low fertilization after conventional ICSI (n = 30)	14 (0–22)	75	<0.001
Nasr-Esfahani et al. (2008)	Single exposure to 10 μM ionomycin for 10 min	Severe teratozoospermia with a split AOA cycle (n = 78)	0	57.8	S
			14.3 (1–33)	58.3	S
			47 (34–65)	63.4	S
			85.8 (66–100)	77.9	NS
Borges et al. (2009a)	Single exposure to 5 μM calcimycin for 30 min, immediately following ICSI	ICSI with spermatozoa from:			
		TESE NOA (n = 29)	44.0	44.7	NS
		TESE OA (n = 24)	65.2	55.0	NS
		PESE OA (n = 49)	65.8	67.0	NS
Borges et al. (2009b)	Single exposure to 5 μM calcimycin for 30 min immediately following ICSI	ICSI with spermatozoa from:			
		Ejaculated (n = 46)	76.2	69.4	NS
		Epididymal (n = 41)	66.6	48.9	NS
		Testicular (n = 70)	56.1	50.6	NS
Montag et al. (2012)	Single exposure to 10 μmol/l calcimycin for 15 min immediately following ICSI	ICSI with previous:			
		Failed fertilization (n = 27)	0	41.6	<0.05
		Low fertilization (n = 38)	19.3 (0–29)	44.4	<0.001
		Very low fertilization (n = 24)	36.8 (30–50)	56.1	<0.001
Vanden Meerschaut et al. (2012)	Injection of 0.1 mol/l $CaCl_2$ together with spermatozoa during ICSI, followed 30 min later by a 2-fold exposure to 10 μmol/l ionomycin for 10 min, 30 min apart	Suspected oocyte-related activation failure with a split AOA cycle and ICSI with previous:			
		Failed fertilization (n = 5)	25.0	72.7	<0.001
		Low fertilization (n = 7)	60.4	75.0	NS
Ebner et al. (2012)	Single exposure to a ready-to-use calcimycin solution for 15 min immediately following ICSI	Azoo- or cryptozoospermia (n = 66)	34.7	56.9	<0.001

Values are in parentheses are 95% CI. Case reports are not included in this overview.
AOA = assisted oocyte activation; ICSI = intracytoplasmic sperm injection; NOA = nonobstructive azoospermia; OA = obstructive azoospermia; NS = no significant; PESE = percutaneous epididymal sperm extraction; S = significant difference but no P-values provided in the paper; split AOA cycle = conventional ICSI and ICSI combined with AOA on sibling oocytes from the same cycle; TESE = testicular sperm extraction.

following ICSI fertilization failure. MOAT and calcium pattern analysis are promising tools; however, the use of mouse oocytes makes extrapolation to the human difficult. Another alternative could be the use of bovine oocytes, since they are easily attainable via ovaries retrieved from abattoirs. However, these oocytes need to be matured *in vitro* after their retrieval from the ovary and it is well known that the oocyte activation rate and further embryo development is related not only to the injected/fertilizing spermatozoon but also to the intrinsic quality of the oocyte

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and thereby to the conditions during (in-vitro) oocyte maturation. When one wants to test the activation capacity of human spermatozoa, a batch of equal-quality oocytes is preferred. Mouse oocytes are therefore a better choice, because mice can easily be superovulated to obtain an equal batch of sibling in-vivo matured oocytes of comparable quality. Moreover, the activation rate after bovine ICSI is still inefficient and requires additional artificial activation, which makes this source of oocytes different from human oocytes and inadequate for testing human sperm activation potential. The best alternative could be the use of human oocytes for calcium pattern analysis. Nevertheless, ethical considerations should be borne in mind when using human oocytes for diagnostic purposes. Human mature donor oocytes are scarce and valuable. An alternative could be the use of in-vitro matured human oocytes; however, it has been shown that calcium release mechanisms do not become fully competent during culture *in vitro* (Herbert et al., 1997). Also, following parthenogenesis, it was shown that human in-vitro matured oocytes have a decreased developmental potential (Versieren et al., 2010).

Calcium pattern analysis is an invasive technique, which imposes technical constraints by the requirement to measure calcium in the same oocyte that is then assessed for embryo development. Therefore, determination of the calcium pattern following fertilization in a clinical setting is impossible. An alternative might be noninvasive particle image velocimetry (PIV), which was proposed for the first time by Ajduk et al. (2011). Fertilization-induced cytoplasmic flows are a conserved feature of eggs in many species. Adjuk et al. (2011) showed that fertilization induces rhythmical cytoplasmic movements and that PIV can predict the viability of oocytes following IVF as well as further pre- and post-implantation embryo development. Using human failed fertilized oocytes, Swann et al. (2012) showed that PIV can also be reliably applied in human oocytes to monitor the pattern of calcium oscillations during activation. Thus, PIV might provide a novel, noninvasive approach to determine in real time the occurrence and frequency of calcium oscillations in human zygotes. Whether PIV is feasible in a clinical setting has not been investigated yet and should be a matter of further investigation. To use PIV as an indicator of successful fertilization or development potential, it is necessary to expose human zygotes to light every 10 s for several hours. Therefore, it should first be determined whether this level of light exposure might have any detrimental effect on the rate of embryo development. Once PIV can be safely applied in the assisted reproduction laboratory, it would be highly interesting to trace back the particular calcium patterns in oocytes that led to pregnancy and live birth.

The goal of AOA is to mimic as closely as possible the physiological calcium changes seen during mammalian fertilization. However, most artificial activating agents, such as calcium ionophores and electrical pulses, are only able to provoke a single calcium rise. Also strontium chloride, which has been shown to provoke multiple calcium rises and egg activation in mouse oocytes, is apparently not able to provoke a physiological calcium oscillation pattern in human oocytes (Rogers et al., 2004; this study centre's experience). The fundamental role of the sperm protein PLCζ in mammalian fertilization has been highlighted by both fundamental and clinical studies (Heytens et al., 2009; Kashir et al., 2012; Saunders et al., 2002; Yoon et al., 2008). The observation of aberrant sperm PLCζ protein expression in infertile males suggests that microinjection of wild-type PLCζ cRNA or protein could be used as a promising therapy to overcome such cases of infertility; however, the rate of synthesis and total amount of PLCζ protein expressed in the egg cannot be readily controlled following the intracellular injection of a bolus of cRNA. Previous studies have shown that successful embryo development requires PLCζ to be present within the oocytes at a precise concentration range to closely match the amount of PLCζ that would be provided physiologically by the entry of a single mature spermatozoon at fertilization (Yu et al., 2008). Also, it has been suggested that mammalian oocytes may contain an endogenous reverse transcriptase that could potentially convert injected cRNA into DNA, which might then be incorporated into the oocyte's genome (Spadafora, 2004). Therefore, the availability of purified, active recombinant human PLCζ protein instead of cRNA appears to represent both a highly practical and the most physiologically therapeutic agent for overcoming failed ICSI cases. Yoon et al. (2012) synthesized recombinant human PLCζ protein using the *Escherichia coli* system. Injection of this protein into both mouse and human oocytes provoked calcium oscillations in a dose-dependent manner, which closely resembled those initiated by the spermatozoa upon fertilization. Moreover, they triggered activation and cleavage in mouse oocytes. However, the rate of development into the morula stage was merely 0–4%, depending on the dose of injected human PLCζ protein. In human in-vitro matured oocytes as well as failed fertilized oocytes following ICSI, injection of human PLCζ protein was able to induce pronuclei formation. Cleavage rates were not assessed. More recently, Nomikos et al. (2013) prepared recombinant human PLCζ protein fused to NusA, a fusion protein known to greatly enhance the solubility and stability of recombinant proteins. These authors showed that, in contrast to the wild-type protein, mutant forms of human spermatozoa PLCζ displayed an aberrant enzyme activity and a total failure to activate unfertilized mouse and human oocytes. Subsequent microinjection of recombinant human PLCζ protein reliably triggered the characteristic pattern of cytoplasmic calcium oscillations at fertilization and was able to induce preimplantation development up to the blastocyst stage in mouse oocytes.

There are still a number of limitations on the usage of recombinant human PLCζ protein, such as the rather low activity, the production in *E. coli* expression systems, the use of fusion proteins and the fact that there might be differences in post-translational modifications between biological *E. coli* systems and human spermatozoa. Also, post-implantation development following the use of recombinant human PLCζ protein has not been assessed yet. Therefore, the production of active recombinant human PLCζ protein in a purified form suitable for clinical use remains an important issue of future investigation. Another concern is linked to the fact that the PLCζ protein activity is very sensitive to calcium (Nomikos et al., 2005). Therefore, the calcium response provoked by injecting recombinant PLCζ protein might vary depending on the cytosolic calcium concentration in the oocyte. The latter in its turn may depend on: (i) oocyte-specific characteristics, such as the amount of ATP and mitochondrial activity (both are

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necessary to sustain calcium ATPase channels to pump calcium from the cytosol into organelles or the extracellular environment); (ii) the ionic characteristics of culture and ICSI media; and/or (iii) the physical conditions of the culture environment (e.g. temperature). The question remains whether one should intervene in biochemical pathways through the manipulation of culture media. Nowadays, exact formulations of culture media are kept secret, which, a priori, impedes such investigations. Also, possible epigenetic effects of such manipulations should be kept in mind.

Data on the follow up of children born following AOA are scarce. Therefore, long-term follow up of children born following AOA remains mandatory, because AOA should still not be considered to be a routine treatment for ICSI fertilization failure. It currently remains to be determined whether AOA represents the safest and most effective method for overcoming certain forms of oocyte activation failure. It is known that an aberrant nonphysiological pattern of calcium oscillations after fertilization in mouse eggs can exert potentially deleterious, downstream, longer-term effects on both gene expression and embryo development (Ozil et al., 2006). ICSI *per se* does influence expression of numerous genes in mouse and bovine models (Bridges et al., 2011; Giritharan et al., 2010). This might also hold true for ICSI combined with artificial oocyte activation. It has been shown that gene activation differs between IVF, ICSI and ICSI plus the application of strontium chloride to activate murine eggs (Bridges et al., 2011). To date, no data on calcium ionophore exposure and gene expression are available.

Conclusion

Oocyte activation is the earliest event of human development and it is undoubtedly during this crucial phase of life that any minor or major adverse event might have long-lasting or persistent effects on the functioning of individual organs and/or the whole human body. As reviewed by Silveira et al. (2007), 'recent studies demonstrate associations between aggressions suffered during the initial phase of somatic development and amplified risks of chronic diseases throughout life, such as obesity, diabetes and cardiovascular diseases'. The latter may apply to (assisted) oocyte activation and the subsequent fertilization process. Currently it is not known if any disturbance of the physiological events during oocyte activation (e.g. calcium oscillatory pattern) and fertilization have an impact on health in later life. Therefore, research in this field is warranted, certainly bearing in mind the recent developments in the field of AOA.

AOA is a highly specialized fertilization technique used in human assisted reproduction treatment. It might be applied when conventional ICSI fails to activate the human oocyte. Recently, AOA has become more popular worldwide and a commercial ionophore solution has been put on the market. On average, success rates are high and comparable with conventional ICSI results. Also, first data on the outcome of children appear to be reassuring. But, despite the high success rates and the application of AOA by experienced fertility centres around the world, AOA should not be considered routine practice yet. Also, one should bear in mind that a clear indication ought to be present before AOA is advised to a couple. Therefore, diagnostic testing following ICSI fertilization failure should be encouraged.

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