





Strontium fails to induce Ca^{2+} release and activation in human oocytes despite the presence of functional TRPV3 channels

Y. Lu ^{1,2}, R. Reddy¹, M. Ferrer Buitrago¹, M. Vander Jeught¹, J. Neupane¹, W.H. De Vos ^{3,4}, E. Van den Abbeel¹, S. Lierman¹, P. De Sutter¹, and B. Heindryckx ^{1,*}

¹Ghent-Fertility and Stem Cell Team (G-FaST), Department for Reproductive Medicine, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium ²Department of Gynecology and Obstetrics, West China Second University Hospital, Sichuan University, Renmin South Road 20, 610041 Chengdu, China ³Cell Systems and Imaging research Group (CSI), Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium ⁴Laboratory of Cell Biology and Histology, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Antwerpen, Belgium

*Correspondence address. Ghent-Fertility and Stem Cell Team, Department for Reproductive Medicine, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium. Tel: +32-9-332-4748; Fax: +32-9-332-4972; Email: Bjorn.Heindryckx@UGent.be  orcid.org/0000-0003-0630-8420

Submitted on September 12, 2017; resubmitted on January 31, 2018; editorial decision on February 23, 2018; accepted on March 15, 2018

STUDY QUESTION: Are the transient receptor potential cation channels vanilloid 3 (TRPV3) present and able to mediate strontium (Sr^{2+}) induced artificial activation in human oocytes?

SUMMARY ANSWER: Sr^{2+} did not induce Ca^{2+} rises or provoke activation in human oocytes, however, mRNA for the TRPV3 channel was present in metaphase II (MII) human oocytes after IVM and TRPV3 agonists induced Ca^{2+} rises and oocyte activation, demonstrating the channels were functional.

WHAT IS KNOWN ALREADY: Selective activation of TRPV3 by agonists induces Ca^{2+} entry and promotes mouse oocyte activation, and the absence of TRPV3 channels in mouse oocytes prevents Sr^{2+} mediated artificial activation. Sr^{2+} is sometimes used to overcome fertilization failure after ICSI in the clinic, but its efficiency is still controversial and the mechanism(s) of how it mediates the Ca^{2+} flux has not been studied yet in human.

STUDY DESIGN, SIZE, DURATION: The protein distribution ($n = 10$) and mRNA expression level ($n = 19$) of the TRPV3 channels was investigated in human MII oocytes after IVM. The Sr^{2+} (10 mM) and TRPV3 agonists (200 μM 2-aminoethoxydiphenyl borate [2-APB] and 200 μM carvacrol)-induced Ca^{2+} response was analyzed in human ($n = 15$, $n = 16$ and $n = 16$, respectively) and mouse oocytes ($n = 15$, $n = 19$ and $n = 26$, respectively). The subsequent embryonic developmental potential following the parthenogenetic activation using these three agents was recorded in human ($n = 10$, $n = 9$ and $n = 9$, respectively) and mouse ($n = 20$ per agent) oocytes, by determining pronucleus, or 2-cell and blastocyst formation rates.

PARTICIPANTS/MATERIALS, SETTING, METHODS: MII oocytes from B6D2F1 mice (6–10 weeks old) as well as human IVM oocytes and IVO oocytes (from patients aged 25–38 years old) with aggregates of smooth endoplasmic reticulum clusters were used. The expression of TRPV3 channels was determined by immunofluorescence staining with confocal microscopy and RT-PCR, and the temporal evolution of intracellular Ca^{2+} concentration was measured by time-lapse imaging after exposure to Sr^{2+} and TRPV3 agonists (2-APB and carvacrol). Artificial activation efficiency was assessed using these agents.

MAIN RESULTS AND THE ROLE OF CHANCE: Sr^{2+} did not promote Ca^{2+} oscillations or provoke activation in human oocytes. Transcripts of TRPV3 channels were present in IVM MII human oocytes. TRPV3 protein was expressed and distributed throughout the ooplasm of human oocytes, rather than particularly concentrated in plasma membrane as observed in mouse MII oocytes. Both agonists of TRPV3 (2-APB and carvacrol), promoted a single Ca^{2+} transient and activated a comparable percentage of more than half of the exposed

human oocytes ($P > 0.05$). The agonist 2-APB was also efficient in activating mouse oocytes, however, significantly fewer mouse oocytes responded to carvacrol than 2-APB in both the Ca^{2+} analysis and activation test ($P < 0.001$).

LIMITATIONS REASONS FOR CAUTION: The availability of fresh IVO matured oocytes in human was limited. Data from TRPV3 knockout model are not included.

WIDER IMPLICATIONS OF THE FINDINGS: The benefit of clinical application using Sr^{2+} to overcome fertilization failure after ICSI requires further validation.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by FWO-Vlaanderen, China Scholarship Council and Special Research Fund from Ghent University (Bijzonder Onderzoeksfonds, BOF). No competing interests are declared.

Key words: failed fertilization / ICSI / assisted oocyte activation / TRPV3 channels / Ca^{2+} / release / human oocytes / embryo development / strontium / ionomycin

WHAT DOES THIS MEAN FOR PATIENTS?

This study looks at a way of trying to overcome fertilization failure after fertility treatment by helping the processes which occur in the egg at this time. Sometimes a substance called strontium is used in clinics to try to initiate fertilization if ICSI is not working. It is still controversial as it is not known how it might work, although there is some evidence that it works in mice.

In this study the researchers looked at both human and mouse eggs. They looked closely at the possible way that strontium initiates fertilization and development of the eggs, and found that strontium did not seem to work with human eggs and that the processes involved were different. They conclude that more evidence is needed about any possible benefits of using strontium when ICSI is not working.

Introduction

Mammalian oocyte activation is triggered by sperm factor phospholipase C zeta (PLC ζ), which initiates a series of oscillations of Ca^{2+} levels within the ooplasm (Saunders et al., 2002; Tesarik, 2002). The PLC ζ hydrolyzes the precursor lipid phosphatidylinositol 4,5-bisphosphate to form both diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Further downstream, the IP_3 binds to its cognate receptors (IP_3Rs) present in the oocyte and thus generates the Ca^{2+} release from endoplasmic reticulum (ER) (Fedorenko et al., 2014; Mak and Foskett, 2015). To enable the series of oscillations, extracellular Ca^{2+} influx is further required to restore the Ca^{2+} concentration in the ER during oocyte activation (Miao et al., 2012; Wakai et al., 2013). As such, the Ca^{2+} signaling mediates successful fertilization and plays a vital role in supporting further embryonic development (Ramadan et al., 2012; Wakai et al., 2013). Irregularities in Ca^{2+} oscillation pattern of the oocyte during activation may thus prevent successful fertilization and reduce embryonic developmental potential (Ajduk et al., 2011; Miao and Williams, 2012).

In IVF centers worldwide, deficiencies during oocyte activation are associated with fertilization failure (FF) or low fertilization rate following treatment involving ICSI (Rawe et al., 2000; Yanagida, 2004; Swain and Pool, 2008; Neri et al., 2014). The oocyte activation potential of sperm from patients who experienced FF can be evaluated in heterologous ICSI models (Araki et al., 2004; Heindryckx et al., 2005). Thereafter, the technology of assisted oocyte activation (AOA) is frequently applied during ICSI, with the principle of inducing a Ca^{2+} increase within the FF oocytes. Thus far, a number of physical, mechanical and chemical AOA methods have been evaluated, with successful oocyte activation and development to term after applying electrical pulses (Egashira et al., 2009), modified ICSI procedures (Tesarik et al.,

2002), Ca^{2+} ionophores (Heindryckx et al., 2008; Ebner et al., 2012; Vanden Meerschaut et al., 2014) and strontium chloride (SrCl_2) (Kim et al., 2012, 2014).

Despite several studies reporting successful pregnancies obtained following AOA treatment with Sr^{2+} (Yanagida et al., 2006; Kyono et al., 2008; Kim et al., 2012, 2014), the efficiency of Sr^{2+} as an activating agent for human oocytes is still under debate. In contrast to mice and rats oocytes studies, in which repetitive Ca^{2+} transients are observed when Sr^{2+} is used (Whittingham and Siracusa, 1978; Roh et al., 2003), there is a lack of evidence supporting the same for human oocytes (Versieren et al., 2010). Recently in mouse, transient receptor potential cation channels, subfamily V, vanilloid 3 (member 3) (TRPV3) were identified to mediate Sr^{2+} induced oocyte activation (Carvacho et al., 2013; Lee et al., 2016). The TRPV3 are highly temperature-dependent channels (Peier et al., 2002; Smith et al., 2002) and are modulated by various stimuli and ligands, including natural compounds, such as carvacrol, thymol and eugenol, as well as 2-aminoethoxydiphenyl borate (2-APB) (Ramsey et al., 2006; Xu et al., 2006). These agonists, in particular 2-APB and carvacrol, showed their capacity to activate TRPV3 channels and promote Ca^{2+} influx, and as consequence, provoked mouse oocyte activation (Carvacho et al., 2013; Lee et al., 2016).

In animal studies, Sr^{2+} has shown to be a highly efficient activating agent in parthenogenetic activation (Kishikawa et al., 1999; Versieren et al., 2010) and somatic cell nuclear transfer of mouse (Otaegui et al., 1999), bovine (Méo et al., 2004; Yamazaki et al., 2005) and porcine oocytes (Che et al., 2007). Moreover, Sr^{2+} was reported to be the most effective AOA method in a mouse model with deficient sperm activation capacity (Vanden Meerschaut et al., 2013) and more recently in a knockout mouse model for PLC ζ (Hachem et al., 2017), compared

to other agents. Since Sr^{2+} induces Ca^{2+} rises similar to the peaks provoked by rodent sperm, it is of interest to investigate the Sr^{2+} triggered Ca^{2+} release and oocytes activation, as well as the expression and functionality of the TRPV3 channels in human oocytes. In addition, the TRPV3 channel agonists, such as 2-APB and carvacrol, could be used as an alternative AOA method in human.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (Bornem, Belgium) unless otherwise indicated.

Ethical approval

The study was approved by the local Ethical Committee of the Ghent University Hospital, Belgium (2009/130, 2010/808 and 2010/182). Written informed consents were obtained from all patients. All procedures involving animal handling and sacrifice were approved by the Ghent University Hospital Ethical Committee for Laboratory Animals (ECD no. 11/41).

Source and culture of human oocytes

Patients (25–38 years old) undergoing ICSI treatment at the Ghent University Hospital between October 2014 and June 2015, were included in this study. Patients undergoing a hormone stimulated cycle were administered a GnRH agonist (Decapeptyl; Ferring, Belgium) or antagonist (Cetrotide; Merck Serono, Germany). Ovarian stimulation was performed by administering hMG (Menopur; Ferring, Belgium) or recombinant (rec) FSH (Gonal-F; Merck Serono, Germany) at a dose of 112.5–300 IU daily and ovulation was induced with 5000 IU hCG (Pregnyl; MSD, Belgium). Oocytes were enzymatically denuded by brief exposure to 80 IU/ml hyaluronidase (Irvine Scientific, Belgium), followed by mechanical denudation prior to ICSI. Nuclear status was assessed and classified as germinal vesicle (GV) (presence of a GV structure), metaphase I (MI) (absence of a both polar body and a GV structure) or metaphase II (MII) stage (presence of a polar body and absence of a GV structure). Donated GV oocytes were further cultured in medium 199, supplemented with 10 ng/ml epidermal growth factor, 1 mg/ml estradiol, 10 mIU/ml recFSH, 0.5 mIU/ml hCG, 1 mM L-glutamine, 0.3 mM sodium pyruvate, 0.8% (v/v) human serum albumin (Red Cross, Belgium), 100 IU/ml penicillin G and 100 mg/ml streptomycin sulfate for 24 h. Immature MI stage oocytes were cultured in Sydney IVF COOK cleavage (CC) medium (Cook Ireland Ltd, Belgium) for 3 h or 24 h based on the first polar body extrusion. All oocytes were cultured at 37°C in 6% CO_2 and 5% O_2 . *In vivo* matured (IVO) fresh MII oocytes showing visible aggregates of tubular smooth ER clusters (SERs) were collected as well, for this study. Although fertilized SER oocytes can lead to healthy live births, the clinical application of them is still under critical review at our Department, owing to the previously reported malformations and impaired pregnancy outcomes (Otsuki *et al.*, 2004; Sa *et al.*, 2011; Itoi *et al.*, 2016). We included a total of 104 oocytes (37 GV–MII, 46 MI–MII and 21 IVO MII with SERs), allocated to the different groups. The distribution of IVM and IVO MII oocytes across the same sets of experiments was analyzed using the Chi-square test, and no significant difference was observed (Table 1, $P > 0.05$).

Source and culture of mouse oocytes

B6D2F1 hybrid female mice (6–10-week-old) were stimulated with 7.5IU pregnant mare's serum gonadotrophin (PMSG, Folligon[®], Intervet, Boxmeer, The Netherlands), followed by 7.5 IU hCG (Chorulon[®], Intervet, Boxmeer, The Netherlands) 46–48 h later. MII oocytes were harvested in HEPES-buffered potassium simplex optimized medium (KSOM-HEPES) supplemented

with 4 mg/ml bovine serum albumin (BSA, Calbiochem, Belgium) 12–14 h after hCG injection. Cumulus cells surrounding the oocytes were removed by treatment with 200 IU/ml hyaluronidase (0.3 mg/ml, type VIII) in KSOM-HEPES. Oocytes were cultured under paraffin oil at 37°C in 6% CO_2 and 5% O_2 in KSOM containing 4 mg/ml BSA until further treatments (Lawitts and Biggers, 1991).

Ca^{2+} imaging in human and mouse oocytes following exposure to Sr^{2+} and TRPV3 agonists. Human ($n = 47$) and mouse oocytes ($n = 67$) were loaded with 7.5 μM of the radiometric Ca^{2+} sensitive dye Fura-2 acetoxy-methyl (AM) ester (Invitrogen, Life Technologies Europe B.V., Belgium) at 37°C in 6% CO_2 , 5% O_2 and for 30 min and then washed extensively. Subsequently, oocytes were placed in individual glass bottom dishes (MatTek, Corporation, Ashland, USA) and Ca^{2+} imaging was performed on an inverted epi-fluorescence microscope (TH4-200, Olympus Soft Imaging Solutions GmbH, Belgium) with a $\times 20$ objective. Fluorescence was recorded at an emission wavelength of ~ 510 nm every 5 s. The ratio of both Ca^{2+} induced signals (340/380 nm) was proportional to the concentration of free intracellular Ca^{2+} (expressed in arbitrary units, AU).

For measuring the Ca^{2+} responses of human oocytes following Sr^{2+} exposure, the fura-2 loaded IVM ($n = 12$) and SERs ($n = 3$) oocytes were transferred to a drop of GIBCO[®] Ca^{2+} / Mg^{2+} free Earle's Balanced Salt Solution (EBSS) supplemented with 10 mM SrCl_2 (Life Technologies, Leuven, Belgium). The Ca^{2+} images were recorded every 5 s for a duration of 6 h. Following the first 2 h of exposure, a group of human oocytes ($n = 5$) that did not respond to the Sr^{2+} was subsequently exposed to 10 μM ionomycin (cat. no. 19657) dissolved in COOK Cleavage medium for 15 min. The Ca^{2+} images were acquired to assess their ability to mobilize the intracellular Ca^{2+} . The Sr^{2+} induced Ca^{2+} oscillations of mouse oocytes were recorded every 5 s for a duration of 2 h, immediately after transferring the eggs to a drop of Ca^{2+} -free KSOM with 10 mM SrCl_2 , in the glass bottom dish.

Following the application of TRPV3 agonists, the Ca^{2+} responses of both human and mouse oocytes were investigated. Ca^{2+} images were recorded from human oocytes exposed to 200 μM 2-APB ($n = 16$) or 200 μM carvacrol ($n = 16$) in 1% polyvinyl alcohol (PVA)-supplemented albumin-free IVFTM medium (Vitrolife, Göteborg, Sweden) for 30 min. The PVA was previously reported to substitute for BSA in mouse embryo culture medium following TRPV3 agonists activation (Carvalho *et al.*, 2013). Mouse oocytes were subjected to the same concentration of the agonists but supplemented in 1% PVA KSOM (BSA-free) medium for 30 min. The stock solutions of TRPV3 agonists were prepared by dissolving them in dimethyl sulphoxide (DMSO), as carried out for ionomycin in our clinic (Heindryckx *et al.*, 2008). The amount of DMSO added to the activation medium was tested and shown not to induce Ca^{2+} release or activation of mouse oocytes.

All oocytes were distributed randomly across the groups and tested within 2 h after assessing the maturation state. A maximum of three oocytes were measured simultaneously. Baseline drifting was adjusted before retrieving values for amplitude (value at maximum increase in fluorescence intensity per peak) expressed in AU. Relative amplitude (RA, amplitude subtracted from the baseline and expressed in AU) and AUC of the Ca^{2+} rise (expressed in AU \times minutes) was calculated.

Parthenogenetic activation of MII human and mouse oocytes

To test the activation efficiency of 10 mM Sr^{2+} , human oocytes were incubated in Ca^{2+} -free EBSS medium and mouse oocytes were activated in Ca^{2+} -free KSOM medium with Sr^{2+} for a duration of 4 h. For creating diploid parthenogenetic embryos, 2 $\mu\text{g}/\text{ml}$ cytochalasin D (CCD) was added to both of the activation media. In the agonist activation tests, human oocytes were treated with 200 μM 2-APB for 30 min or 200 μM carvacrol for 10 min dissolved in IVF medium at 37°C, while mouse oocytes were exposed at

the same conditions as the human oocytes but within KSOM medium (albumin-free 0.1% PVA). CCD was added to the subsequent culture medium for 4 h. Following activation, human oocytes were further cultured in CC medium for 16 h, and mouse oocytes were incubated in KSOM for 60–72 h followed by a 24 h extended culture in COOK Blastocyst medium. The activation of human oocytes was evaluated by formation of a single pronucleus (IPN) 16 h after treatment. Mouse embryo development was assessed at 24 (two-cell), 72 (morula/early blastocyst) and 96 h (blastocyst) post-activation time.

Immunofluorescence staining of TRPV3 channels

Oocytes were fixed and stained as previously reported (Carvacho et al., 2013). Briefly, the zona pellucida (ZP) of the oocytes was removed with Tyrode's solution. The zona-free oocytes were washed intensively in 2% goat serum and 1% BSA-supplemented PBS and fixed in PBS–BSA containing 2% paraformaldehyde for 45 min at room temperature. Oocytes were washed and blocked in PBS containing 0.1 M glycine, 2% goat serum and 0.01% Tween 20 for at least 1 h. Oocytes were then permeabilized with 0.1% Triton X-100 for 15 min at room temperature. Subsequently, samples were washed with PBS supplemented with 2% goat serum and 1% PVA followed by incubation at 4°C with the primary antibody against TRPV3 (1:100, 10 µg/ml, Neuromab, Davis, CA, USA). After washing, oocytes were treated with the secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) (1:200, Molecular Probes, Eugene, OR, USA) for 1 h at room temperature, followed by extended washings. In addition, chromosomes were stained with ethidium homodimer-2 (1:500, Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature. The negative controls were treated with the secondary antibodies alone. Finally, the oocytes were mounted in Mowiol containing 0.01% phenylenediamine. To validate the immunostaining of TRPV3 protein, a different fixation process and antigen retrieval experiment was performed (Supplementary Data), however, no protocol showed improved immunostaining results (Supplementary Fig. S1). All samples were imaged using a laser scanning microscope, Nikon AIR confocal microscope (Nikon Instruments, Paris, France) with a x60 Plan Apo VC oil immersion objective. The TRPV3 distribution and chromosome alignments were obtained from Z-stacks (0.5–0.75 µm/Z-step), using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RT-PCR on pooled IVM human oocytes

RNA was extracted from pooled IVM MII and IVO SER human oocytes (Group I, $n = 11$ and Group II, $n = 8$, Table I), using a PicoPure RNA isolation kit, as described by the manufacturer (Life Technologies, CA, USA). The following cDNA synthesis was performed using the Superscript VILO cDNA synthesis kit (Invitrogen, USA). Primers (5'–3') were designed to amplify the fragment spanning exons 6 and 7 of TRPV3 (Forward AGGCTTCTACTTCGGTGAGAC, Reverse AGGG CGTGAAGGATGTTGTTG) using Primer3 program.

The RT-PCR was performed on the cDNA of these two groups of human oocytes and Day 5 arrested human embryos ($n = 4$), while GAPDH was used as a positive control. Briefly the PCR conditions are as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 45 s and a final extension at 72°C for 10 min. The RT-PCR products were subsequently loaded onto the fragment analyzer and analyzed using PROsize program (Advanced Analytical Technologies, Ankeny, IA, USA). Subsequently, the RT-PCR products was purified using Exo-AP and bi-directional Sanger sequencing was performed on the purified RT-PCR products using the Big Dye Terminator kit (ABI, USA). The Sanger sequencing products were further cleaned using magnetic beads with 85% alcohol and analyzed using the ABI 3130 genetic analyzer (ABI, USA).

Statistical analysis

The Statistical Package for the Social Sciences version 21 (SPSS® Statistics, IBM corp, NY, USA) was used for statistical analysis. Proportions were compared by a contingency table analysis followed by a chi-square or Fisher's exact test. Means (RA and AUC) from multiple groups were compared using ANOVA and Bonferroni's multiple comparison test. Differences yielding a P value <0.05 were considered as being statistically significant.

Table I The allocations of IVM and IVO human oocytes across all groups in the study.

		No. oocytes (n)	GV-MII n (%)	MI-MII n (%)	IVO with SERs n (%)
Ca ²⁺ imaging	Sr ²⁺	15	6 (40%)	6 (40%)	3 (20%)
	2-APB	16	6 (38%)	6 (38%)	4 (25%)
	Carvacrol	16	8 (50%)	5 (31%)	3 (19%)
Immunostaining	TRPV3	10	4 (40%)	6 (60%)	n/a
RT-PCR	Group I	11	3 (27%)	8 (73%)	n/a
	Group II	8	2 (25%)	5 (63%)	1 (12%)
Activation test	Sr ²⁺	10	2 (20%)	4 (40%)	4 (40%)
	2-APB	9	3 (33%)	3 (33%)	3 (33%)
	Carvacrol	9	3 (33%)	3 (33%)	3 (33%)

n/a, Not applicable.

IVO: *in vivo* matured.

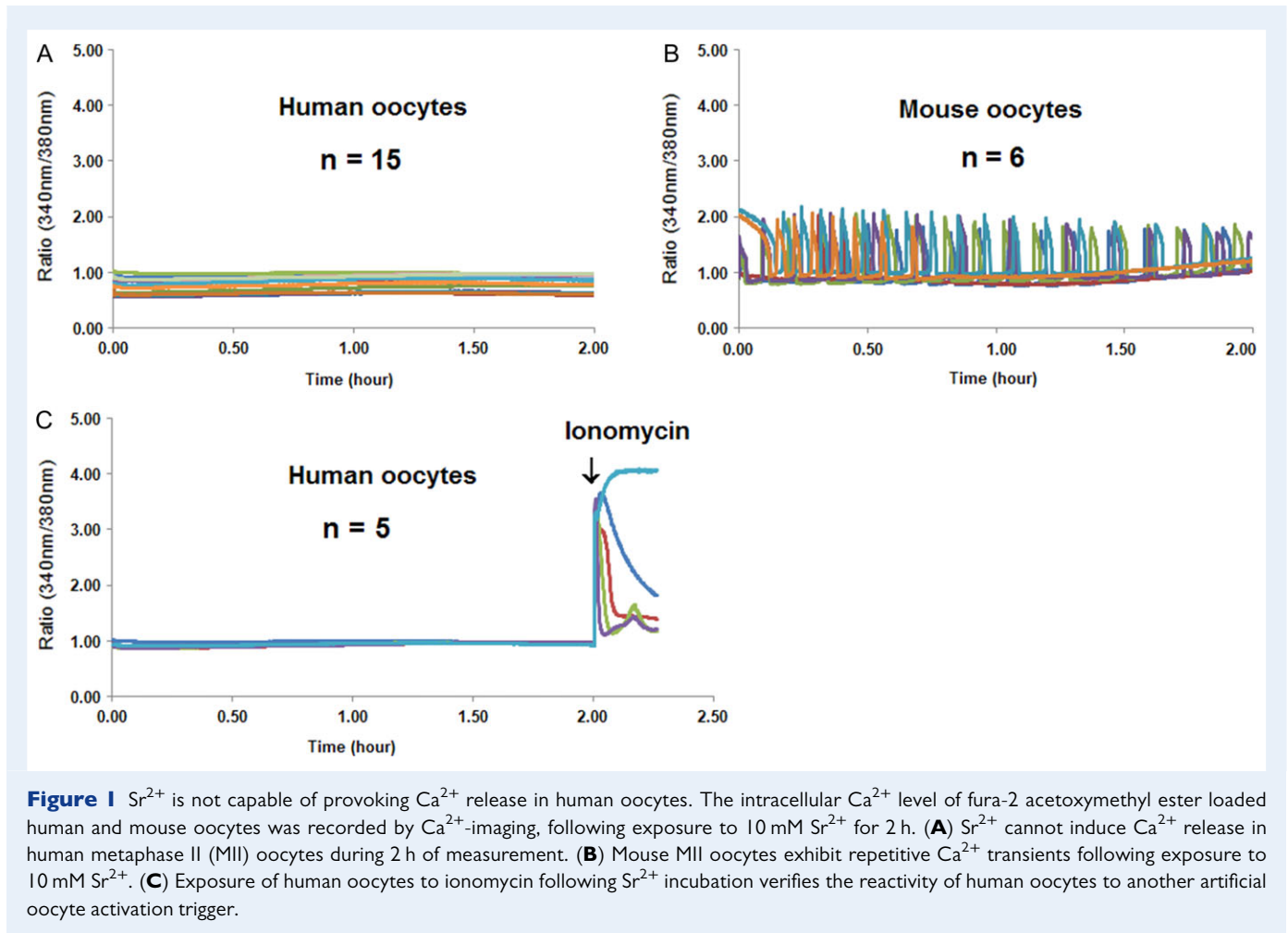
SER: aggregates of tubular smooth endoplasmic reticulum clusters.

2-APB: 2-aminoethoxydiphenyl borate.

TRPV3: transient receptor potential cation channels vanilloid 3.

MI: metaphase I.

MII: metaphase II.



Results

Sr^{2+} failed to induce Ca^{2+} release and activation in human MII oocytes

To verify the Ca^{2+} releasing and activating ability of Sr^{2+} exposure, IVM and IVO MII human oocytes ($n = 25$, Table I) were subjected to 10 mM Sr^{2+} . Following the exposure, the changes of intracellular Ca^{2+} levels of analyzed oocytes were recorded by Ca^{2+} imaging. However, no increases in the intracellular Ca^{2+} concentrations were observed in a total number of 15 oocytes within a duration of 6 h (Fig. 1A shows records from the first 2 h). In contrast, all mouse oocytes ($n = 15$) exposed to the same concentration of Sr^{2+} showed dynamic intracellular Ca^{2+} oscillations during the 2 h analysis (five traces are presented in Fig. 1B). To test the reactivity to Ca^{2+} triggers of the human oocytes, five IVM human oocytes were further subjected to 10 μM ionomycin following a 2 h exposure of Sr^{2+} . All five IVM oocytes exhibited a single Ca^{2+} rise with a rapid upstroke followed by a slower decline towards the baseline immediately after the ionomycin exposure (Fig. 1C and Table II). Activation capacities of the human oocytes ($n = 10$) were further evaluated following Sr^{2+} exposure. None of the human oocytes showed IPN after 16 h following Sr^{2+} exposure while, in contrast, all of the mouse oocytes activated and cleaved, 16 h post Sr^{2+} activation (Table III).

TRPV3 channels are expressed in human oocytes

The TRPV3 channels were recently identified as the major channels to conduct Sr^{2+} influx and induce activation of mouse oocytes (Carvacho *et al.*, 2013; Lee *et al.*, 2016). Since Sr^{2+} failed to activate human oocytes or elicit Ca^{2+} transients, we then examined the expression and the function of the channels. Following the fluorescence staining, we observed a non-specific distribution of TRPV3 channels throughout the ooplasm of the analyzed human oocytes ($n = 10$) (Fig. 2A and B). To validate the staining, we further examined the TRPV3 localization in mouse oocytes ($n = 10$), which (unlike human) showed a distribution that was particularly concentrated at the plasma membrane, as reported previously (Carvacho *et al.*, 2013; Lee *et al.*, 2016) (Fig. 2C and D). To further confirm this result, TRPV3 mRNA expression levels in human oocytes were investigated at transcriptional level by RT-PCR. The transcripts of TRPV3 were detected in pooled IVM MII and IVO SERs human oocytes (Group I, $n = 11$ and Group II, $n = 8$, Table I), as demonstrated by the expected mRNA products in both groups (Fig. 3A). Sanger sequencing of the RT-PCR products further revealed that the sequencing reads were aligned with the TRPV3 cDNA sequence downloaded from the University of California Santa Cruz genome browser with the transcript containing 791 amino acids (NM_001258205) using DNASTAR (Fig. 3B).

Table II The Ca²⁺ response of human oocytes following exposure to various activation agents.

Activation agent	No. oocytes	Survived oocytes, n (%)	Responded oocytes, n (%)	RA of the Ca ²⁺ rise	AUC of the Ca ²⁺ rise
Sr ²⁺	15	15 (100%)	0	n/a	n/a
2-APB	16	16 (100%)	4 (25%)	0.70 ± 0.07 ^a	2.36 ± 0.90 ^b
Carvacrol	16	16 (100%)	9 (31%)	2.32 ± 0.73 ^a	4.98 ± 1.93 ^b
Ionomycin post Sr ²⁺	5	5 (100%)	5 (100%)	2.44 ± 0.20 ^c	13.14 ± 9.32 ^d
Ionomycin post 2-APB	9	8 (89%)	8 (100%)	1.91 ± 0.74	6.45 ± 5.50
Ionomycin post carvacrol	7	7 (100%)	7 (100%)	1.14 ± 0.90 ^c	2.30 ± 0.80 ^d

Oocytes are either exposed to 10 mM Sr²⁺ for 2 h or subjected to 200 μM 2-APB or 200 μM carvacrol for 30 min. The subsequent exposure of 10 μM ionomycin is for 15 min. ANOVA and Bonferroni's multiple comparison test. ^aP < 0.01, ^{b,c,d}P < 0.05, n/a, not applicable. Values with the same superscripts differ significantly. Data are shown as mean ± SD. 2-APB, 2-aminoethoxydiphenyl borate. RA, relative amplitude.

Table III The response of human and mouse oocytes following exposure to Sr²⁺, 2-APB and carvacrol.

Oocytes	Activating agent	No.	Survived, n (%)	IPN, n (%)	2-cell, n (%)	Blastocyst, n (%)
Human	Sr ²⁺	10	10 (100)	0 ^{a,b}	n/a	n/a
	2-APB	9	8 (89)	5 (63) ^a	n/a	n/a
	Carvacrol	9	7 (78)	5 (71) ^b	n/a	n/a
Mouse	Sr ²⁺	20	19 (95)	n/a	19 (100) ^d	18 (95)
	2-APB	20	14 (70) ^c	n/a	13 (93) ^e	12 (92)
	Carvacrol	20	20 (100) ^c	n/a	1 (5) ^{d,e}	1 (100)

Oocytes are exposed to 10 mM Sr²⁺ for 4 h, or subjected to 200 μM 2-APB for 30 min or 200 μM carvacrol for 10 min. Chi-square and Fisher's exact test: ^{a,b,d,e}P < 0.01, ^cP < 0.05, n/a, not applicable. Values with the same superscripts differ significantly. PN, pronucleus.

TRPV3 agonists triggered Ca²⁺ release and provoked oocyte activation of human and mouse oocytes

The Ca²⁺ releasing and activating ability of the TRPV3 channels were further investigated by subjecting human oocytes to the agonists 2-APB and carvacrol. Following the exposure of 16 MII human oocytes to 200 μM 2-APB, the intracytoplasmic Ca²⁺ increased in 25% (4 out of 16) of them, displaying a small rise during the first 5 min of measurement (Fig. 4A and Table II). When subjecting 16 human oocytes to the same concentration of carvacrol, a similar proportion (31%, 5/16) of the oocytes responded, and exhibited a significant increase in Ca²⁺ level compared to the Ca²⁺ rise triggered by 2-APB ($P = 0.0034$), during the first 10 min of exposure (Fig. 4B and Table II). Moreover, four more IVM oocytes showed much lower Ca²⁺ fluctuations (RA 0.28 ± 0.14, AUC 1.54 ± 0.56) which were not defined as responding oocytes (Fig. 4B and Table II). All of the human oocytes survived following these chemical exposures and imaging. The groups of human oocytes which failed to respond to these TRPV3 agonists, were further exposed to ionomycin to evaluate the response of the oocytes to other Ca²⁺ triggers. All oocytes exhibited a sharp Ca²⁺ rise following the ionomycin exposure, showing a positive reactivity to the Ca²⁺ ionophore (Table II).

The addition of 200 μM 2-APB to MII mouse oocytes ($n = 26$) dramatically increased Ca²⁺ levels and immediately provoked a protracted peak during the 30 min exposure (Fig. 4C). Following the exposure, 73%

(19/26) oocytes survived and 89% (17/19) showed intracellular Ca²⁺ release (Fig. 4C). The seven damaged oocytes showed a specific protracted Ca²⁺ pattern (Fig. 4C, inset picture). Carvacrol (200 μM) evoked increases of intracellular Ca²⁺ in all subjected mouse oocytes ($n = 26$), however, only displaying a small peak during the 10 min of exposure (Fig. 4D). Moreover, the 54% (14/26) that responded to carvacrol exhibited a minor Ca²⁺ rise fluctuation (absolute amplitude <0.2) in the first 5 min of exposure (Fig. 4D, inset picture).

Furthermore, the activation potential of human and mouse MII oocytes was investigated as well, following the application of the two TRPV3 agonists. We allocated in total 18 IVM and IVO human oocytes (Table I) and 40 mouse oocytes to the 2-APB and carvacrol (each 200 μM) activation tests. Interestingly, more than half of the human oocytes activated and formed IPN, observed at 16 h post stimulation (63 and 71% for 2-APB and carvacrol, respectively, Table III). When exposing mouse oocytes to 200 μM 2-APB, 70% of them survived following the stimulation, and subsequently, more than 90% of them cleaved and developed to blastocysts (Table III). In contrast, carvacrol induced significantly less (5%) cleavage to the two-cell stage compared to the 2-APB group ($P < 0.0001$, Table III).

Discussion

To date, Sr²⁺ has been applied as an AOA method to overcome FF or low fertilization rates (Kyono et al., 2008; Kim et al., 2012, 2014) in

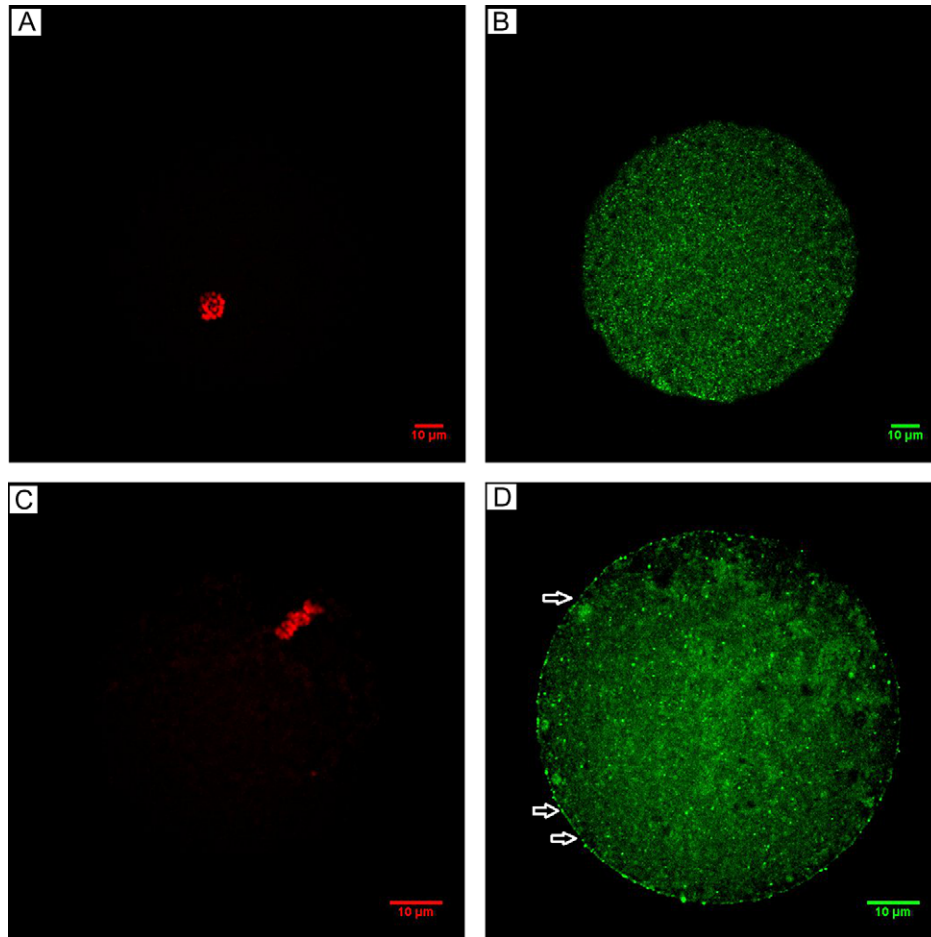


Figure 2 The localization of TRPV3 channels in human MII oocytes. Human and mouse oocytes were stained with Transient receptor potential cation channels vanilloid 3 (TRPV3) antibody and analyzed by confocal microscope. **(A)** Chromosomes of IVM human oocytes are encompassing two regular rings, observed from the optical axis passing through the spindle poles. **(B)** TRPV3 protein of human IVM MII oocyte shows a diffused, non-specific pattern. **(C)** Chromosomes of mouse *in vivo* matured (IVO) oocytes are aligned at the equatorial plate. **(D)** Mouse TRPV3 channels are expressed and concentrated at the cytoplasmic membrane.

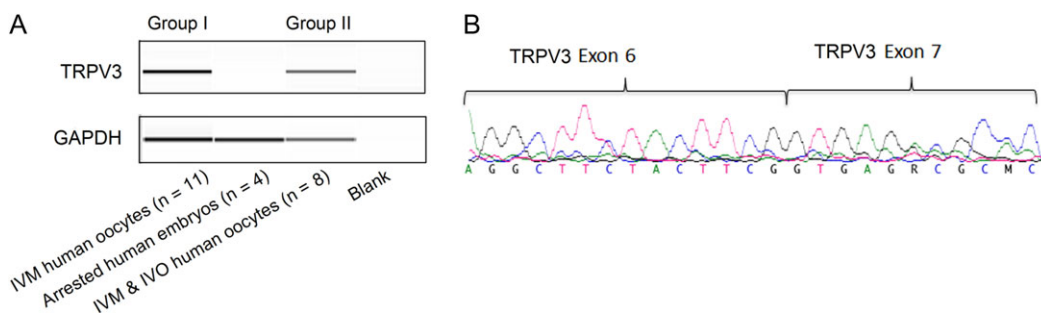


Figure 3 TRPV3 mRNA expression levels in IVM MII and IVO human oocytes with aggregates of smooth endoplasmic reticulum clusters. The mRNA expression of TRPV3 was analyzed by RT-PCR and verified by Sanger sequencing in pooled IVM and *in vivo* matured (IVO) human oocytes. **(A)** RT-PCR products from the agarose gel show the transcripts of TRPV3 are expressed in a group of pooled IVM human oocytes and a group of pooled IVM and IVO human oocytes. **(B)** Chromatogram showing the exons 6–7 junction of the TRPV3 gene after Sanger sequencing of the RT-PCR product from pooled IVM human oocytes.

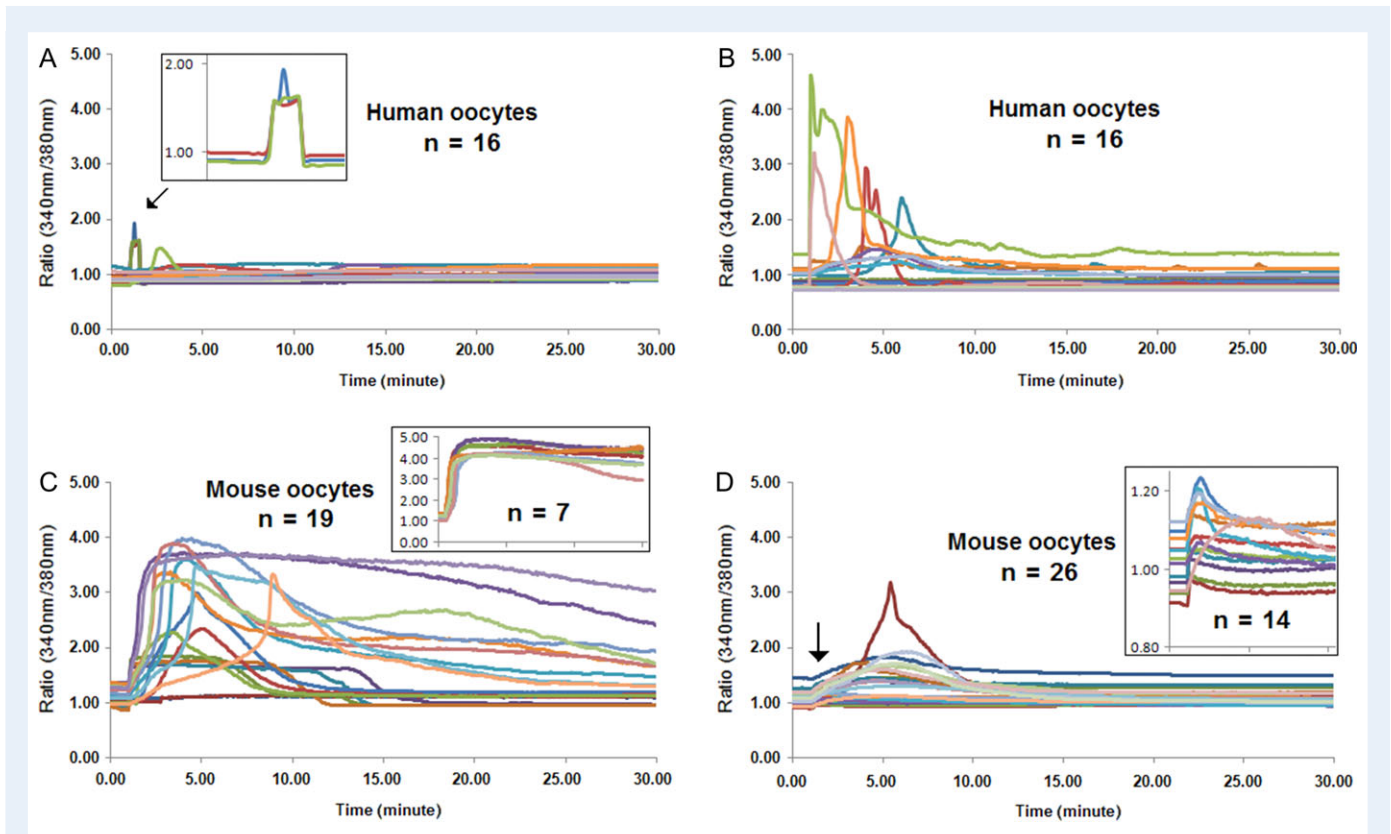


Figure 4 The TRPV3 agonists, 2-APB and carvacrol, induced a Ca^{2+} response in both human and mouse MII oocytes. The intracellular Ca^{2+} level of fura-2 acetoxymethyl ester loaded human and mouse oocytes was recorded by Ca^{2+} -imaging, following exposure to 200 μM 2-aminoethoxydiphenyl borate (2-APB) and 200 μM carvacrol for 30 min. **(A)** Increased intracellular Ca^{2+} was provoked by 2-APB in 4 out of 16 IVM human MII oocytes, displaying a small peak. The three overlapped Ca^{2+} rises of the responding oocytes are shown in the inset picture. **(B)** Five out of 16 human MII oocytes responded to carvacrol, showing a sharp Ca^{2+} rise. **(C)** Elevated intracellular Ca^{2+} in 17 out of 19 alive mouse oocytes following the exposure to 2-APB, displaying a large Ca^{2+} peak. The inset picture showed the Ca^{2+} pattern of another seven damaged oocytes during 2-APB stimulation. **(D)** A lower Ca^{2+} response was observed in all mouse oocytes ($n = 26$) in response to carvacrol, 54% of which (14/26) exhibited a minor Ca^{2+} rise fluctuation (absolute amplitude <0.2) in the first 5 min of exposure (picture inset).

several IVF centers. However, the efficiency and the exact mechanism of Sr^{2+} as an activation agent in human oocytes remains largely unknown. In the present study, we investigated the activation capacity and Ca^{2+} response of human oocytes after Sr^{2+} exposure. Since it was recently shown that the TRPV3 channel mediates Sr^{2+} induced artificial activation in mouse oocytes (Carvacho et al., 2013), we evaluated the presence and functionality of these TRPV3 channels in human oocytes. We demonstrated that Sr^{2+} failed to mediate Ca^{2+} release and induce activation in human oocytes, despite the presence and functioning of TRPV3 channels.

In contrast to other artificial activation agents that induce a single Ca^{2+} transient of mammalian oocytes (Vanden Meerschaut et al., 2014; Nikiforaki et al., 2016), Sr^{2+} activates rodent eggs by inducing a series of Ca^{2+} like oscillations (Whittingham and Siracusa, 1978; Roh et al., 2003), which closely mimic the pattern of Ca^{2+} rises triggered by PLC ζ at fertilization. As such, Sr^{2+} has been applied initially as an AOA agent in the clinic to overcome FF after ICSI. Consequently, improved fertilization rates and embryo qualities were reported for several couples with repetitive FF (Kyono et al., 2008; Chen et al., 2010), frozen-thawed testicular spermatozoa (Kim et al., 2012) and a globozoospermia case (Yang et al., 2012), with resulting healthy live

births. Despite these studies showing the potential of Sr^{2+} to overcome activation failure in human, we demonstrated that Sr^{2+} was not capable of inducing a Ca^{2+} increase or provoking activation of human oocytes. These discrepancies could be due to the lack of diagnostic methods used in these studies to show they were real sperm-related activation deficiencies (Kyono et al., 2008; Chen et al., 2010; Kim et al., 2014). Still, one patient with globozoospermia (Yang et al., 2012) was also successfully treated with Sr^{2+} , which could be explained by the fact that some globozoospermic patients can achieve successful fertilization even after the application of routine ICSI in the absence of AOA (Huang et al., 2010). Therefore, it is difficult to know whether the patients enrolled in those AOA studies using Sr^{2+} as the activating agent really required AOA in first instance, as diagnostic evidence (heterologous ICSI, genetic screening of PLC ζ , Ca^{2+} pattern analysis) was not obtained to show a lack of activation capacity in their sperm. However, it is still possible that the Sr^{2+} might act through unknown Ca^{2+} -irrelevant pathways or through the mechanical permeabilization created during ICSI. More studies are required to verify this further.

To enable activation of mouse oocytes, extracellular Sr^{2+} influxes are transported across its major membrane channel, TRPV3 (Carvacho et al., 2013), promoting downstream oscillations in $[\text{Ca}^{2+}]_i/[\text{Sr}^{2+}]$ of

the oocytes, probably by sensitizing IP₃Rs and thus facilitating Ca²⁺ oscillations (Zhang *et al.*, 2005), or substituting for Ca²⁺ in the potentiation of IP₃Rs (Girard and Clapham, 1993; Marshall and Taylor, 1994; Lee, 2016). Thus, the failure to activate human oocytes with Sr²⁺ alone could be attributed to the absence of membrane TRPV3 channels (which was proven not to be the case in the present study) or, unlikely, the inability of the residues to conduct Sr²⁺, which localize at position 412 on the loop of the membrane proximal domain on the N terminus responsible for switching the temperature dependence of the channel (Latorre *et al.*, 2007; Liu and Qin, 2017), as well as the insensitivity of IP₃R1 in responding to Sr²⁺ in human oocytes. Further investigations are required to verify these options.

Furthermore, we did confirm that the transcripts of TRPV3 channels were present in IVM human oocytes. The TRPV3 channels were also recently demonstrated to be expressed in pooled fresh IVO human oocytes by a whole transcriptome analysis, albeit at low levels (Kocabas *et al.*, 2006). The diffuse cytoplasmic localization of TRPV3 protein might contribute to the observed inability of conducting Sr²⁺ influx by TRPV3 or the aberrant Ca²⁺ release pattern following 2-APB treatment, owing to failure of trafficking functional TRPV3 protein to the membrane during *in vitro* culture, as the cytoskeleton modulates the function of TRPV3 channels (Kuipers *et al.*, 2012; Smani *et al.*, 2014; Lee *et al.*, 2016). Unfortunately, we could not rule out the possibility of low specificity of the immunostaining TRPV3 antibody that was used in the present setting. Moreover, the TRPV3 protein distribution in human oocytes could be impaired by IVM, as no TRPV3 channels are expressed on the plasma membrane of mouse GV oocytes and the TRPV3 proteins are transferred from the ooplasm to the plasma membrane during oocyte maturation in mouse (Lee *et al.*, 2016). Future analysis, for example tracking the TRPV3 protein distribution in IVM and IVO human oocytes by TRPV3 tagging with ruby fluorescent protein (Lee *et al.*, 2016), is required to verify this.

Although the expressed TRPV3 channels in human oocytes failed to conduct Sr²⁺ influx or the conducted Sr²⁺ was not sufficient to promote Ca²⁺ release from the ER, they were shown to be functional in supporting an agonist triggered Ca²⁺ rise and oocyte activation in both mouse and human oocytes. In view of the efficiency of activating rodent oocytes, 2-APB was suggested as a potential AOA agent targeting TRPV3 channels (Lee *et al.*, 2016; Lee, 2016). However, in our view, it is too soon to encourage its use at this moment, as both 2-APB and carvacrol activate multiple Ca²⁺ related channels (Bilmen and Michelangeli, 2002; Bilmen *et al.*, 2002; Colton and Zhu, 2007; Pires *et al.*, 2015) and the exact mechanism remains largely unclear. Moreover, 2-APB mediated Ca²⁺ influx exclusively from the external culture medium (Xu *et al.*, 2006; Lee *et al.*, 2016), whereas, Ca²⁺ ionophore promoted Ca²⁺ increase from both the ER and extracellular Ca²⁺ influx (Lu *et al.*, 2018). Currently, ionomycin is still the most recommended agent to overcome FF after ICSI (Heindryckx *et al.*, 2005; Vanden Meerschaut *et al.*, 2014; Nikiforaki *et al.*, 2016) due to its high efficiency in provoking a Ca²⁺ rise and inducing activation, as demonstrated in both mouse and human oocytes (Vanden Meerschaut *et al.*, 2014; Nikiforaki *et al.*, 2016).

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

Authors' roles

Y.L., B.H. and P.D.S. conceived and designed the study. Y.L., M.F.B., J. N. and W.H.D. performed experiments reported in Tables I–III and Figs 1–2, 4. R.R. performed the RT-PCR and Sanger sequencing leading to Fig. 3. Y.L., B.H., M.F.B. wrote the article and assembled the figures. M.V.d.J. assisted in English editing. All authors contributed to the interpretation of the results and the editing of the article.

Funding

This study was supported by grants from FWO-Vlaanderen (Research Foundation Flanders, Fonds Wetenschappelijk Onderzoek—Vlaanderen, Grant no. G060615N); China Scholarship Council (no.2010616105); and BOF of Ghent University (Special Research Fund, Bijzonder Onderzoeksfonds, Grant no. 01SC3712).

Conflict of interest

None declared.

References

- Ajduk A, Ilozue T, Windsor S, Yu Y, Seres KB, Bompfrey RJ, Tom BD, Swann K, Thomas A, Graham C *et al.* Rhythmic actomyosin-driven contractions induced by sperm entry predict mammalian embryo viability. *Nat Commun* 2011;**2**:417.
- Araki Y, Yoshizawa M, Abe H, Murase Y. Use of mouse oocytes to evaluate the ability of human sperm to activate oocytes after failure of activation by intracytoplasmic sperm injection. *Zygote* 2004;**12**:111–116.
- Bilmen JG, Michelangeli F. Inhibition of the type I inositol 1,4,5-trisphosphate receptor by 2-aminoethoxydiphenylborate. *Cell Signal* 2002;**14**:955–960.
- Bilmen JG, Wootton LL, Godfrey RE, Smart OS, Michelangeli F. Inhibition of SERCA Ca²⁺ pumps by 2-aminoethoxydiphenyl borate (2-APB). 2-APB reduces both Ca²⁺ binding and phosphoryl transfer from ATP, by interfering with the pathway leading to the Ca²⁺-binding sites. *Eur J Biochem* 2002;**269**:3678–3687.
- Carvacho I, Lee HC, Fissore RA, Clapham DE. TRPV3 channels mediate strontium-induced mouse-egg activation. *Cell Rep* 2013;**5**:1375–1386.
- Che L, Lalonde A, Bordignon V. Chemical activation of parthenogenetic and nuclear transfer porcine oocytes using ionomycin and strontium chloride. *Theriogenology* 2007;**67**:1297–1304.
- Chen J, Qian Y, Tan Y, Mima H. Successful pregnancy following oocyte activation by strontium in normozoospermic patients of unexplained infertility with fertilisation failures during previous intracytoplasmic sperm injection treatment. *Reprod Fertil Dev* 2010;**22**:852.
- Colton CK, Zhu MX. 2-Aminoethoxydiphenyl borate as a common activator of TRPV1, TRPV2, and TRPV3 channels. *Handb Exp Pharmacol* 2007;**179**:173–187.
- Ebner T, Koster M, Shebl O, Moser M, Ven H, Van der, Tews G, Montag M. Application of a ready-to-use calcium ionophore increases rates of fertilization and pregnancy in severe male factor infertility. *Fertil Steril* 2012;**98**:1432–1437.
- Egashira A, Murakami M, Haigo K, Horiuchi T, Kuramoto T. A successful pregnancy and live birth after intracytoplasmic sperm injection with globozoospermic sperm and electrical oocyte activation. *Fertil Steril* 2009;**92**:2037.e5–9.
- Fedorenko OA, Popugaeva E, Enomoto M, Stathopoulos PB, Ikura M, Bezprozvanny I. Intracellular calcium channels: inositol-1,4,5-trisphosphate receptors. *Eur J Pharmacol* 2014;**739**:39–48.

- Girard S, Clapham D. Acceleration of intracellular calcium waves in *Xenopus* oocytes by calcium influx. *Science* 1993;**260**:229–232.
- Hachem A, Godwin J, Ruas M, Lee HC, Buitrago MF, Ardestani G, Bassett A, Fox S, Navarrete F, de Sutter P et al. PLC ζ is the physiological trigger of the Ca²⁺ oscillations that induce embryogenesis in mammals but offspring can be conceived in its absence. *Development* 2017;**144**:2914–2924. <http://www.ncbi.nlm.nih.gov/pubmed/28694258>.
- Heindryckx B, Van der Elst J, de Sutter P, Dhont M. Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. *Hum Reprod* 2005;**20**:2237–2241.
- Heindryckx B, Gheselle S De, Gerris J, Dhont M, de Sutter P. Efficiency of assisted oocyte activation as a solution for failed intracytoplasmic sperm injection. *Reprod Biomed Online* 2008;**17**:662–668.
- Huang D, Jiang L, Xu W, Tong X, Zhu H, Li C, Zhou F, Liu L, Lin X, Zhang S. [Fertilizing ability, cleavage potential and inheritance risk of globozoospermia]. *Zhonghua Yi Xue Za Zhi* 2010;**90**:2351–2353.
- Itoi F, Asano Y, Shimizu M, Honnma H, Murata Y. Embryological outcomes in cycles with human oocytes containing large tubular smooth endoplasmic reticulum clusters after conventional in vitro fertilization. *Gynecol Endocrinol* 2016;**32**:315–318. <http://www.ncbi.nlm.nih.gov/pubmed/26607857>.
- Kim J-W, Choi J-L, Yang S-H, Yoon S-H, Jung J-H, Lim J-H. Live birth after SrCl₂ oocyte activation in previous repeated failed or low fertilization rates after ICSI of frozen-thawed testicular spermatozoa: case report. *J Assist Reprod Genet* 2012;**29**:1393–1396.
- Kim JW, Kim SD, Yang SH, Yoon SH, Jung JH, Lim JH. Successful pregnancy after SrCl₂ oocyte activation in couples with repeated low fertilization rates following calcium ionophore treatment. *Syst Biol Reprod Med* 2014;**60**:177–182.
- Kishikawa H, Wakayama T, Yanagimachi R. Comparison of oocyte-activating agents for mouse cloning. *Cloning* 1999;**1**:153–159.
- Kocabas AM, Crosby J, Ross PJ, Otu HH, Beyhan Z, Can H, Tam WL, Rosa GJM, Halgren RG, Lim B et al. The transcriptome of human oocytes. *Proc Natl Acad Sci USA* 2006;**103**:14027–14032.
- Kuipers AJ, Middelbeek J, van Leeuwen FN. Mechanoregulation of cytoskeletal dynamics by TRP channels. *Eur J Cell Biol* 2012;**91**:834–846.
- Kyono K, Kumagai S, Nishinaka C, Nakajo Y, Uto H, Toya M, Sugawara J, Araki Y. Birth and follow-up of babies born following ICSI using SrCl₂ oocyte activation. *Reprod Biomed Online* 2008;**17**:53–58.
- Latorre R, Brauchi S, Orta G, Zaelzer C, Vargas G. ThermoTRP channels as modular proteins with allosteric gating. *Cell Calcium* 2007;**42**:427–438.
- Lawitts JA, Biggers JD. Optimization of mouse embryo culture media using simplex methods. *J Reprod Fertil* 1991;**91**:543–556.
- Lee HC. Mammalian Egg Activation: The Roles of TRPV3 Channels and PLC ζ . 2016.
- Lee HC, Yoon S-Y, Lykke-Hartmann K, Fissore RA, Carvacho I. TRPV3 channels mediate Ca²⁺ influx induced by 2-APB in mouse eggs. *Cell Calcium* 2016;**59**:21–31.
- Liu B, Qin F. Single-residue molecular switch for high-temperature dependence of vanilloid receptor TRPV3. *Proc Natl Acad Sci USA* 2017;**114**:1589–1594.
- Lu Y, Bonte D, Ferrer-Buitrago M, Popovic M, Neupane J, Jeught M, Van der, Leybaert L, de Sutter P, Heindryckx B. Culture conditions affect Ca²⁺ release in artificially activated mouse and human oocytes. *Reprod Fertil Dev* 2018; doi:10.1071/RD17145 [Epub ahead of print].
- Mak DO, Foskett JK. Inositol 1,4,5-trisphosphate receptors in the endoplasmic reticulum: a single-channel point of view. *Cell Calcium* 2015;**58**:67–78.
- Marshall IC, Taylor CW. Two calcium-binding sites mediate the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states. *Biochem J* 1994;**301**:591–598.
- Miao YL, Stein P, Jefferson WN, Padilla-Banks E, Williams CJ. Calcium influx-mediated signaling is required for complete mouse egg activation. *Proc Natl Acad Sci USA* 2012;**109**:4169–4174.
- Miao Y-L, Williams CJ. Calcium signaling in mammalian egg activation and embryo development: the influence of subcellular localization. *Mol Reprod Dev* 2012;**79**:742–756.
- Méo SC, Leal CLV, Garcia JM. Activation and early parthenogenesis of bovine oocytes treated with ethanol and strontium. *Anim Reprod Sci* 2004;**81**:35–46.
- Neri QV, Lee B, Rosenwaks Z, Machaca K, Palermo GD. Understanding fertilization through intracytoplasmic sperm injection (ICSI). *Cell Calcium* 2014;**55**:24–37.
- Nikiforaki D, Vanden Meerschaut F, de Roo C, Lu Y, Ferrer-Buitrago M, de Sutter P, Heindryckx B. Effect of two assisted oocyte activation protocols used to overcome fertilization failure on the activation potential and calcium releasing pattern. *Fertil Steril* 2016;**105**:798–806e2.
- Otaegui PJ, O'Neill GT, Wilmut I. Parthenogenetic activation of mouse oocytes by exposure to strontium as a source of cytoplasts for nuclear transfer. *Cloning* 1999;**1**:111–117.
- Otsuki J, Okada A, Morimoto K, Nagai Y, Kubo H. The relationship between pregnancy outcome and smooth endoplasmic reticulum clusters in MIH human oocytes. *Hum Reprod* 2004;**19**:1591–1597.
- Peier AM, Reeve AJ, Andersson DA, Moqrich A, Earley TJ, Hergarden AC, Story GM, Colley S, Hogenesch JB, McIntyre P et al. A heat-sensitive TRP channel expressed in keratinocytes. *Science* 2002;**296**:2046–2049.
- Pires PW, Sullivan MN, Pritchard HA, Robinson JJ, Earley S. Unitary TRPV3 channel Ca²⁺ influx events elicit endothelium-dependent dilation of cerebral parenchymal arterioles. *Am J Physiol Heart Circ Physiol* 2015;**309**:H2031–H2041.
- Ramadan WM, Kashir J, Jones C, Coward K. Oocyte activation and phospholipase C zeta (PLCzeta): diagnostic and therapeutic implications for assisted reproductive technology. *Cell Commun Signal* 2012;**10**:12.
- Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. *Annu Rev Physiol* 2006;**68**:619–647.
- Rawe VY, Olmedo SB, Nodar FN, Doncel GD, Acosta AA, Vitullo AD. Cytoskeletal organization defects and abortive activation in human oocytes after IVF and ICSI failure. *Mol Hum Reprod* 2000;**6**:510–516.
- Roh S, Malakooti N, Morrison JR, Trounson AO, Du ZT. Parthenogenetic activation of rat oocytes and their development (in vitro). *Reprod Fertil Dev* 2003;**15**:135–140.
- Sa R, Cunha M, Silva J, Luis A, Oliveira C, Teixeira da Silva J, Barros A, Sousa M. Ultrastructure of tubular smooth endoplasmic reticulum aggregates in human metaphase II oocytes and clinical implications. *Fertil Steril* 2011;**96**:143–149 e7.
- Saunders CM, Larman MG, Parrington J, Cox LJ, Royle J, Blayney LM, Swann K, Lai FA. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* 2002;**129**:3533–3544.
- Smani T, Dionisio N, Lopez JJ, Berna-Erro A, Rosado JA. Cytoskeletal and scaffolding proteins as structural and functional determinants of TRP channels. *Biochim Biophys Acta* 2014;**1838**:658–664.
- Smith GD, Gunthorpe MJ, Kelsell RE, Hayes PD, Reilly P, Facer P, Wright JE, Jerman JC, Walhin JP, Ooi L et al. TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature* 2002;**418**:186–190.
- Swain JE, Pool TB. ART failure: oocyte contributions to unsuccessful fertilization. *Hum Reprod Update* 2008;**14**:431–446.
- Tesarik J. Calcium signalling in human oocytes and embryos: two-store model revival. *Hum Reprod* 2002;**17**:2948–2949.
- Tesarik J, Rienzi L, Ubaldi F, Mendoza C, Greco E. Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne and oocyte-borne oocyte activation failures. *Fertil Steril* 2002;**78**:619–624.
- Vanden Meerschaut F, Nikiforaki D, de Roo C, Lierman S, Qian C, Schmitt-John T, de Sutter P, Heindryckx B. Comparison of pre- and post-implantation development following the application of three artificial activating stimuli in a mouse model with round-headed sperm cells deficient for oocyte activation. *Hum Reprod* 2013;**28**:1190–1198.

- Vanden Meerschaut F, Nikiforaki D, Heindryckx B, de Sutter P. Assisted oocyte activation following ICSI fertilization failure. *Reprod Biomed Online* 2014;**28**:560–571.
- Versieren K, Heindryckx B, Lierman S, Gerris J, de Sutter P. Developmental competence of parthenogenetic mouse and human embryos after chemical or electrical activation. *Reprod Biomed Online* 2010;**21**:769–775.
- Wakai T, Zhang N, Vangheluwe P, Fissore RA. Regulation of endoplasmic reticulum Ca(2+) oscillations in mammalian eggs. *J Cell Sci* 2013;**126**:5714–5724.
- Whittingham DG, Siracusa G. The involvement of calcium in the activation of mammalian oocytes. *Exp Cell Res* 1978;**113**:311–317.
- Xu H, Delling M, Jun JC, Clapham DE. Oregano, thyme and clove-derived flavors and skin sensitizers activate specific TRP channels. *Nat Neurosci* 2006;**9**:628–635.
- Yamazaki W, Ferreira CR, Méo SC, Leal CLV, Meirelles FV, Garcia JM. Use of strontium in the activation of bovine oocytes reconstructed by somatic cell nuclear transfer. *Zygote* 2005;**13**:295–302.
- Yanagida K. Complete fertilization failure in ICSI. *Hum Cell* 2004;**17**:187–193.
- Yanagida K, Morozumi K, Katayose H, Hayashi S, Sato A. Successful pregnancy after ICSI with strontium oocyte activation in low rates of fertilization. *Reprod Biomed Online* 2006;**13**:801–806.
- Yang X-Y, Wang J, Liu J-Y, Gao Y, Zhou Z-M, Sha J-H, Zhang W, Cui Y-G, Qian X-Q. Pregnancy outcome after intracytoplasmic sperm injection with strontium oocyte activation in a globozoospermic patient. *Asian J Androl* 2012;**14**:341–343.
- Zhang D, Pan L, Yang LH, He XK, Huang XY, Sun FZ. Strontium promotes calcium oscillations in mouse meiotic oocytes and early embryos through InsP3 receptors, and requires activation of phospholipase and the synergistic action of InsP3. *Hum Reprod* 2005;**20**:3053–3061.